Plasmodium falciparum **drug resistance, molecular genotyping and generation of a malaria resistance genogram by DNA microarray-based technology**

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Prof. Dr. Hans-Jakob Wirz

Dekan

to my wife, Vera and Sons, Kelvin and Dereck.

Acknowledgement

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Summary

Prior to the 2001 malarial treatment policy change in Tanzania, studies were conducted to assess the efficacy of sulfadoxine-pyrimethamine (SP) and usefulness of molecular markers in monitoring SP resistance. In these studies the 1996 WHO protocol (with 14 days follow-up) was used to assess treatment responses. The findings show that SP failure rates were 6.8 – 13.5% and *P. falciparum* triple-*Pfdhfr* mutant genotype (18.6 – 21.8 %) were already prevalent prior to the change. Mkuzi site, which due to high CQ failure rate, had been using SP against pediatric malaria since 1984, had exceptionally high failure rate (23.6%) and prevalence of triple-*Pfdhfr* mutant genotype (80%). Therefore, the study suggested that the drug may have a short useful therapeutic life (UTL) in Tanzania. Hence SP was adopted as an interim first line antimalarial drug in 2001 while combination therapies were being evaluated for long-term use. The molecular findings also pointed to the potential of the triple-*pfdhfr* mutant genotype as an early warning tool for increasing SP resistance. These data formed the baseline SP efficacy and molecular markers profile in Tanzania prior to the policy change.

SP efficacy monitoring studies conducted using the WHO 2002 protocol (with 28 days follow-up) after widespread use of SP showed high (~40%) SP failure rates in Tanzania. Therefore, these findings provided evidence for withdrawing SP use in Tanzania and highly justified the recommendation of Ministry of Health of switching the first line treatment to artemether-lumefantrine (AL). Concurrent with efficacy studies, community surveys were conducted in the health facilities' catchment areas to assess the profile of all molecular markers of *P. falciparum* resistance to withdrawn and in-use antimalarial drugs as well as those that have never been officially deployed for use. Results show that molecular markers of SP resistance are more prevalent compared to those recorded prior to adoption of SP, with triple-*Pfdhfr* mutant genotype ranging from 54 – 74%. The triple-*Pfdhfr* mutant genotype showed some little evidence of depiction of SP failure rate observed at health facilities. This marker may be applicable as a tool in community-based surveillance of dynamics of SP resistance. However, its usefulness must be further explored by assessing its dynamics relative to SP failure rates in many sites, preferably

with different failure rates while taking into account linkage disequilibria of the observed mutations.

The frequencies of the main mediators of CQ resistance markers (*Pfcrt* 76 and *Pfmdr1* 86 Asn) have decreased following suspension of CQ use in Tanzania, but seems to be influenced by uncontrolled use of QN and AQ for uncomplicated malaria. The lack of the ATPase6 mutation suggest that resistance to artemisinin has not been selected in Tanzania. This observation further supports the decision to adopt AL. The capacity to detect many SNPs in many resistance conferring genes makes DNA micorarray technology a potential tool for monitoring dynamics of *P falciparum* resistance to both withdrawn, "in-use" and undeployed drugs. However, in order to better appreciate parasite genotypic dynamics following alteration of drug pressure, the interval between assessments should be relatively longer than the one used in this study.

In the framework of WHO/TDR combination therapy (ACT) trials of uncomplicated *P. falciparum* malaria in Sub-Sahara African children, we assessed genotyping performance and use-effectiveness. Parasitological failures were adjusted by stepwise genotyping the *P. falciparum glutamine rich protein* (*glurp*), *merozoite surface protein* 1 (*msp1*) and *2* (*msp2*) in Day 0 and post-Day14 recurrent parasitaemias. Recurrences on or before Day 14 were assumed to be recrudescent and were not genotyped. Molecular genotyping refined parasitological outcomes, with differences between crude and adjusted outcomes in most sites >10%. The overall and laboratory performances, (69%) and (78%), respectively, of the stepwise genotyping system were better. However, diligence is needed in sample collection and analysis in order to reduce loss of genotyping data and hence failure to resolve recurrences. Additional genotyping of pre-Day 14 recurrences in Uganda site identified many more new infections and further reduced the PCR-adjusted parasitological failure rate by 8%. Therefore, the study recommends that all recurrent infections in malaria treatment trials/studies in high transmission areas should be genotyped. The stepwise genotyping approach, coupled with more advanced DNA extraction methods needs to be validated and considered for adoption as a standard integral part in malaria drug efficacy studies.

Zusammenfassung

Bevor Tansanien 2001 seine Standards zur Malariabehandlung geändert hat, wurden Studien durchgeführt, um die Effizienz von Sulphadoxin-Pyrimethamine (SP), sowie den Nutzen molekularer Marker zur Überwachung der SP-Resistenz zu testen. Um den Behandlungserfolg mit SP zu testen, wurde für diese Studien das WHO Protokol von 1996 (mit 2-wöchiger Überwachungsperiode) angewendet. Hierbei zeigte sich, das die Behandlungsmisserfolge mit SP zwischen 6.8 und 13.5 % lagen und die 3-fach Mutante des *Plasmodium falciparum dhf*-Gens (18.6 – 21.8%) bereits vor dem Medikamentenwechsel auftrat. In Mkuzi wurden, wo bereits seit 1984 wegen Behandlungsversagen bei Chloroquine SP bei pädiatrischer Malaria verwendet wurde, aussergewöhnliche hohe Behandlungsmisserfolge (23.6%) und eine hohe Prävalenz der *dhfr* Triplemutante festgestellt. Daher weist diese Studie auf einen sehr kurzen therapeutisch nützlichen Zeitraum von SP in Tansanien hin (useful therapeutic life, UTL). Dennoch wurde SP als Interims-Medikament zur Behandlung unkompolizierter Malaria gewählt, während seit 2001 Kombinationstherapien für die dauerhafte Behandlung evaluiert werden. Die molekularen Daten unterstreichen das Potential, die *dhfr* Triplemutante als Frühwarnsystem für zunehmenden Resistenz gegen SP zu verwenden. Die hier vorgestellten Daten sind die Basis der SP Effizienz und stellen das molekulare Markerprofil in Tansanien vor dem Wechsel der Behandlungsstrategie dar.

Effizienstudien mit SP nach weitläufigem Gebrauch von SP wurden mit dem WHO Protokoll von 2002 (mit 28-tägiger Überwachungsperiode) durchgeführt und zeigten eine hohe Rate an Behandlungsmisserfolgen (~40%) in Tansanien. Dieser Befund verstärkt die Empfehlung SP abzusetzen und begründet die Empfehlung des Gesundheitsministeriums, Artemether-Lumefantrine (AL) als Standardmedikament zu verwenden. Gleichzeitig zu Studien an den Gesundheitszentren wurden Untersuchungen in den Dorfgemeinschaften im Einzugsgebiet der Gesundheitszentren durchgeführt, um das Profil aller molekularen Marker, die mit Resistenz assoziert sind, zu erstellen. Dies umfasste auch Marker für bereits zurückgezogene oder bisher offiziell nicht verwendete Antimalaria-Medikamente. Dabei zeigte sich, dass die molekularen Marker für SP-Resistenz, insbesondere die *dhfr* Triplemutante mit einer Prävalenz von 54 – 74% eine

höhere Prävalenz haben als vor der Einführung von SP als Standardbehandlung. Es gab jedoch keine Beziehung zwischen der Anzahl der Behandlungs-misserfolge im Gesundheitszentrum und der individuellen Allel-Frequenz eines bestimmten mutierten Gens im Einzugsgebiet. Nur die *dhfr* Triplemutante deutete ansatzweise auf eine Verbindung zur Misserfolgsrate im Gesundheitszentrum hin. Dieser Marker könnte daher eventuell als Marker für eine *community-based* Überwachung der SP Resistenzdynamik nützlich sein. Die tatsächliche Verwendbarkeit dieses Markers zur Bestimmung der relativen SP Resistenz muss jedoch in anderen Gebieten, wenn möglich mit anderen Behandlungsmiss-erfolgsraten, untersucht werden.

Die Häufigkeit der Marker für Chloroquinresistenz (*Pfcrt* 76 and *Pfmdr1* 86) ist nach dem Absetzen von Chloroquin in Tansanien zurückgegangen, scheint jedoch weiterhin von unkrontrolliertem Einsatz von Chinin und Amodiaquin zur Behandlung von leichter Malaria beinflusst zu werden. Die Absenz von Mutationen im ATPase6-Gen weist darauf hin, dass Artemisinin-Resistenz in Tansanien noch nicht selektioniert wurde. Dies unterstützt die Entscheidung zu AL zu wechseln. Die Möglichkeit, gleichzeitig viele verschiedene Punktmutationen (SNPs) in resistenz-assoziierten Genen nachzuweisen, macht den DNA-Mikroarray zum idealen Werkzeug bei der Überwachung der *P. falciparum* Resistenzdynamik gegen bereits verwendete und zurück gezogene Medikamente, momentan empfohlene, und bisher noch nicht verwendete Medikamente. Um jedoch die Dynamik Parasitenresistenz nach Veränderung des Selektionsdrucks durch Medikamente besser zu verstehen, sollten die Abstände zwischen den Untersuchungen länger sein, als in dieser Studie.

Im Rahmen der, von der WHO durchgeführten, Studien mit Kombinationspräparaten (ACT) zur Behandlung unkomplizierter Malaria bei Kindern in Afrika südlich der Sahara, haben wir untersucht, welchen Nutzen und Effekt die molekulare Genotypisierung in diesen Studien hat. Die Rate der parasitologischen Behandlungsmisserfolge wurde nach stufenweiser Genotypisierung und Vergleich der Parasiten am Tag 0 und am Tag des Wiederauftretens zuerst auf Basis des *P. falciparum glutamine-rich proteins* (*glurp*), und danach mit den *merozoite surface proteins 1* und *2*

entsprechend korrigiert. Wiederauftretende Parasiten vor oder am Tag 14 wurden als rekurierende Parasiten angeschaut und wurden nicht genotypisiert. Das molekulare Genotypisieren verbesserte die parasitologische Ergebnisse, wonach die Medikamenteneffizienz in den meisten Studiengebieten um >10% verbessert wurde. Die allgemeine Durchführungserfolg der Genotypisierungssudien war mit 69%, und die spezifische Labordurchführung des stufenweisen Genotypisierens mit 78%, zufriedenstellend. Besondere Aufmerksamkeit sollte jedoch auf die Sample-Gewinnung und Analyse gelegt werden um Datenverlust bei der Genotypisierung zu reduzieren, wodurch eine Untersuchung wiederauftretender Parasiten verunmöglicht wird. Die Genotypisierung von Proben vor Tag 14 in Uganda erlaubte die Identifikation einer grosse Anzahl von neuen Infektionen, wodurch die Rate PCR angepasster parasitologischer Behandlungsversagen um 8% reduziert wurde. Die Annahme, dass Parasiten vor Tag 14 immer rekurierende Parasiten sind, muss somit als falsch angesehen werden. Diese Studie empfiehlt deshalb, alle wieder auftretenden Parasiten in Medikamentenstudien in Gebieten mit hoher Malaria-Transmission zu genotypisieren. Die stufenweise Genotypisierung mit verbesserten DNA Aufarbeitungsmethoden sollte weiter validiert werden, um als intergraler Standarttest in Effizienzstudien von Antimalaria-Medikamenten aufgenommen zu werden.

1 Chapter 1

1.1 General introduction

1.2 Background

1.2.1 The burden of Malaria

Malaria is a tropical protozoan parasitic disease caused by *Plasmodium falciparum*, *P. vivax*, *P. ovale* and *P. malariae*. The disease is transmitted to human by the female *Anopheles* mosquito, during a blood meal. Of the four species *P. falciparum* is the most pathogenic responsible for about 40,000 million disability adjusted life years and an estimated 350–500 million malaria clinical episodes occur annually world-wide. Around 60% of the cases and over 80% of the deaths due to malaria occur in Sub-Saharan Africa. This is because the majority of infections in Africa are caused by *P. falciparum* and the most effective malaria vector, the mosquito *Anopheles gambiae*, is the most widespread in Africa and the most difficult to control. More than 1 million Africans, mostly children under 5 years of age, die from malaria each year. Malaria also contributes significantly to anemia in children and pregnant women, adverse birth outcomes such as spontaneous abortion, stillbirth, premature delivery and low birth weight, and overall child mortality. The disease contributes approximately 1.3% annual reduction of in economic growth in the region (World Malaria Report 2005). In Tanzania malaria transmission is intense and occurs year round in at least 100 of the country's 121 Districts. More than 18 million cases of malaria are diagnosed and treated each year. Many more are treated outside the formal health facilities. Malaria is the leading cause of childhood mortality in Tanzania and the single leading reason for outpatient consultation at health facilities for all ages and the leading cause of deaths, accounting for 20% of all life-years lost. In Tanzania the disease is responsible for 45% of the disease burden for the children under five and for 16 - 20% of hospital death among this age group (Local Initiative for Integrated Malaria Control, (LIIMCO) - National Malaria Control Program (NMCP), unpublished data).

Resistance to antimalarials is a major drawback in effective malaria control in Sub-Saharan Africa. Efficacy data collected in southern Africa between 1996 and 2000 using

the 14 day protocol showed high chloroquine (CQ) failure rates. Most of the region being above the critical value of total treatment failure = 25% (Talisuna *et al.* 2004; EANMAT 2003). At that period, sulfadoxine-pyrimethamine (SP) showed high adequate clinical response (ACR) ranging from 71.8 - 93% and > 90% for amodiaquine (AQ). Thus, around the year 2000, most southern African countries withdrew CQ and adopted either SP alone, SP+CQ or AQ in combination with artesunate (EANMAT 2003). In 2001 Tanzania adopted SP as an interim first line drug for the treatment of uncomplicated malaria. Therefore, there was a need to monitor SP efficacy after its widespread use as first line malaria treatment drug in Tanzania. In contrary between 1999 and 2000, CQ efficacy in western Africa was generally high with only Ghana recording CQ treatment failure rate above the critical value (Talisuna *et al.* 2004). CQ has been the most commonly used drug for uncomplicated malaria in this region (Evans *et al.* 2005; Happi *et al.* 2005). It was earlier shown that artemisinin-based combination therapy (ACT) may slow down the development and spread of resistance to the drug accompanying it. Thus WHO began testing for tolerance and efficacy of combinations of CQ, SP and AQ with artesunate in 1999, by conducting trials in 9 sites in Sub-Saharan Africa. In these studies treatment responses were assessed for 28 days and genotyping was done to distinguish recrudescence from new infections.

The mode of action and mechanism of parasite resistance to antimalarial drugs has been extensively studied but remains to be partially characterized. A number of genes have been associated to resistance to quinoline [*Pfcrt* (Fidock *et al.* 2000b) and *Pfmdr1* (Foote *et al.* 1990)], antifolates [*Pfdhfr* (Cowman *et al.* 1988; Reeder *et al.* 1996) and *Pfdhps* (Triglia *et al.* 1997)] and artemisinins [PfATPase6 (Jambou *et al.* 2005)]. Mutations in these resistance conferring genes alters the respective protein structural conformation leading to reduced binding or altered molecular transport system, hence parasite surviving the drug effect. There is a large body of data showing that a combination of mutations in *pfdhfr* (51Ile, 59Arg, and 108Asn) and *pfdhps* (437Gly and 540Glu) might form a useful marker for field surveillance of SP resistance in Africa (Kyabayinze *et al.* 2003; Kublin *et al.* 2002; Happi *et al.* 2005). However, the usefulness of these markers remains controversial because other investigators (Jelinek *et al.* 1997; Rallon *et al.* .

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1999; Francis *et al.* 2006) did not establish association with treatment outcome. Nonetheless, the mutations in these resistance-associated genes are considered as markers for resistance and may be useful as early warning signals for increasing resistance (Wongsrichanalai *et al.* 2002)

Therefore, the studies described here were conducted in order to establish the baseline SP clinical and molecular marker profiles prior to the adoption of SP as first line drug and to monitor efficacy after its widespread use in Tanzania. In framework of WHO-CT trials conducted in Sub-Sahara Africa countries, the study also evaluated the use-effectiveness of molecular genotyping in discriminating recrudescence and new infections in recurrent infections. Finally the studies assess the usefulness of a novel high throughput SNP detection technique as a tool for large-scale community-based surveillance of dynamics of parasite resistance to both withdrawn and in use drugs.

2 Chapter 2

2.1 Literature review

2.2 Malaria Chemotherapy and Assessment of antimalarial efficacy

Correct diagnosis and prompt treatment with an effective antimalarial drug is of paramount importance in determining the outcome of the malaria treatment. Malaria can be diagnosed by clinical signs (i.e. presumptive diagnosis), observation of presence of parasites by microscopy or parasite antigens by rapid diagnostic tests (RDTs) or DNA by polymerase chain reaction (PCR) (Arrow *et al.* 2004; WHO, 2006). Various drugs are used for management of malaria including the antifolates (e.g. sulphadoxine, pyrimethamine, proguanil, chlorproguanil and trimethoprim), quinolines (e.g. mefloquine, halofantrine, lumefantrine, amodiaquine, piperaquine, tafenoquine chloroquine, quinine and quinidine), artemisinins (e.g. artemisinin, dihydroartemisinin artemether, artesunate), atovaquone (falls into its own class with specific mode of action) and several antibacterial drugs (e.g. tetracycline, clindamycin) also have weak antiplasmodial activities (Arrow *et al.* 2004).

Shortly after the first report of CQ resistance in 1965, standardized *in vivo* antimalarial drug efficacy testing systems were developed, used, and updated in 1972. These protocols remained in use until 1996 when a specific protocol for intense transmission areas (WHO, 1996) was developed. In this protocol, *in vivo* treatment responses were assessed for 14 days and classified on the basis of either clearance of clinical signs/symptoms as adequate clinical response (ACR), early treatment failure (ETF), and late treatment failure (LTF) or parasite as resistance level 1 (RI) level 2 (RII) and level 3 (RIII). Experience gained showed that the 14 days protocol underestimates treatment failure rates. This led to the suggestion that post-treatment follow up should be long enough to detect recrudescent infections emerging later after initial parasite clearance. Hence the 1996 protocol was revised in 2002 incorporating *in vitro* parasite susceptibility testing and drug resistance molecular markers assessment protocols as supporting

methods (WHO, 2002). This new protocol recommends that assessment of response should be done for 28 - 63 days, depending on the half life of the drug under study. In addition, the protocol combines clinical and parasitological observations in assessing treatment responses. Therefore, treatment outcomes are classified as adequate clinical and parasitological response (ACPR), ETF, late clinical failure (LCF) and late parasitological failure (LPF). Thus it is redundant now to report clinical and parasitological responses separately (Ringwald, 2004).

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

In vitro efficacy tests and molecular genotyping of resistance markers (mutations in resistance conferring genes) are supplementary methods used in the assessment of *P. falciparum* resistance to antimalarial drugs. The former involve testing the susceptibility of parasite to drug in culture whereas the latter measures the SNPs at various positions in resistance-associated genes. If reliable evidence on their *in vivo* resistance predictive value is established, the two methods may replace the former method which is labour and time intensive. In order to able to fully exploit makers of antimalarial drug resistance we need to a better understanding of how drugs work and how resistance come about. A

detailed review of mode of action, mechanism of resistance and their respective molecular markers of resistance is provided in chapter 2.

2.3   Antimalarial Drug Resistance situation in Sub-Sahara Africa

Drug resistance is defined as the ability of a parasite to survive and/or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended, but within the limits of tolerance of the subject (WHO, 1965; WHO, 1973). Resistance to antimalarials is a major drawback in effective malaria control in Sub-Saharan Africa. In this region, *P. falciparum* has developed resistance to the cheap and safe antimalarials such as CQ and sulfadoxine-pyrimethamine (SP). Efficacy data collected in southern Africa [Tanzania (Mainland and Zanzibar), Kenya, Uganda and Rwanda] showed high clinical failure to CQ (10% 71%) (EANMAT 2003), with most of the region being above the critical value of total treatment failure = 25% (Talisuna *et al.* 2004). However, SP and AQ both showed high ACR ranging from 71.8% - 93%. Thus around 2000 most southern African countries replaced CQ with either SP monotherapies, AQ+AS or non-artemisinin combination therapies (non-ACTs) such as SP+AQ as first line malaria treatment drugs (EANMAT 2003). In contrary, within the same period CQ was efficacious in western Africa (Nigeria, Mali, Senegal, Ghana, Ivory Coast and The Gambia). Only Ghana had CQ treatment failure rate above the critical value (Talisuna *et al.* 2004). Thus the drug has been the most commonly used drug for uncomplicated malaria in this region (Evans *et al.* 2005; Happi *et al.* 2005). However, recent data show high levels of CQ resistance in Nigeria (39% at day 14, Sowunmi *et al.* 2005), Senegal (21%, at day 28, Sarr *et al.* 2005) and Ghana (25%, day 28 PCR corrected Koram *et al.* 2005).

Due to spread of resistance to SP and CQ monotherapies, the use of artemisinin-based combination antimalarial therapies (ACT) is now highly advocated (Arrow *et al.* 2004; WHO, 2006). However, for an effective combination therapy, both partner drugs must be reasonably efficacious and deployed preferably prior to their use as monotherapies (Watkins *et al.* 2005). Indeed the high background of SP and CQ failure rates observed in

various countries made these drugs unsuitable partners in ACT (Obonyo *et al.* 2003, Prioto *et al.* 2003, Sirima *et al.* 2003, Gill *et al.* 2003). Recommended ACTs include artemether+lumefantrine (AL), artesunate+amodiaquine (AS+AQ) and artesunate+mefloquine (AS+MQ) (WHO, 2006). Most Sub-Saharan African countries are revising malaria treatment policies to adopt ACT. So far 15 out of 43 Sub-Saharan countries have already adopted the policy of AS-AQ as first-line, and the rest are at various stages of preparation for switching to ACT. Tanzania will revise malaria treatment policy to replace SP with AL in 2006 (NMCP, unpublished data). Artemisinins are highly potent antimalarial drugs and are also active against early-stage gametocytes (Mehra and Bhasin 1993). To date no relevant clinical resistance has been reported since they were firstly introduced in 1972. The drugs have short half-lives and act very fast. They clear over 90% of parasite load within the first 6 hours of administration and the rest of the load is slowly eliminated by the partner drug that usually has long half-life and acts slowly. Hence fewer parasites are exposed to sub-therapeutic levels, a potential factor for the selection and spread of resistance (White and Olliaro 1996; Bloland *et al.* 2000).

2.4   Mode of action and mechanisms of resistance to antimalarial drugs

2.4.1   Antifolates

Prokaryotic and eukaryotic cells require reduced folate cofactors for the biosynthesis of many cellular components. In plants and most microorganisms folate must be synthesized *de novo* through the folate biosynthesis pathway. However, higher eukaryotic cells including mammal can not synthesize folate *de novo* and are totally dependent on exogenous (dietary supplied) folate as the only source for tetrahydrofolate (THF) production by dihydrofolate reductase (DHFR). These differences in folate biosynthesis capacity between mammals and microorganisms makes the pathway an attractive antimicrobial target (Bermingham and Derrick 2002; Djapa *et al.* 2006). In normal physiological state the parasite's dihydropteroate synthase (DHPS) catalyses the condensation of *p*-aminobenzoic acid (*p*-ABA) with 2-amino-4-hydroxy-6-hydroxymethyl-7, 8 dihydropteridine pyrophosphate (DHPPP) to form dihydropteroate

(DHP). Subsequently the dihydrofolate synthase (DHFS) adds a glutamate to DHP to form dihydrofolate (DHF) which is finally reduced by DHFR to form THF (Figure 1). THF and its derivatives are used as cofactors in biosynthesis of amino acids (e.g. serine, methionine, glycine and histidine) and purines and thymidylate for normal cell growth and function. Sulfa drugs and *p*-ABA show high degree of structural similarities, thus competitively bind to DHPS. Therefore, by binding to DHPS sulfa drugs competitively inhibits the activity of this enzyme. Pyrimethamine selectively binds with several folds higher affinity to DHFR of the parasite than the human host, preventing its activity of DHP. Hence sulfadoxine and pyrimethamine exert their parasitocidal effect by synergistically inhibiting the parasite's folic acid biosynthesis pathway. On the other hand, it was earlier shown that DHPS catalyses the formation of sulfa-DHP (Dieckmann *et al.* 1986). This complex was thought to play a role in parasitocidal effect of sulfadoxine (Mberu *et al.* 2002) and was recently confirmed to be inhibitory to parasite growth (Patel *et al.* 2004). The proposed sulfa-DHP complex formation is indicated in the folic acid biosynthesis pathway in Figure 1.

Therefore, point mutations at the amino acid position in the *dhfr* 16 Val, 51 Ile, 59 Arg, 108 Asn/Thr and 164 Leu (Cowman *et al.* 1988; Peterson *et al.* 1988) and *dhps* 436 Ala/Phe, 437 Gly, 540 Glu, 581 Gly and 613 Thr/Ser (Triglia *et al.* 1997) result in structural changes on the two proteins' active site cavities and subsequently reduced binding affinity, consequently inhibiting folic acid synthesis. Accumulation of mutations, in a stepwise fashion is incriminated for increased resistance to antifolate. (Plowe *et al.* 1998; Plowe *et al.* 1997). These mutations are considered as molecular markers for surveillance of antifolate resistance. Several studies have shown their association with SP treatment failure (Kublin *et al.* 2002; Nzila *et al.* 2000; Kyabayinze *et al.* 2003; Happi *et al.* 2005). The ratios of prevalence of these markers to SP treatment failure rates, termed genotype failure index (GFI) (Kublin *et al.* 2002; Kyabayinze *et al.* 2003) point to the existence of association between treatment failure and SP resistance markers. However, the *dhps* genotype has not consistently been able to completely account for *P. falciparum in vivo* failure to SP (Alifrangis *et al.* 2003; Mutabingwa *et al.* 2001; Mockenhaupt *et al.* 2005) or *in vitro* sulfadoxine or dapsone resistance in (Mberu *et al.* 2002). These

observations unequivocally support the suggestion (Patel *et al.* 2004) that resistance to sulfadoxine may primarily be attributable to other determinants, such as formation of sulfa-DHP complex. *Pfdhfr/Pfdhps g*ene amplification has not been demonstrated in nature and appears to play no role in antifolate resistance (Plowe, 2005).
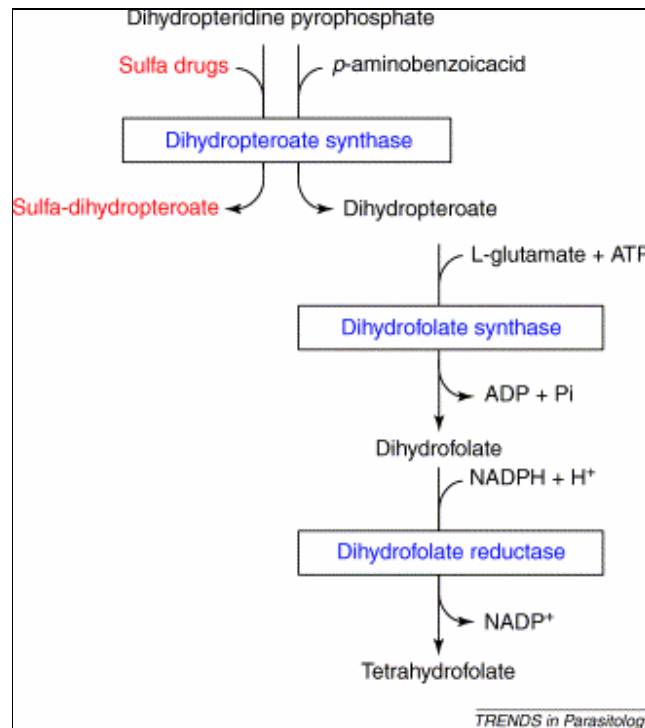
Source: Patel *et al.* 2004

Figure 1. The folate biosynthesis pathway. The condensation of *p*-aminobenzoic acid to dihydropterin pyrophosphate is catalyzed by dihydropteroate synthase (DHPS) to form dihydropteroate, which is then reduced to dihydrofolate by dihydrofolate synthase. Dihydrofolate is reduced to tetrahydrofolate by dihydrofolate reductase. DHPS also catalyzes the formation of sulfa-dihydropteroate in the presence of a sulfa drug. Sulfa drugs and the sulfa-DHP formed are shown in red. Enzymes are indicated in blue and normal metabolites are shown in black. Reproduced from Patel *et al.* 2004.

2.4.2 4-aminoquinolines

This class includes chloroquine (CQ) and amodiaquine (AQ). Many studies have been done to elucidate the parasitocidal activity of these quinolines. However, to date their modes of action remain largely unclear. Nonetheless, a large body of knowledge accumulated for over 30 years shows that the drugs act primarily in the parasite digestive vacuole (DV) by interfering with detoxification of heme, a by-product of hemoglobin digestion (Ginsburg *et al.* 1998; Zhang *et al.* 1999). In this compartment CQ is considered to have several target sites including heme dimerisation activity, aspartic and cysteine protease activity and intravesicular pH (Krogstad and De 1998)

In order to comprehend the current and widely accepted mode of CQ action, it is important to understand some *P. falciparum* ultra-structures and feeding processes. The intraerythrocytic parasites are surrounded by a parasitophorous vacuolar membrane (PVM). Thus together with the parasite plasma membrane (PPM), intraerythrocytic parasites are surrounded by a double membrane layer. The parasites feed on the hemoglobin found in the host RBC via a cytostome by forming a localized invagination of the PVM and PPM. The double membrane hemoglobin-laden endocytic vesicles [transport vesicles (TV)] are then pinched off from the cytostome (Figure 2). The first formed TV matures into DV whose PVM is digested leaving only the PPM (Hempelmann *et al.* 2003). The DV is an equivalent of lysosomes in other eukaryotic cells, hence sometime called secondary lysosomes. In the DV (Yoyan *et al.* 1984, in Hempelmann *et al.* 2003) and/or TV (Slomianny *et al.* 1990) hemoglobin is broken down by several enzymes including cysteine and aspartic proteases, into peptides and/or amino acids and ferrous protoporphyrin (FeIII IX) which is quickly oxidized to ferric protoporphyrin (FeII IX) (heme) (Barnerjee and Goldenberg 2001; Eagan *et al.* 2002). The amino acids (AA) are believed to undergo protonation (AAH+) and exported into the cytosol (Figure 2 & 3) where they are utilized for protein synthesis and parasite growth. The heme is membrane-toxic, it rapidly intercalate with lipid bilayers and interferes with electron transport chains, leading to peroxidative damage to unsaturated lipids and/or membrane-embedded proteins (Zhang *et al.* 1999). The parasite lacks a heme oxygenase pathway but protects itself from heme toxicity by crystalising free heme into non-toxic

hemozoin (malaria pigment) which accumulates in the DV (Banerjee and Goldberg 2001; Slater *et al.* 1991; Slater and Cerami 1992 in Waters and Janse). Chemically hemozoin is identical to synthetic β-hematin and comprised of dimers of β-hematin linked by hydrogen bonds (Pagola *et al.* 2000). It is believed that heme dimerization process is promoted mainly by the lipid (linoleic acid) fraction of the erythrocyte membrane forming the inner membrane of endocytic vesicles (Fitch *et al.* 2000; Orjih, 2001; Hempelmann *et al.* 2003) and the acid environment (pH around 5) inside the vesicles (Orjih, 2001).
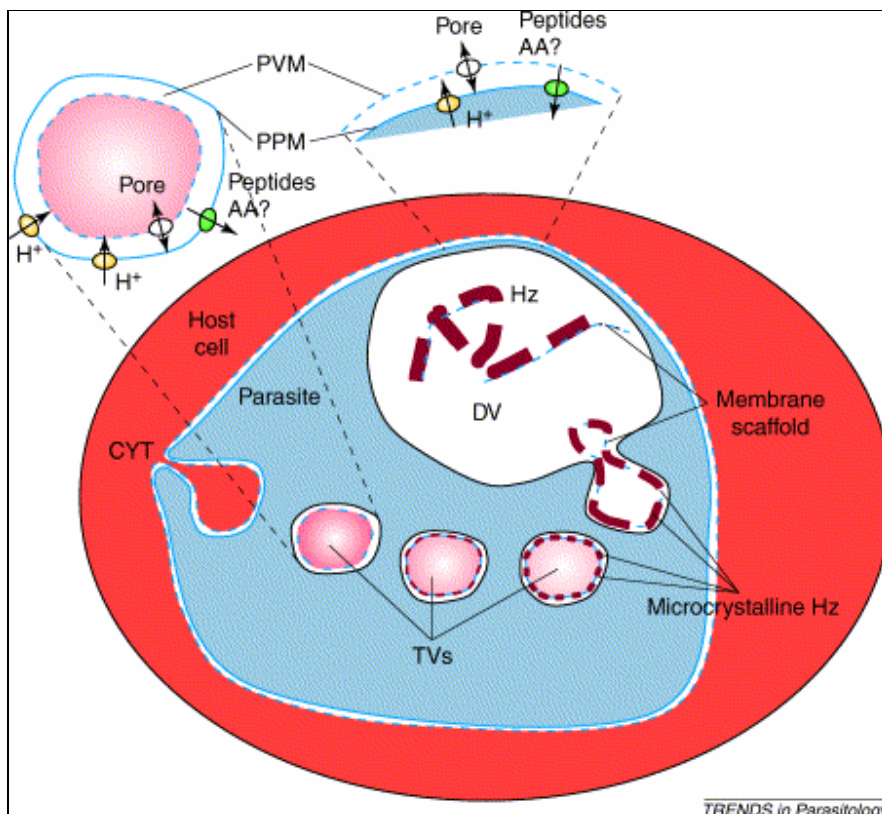
Source: Hempelmann *et al.* 2003

Fig. 2. Proposed mechanism of haemozoin biogenesis. Host cell cytoplasm is ingested by the cytostome (CYT) and packaged in unique double-membrane transport vesicles (TV). The inner (solid blue line) and outer (broken blue line) membrane of the TV is derived from the parasitophorous vacuolar membrane (PVM) and parasite plasma membrane (PPM), respectively. The TV is acidified by the action of the vacuolar proton pump (H^+).

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Degradation of hemoglobin takes place inside the inner membrane, and the heme is deposited on the inner membrane. Small peptides and/or amino acids (AA) pass through the pore and are transported through the outer TV membrane into the parasite cytoplasm. The high concentration of heme on the inner TV membrane promotes formation of microcrystalline haemozoin (Hz, brown) or 'malarial pigment'. On completion of digestion, the vesicles fuse with the digestive vacuole (DV), and the maturing crystals and residual membrane scaffold (MS) are delivered to the interior of the DV. Reproduced from Hempelmann *et al.* 2003

Treatment with CQ or AQ results in swelling of the DV as a result of drug accumulation (Aikawa 1972; Jacobs *et al.* 1988 in Waller *et al.* 2004). Further studies showed that CQ-resistant (CQR) parasites accumulate less drug than CQ-sensitive (CQS) (Saliba *et al.* 1998). In the DV these drugs binds to heme preventing its detoxification (Ginsburg *et al.* 1998; Zhang *et al.* 1999). Search for genetic determinants of CQ resistance mapped CQR to a 36 kb region that contains 8 putative genes and identified cg1 and cg2 gene as responsible for CQ resistance (Su *et al.* 1997). These genes were shortly shown to have no role in resistance and it was proposed that other nearby genes may be more important in CQ resistance (Fidock *et al.* 2000a). Further screening of the region identified a *P. falciparum* CQ resistance transporter gene (*Pfcrt*) on chromosome 7 as the most important determinant of CQ resistance and mutations were identified and associated with increased CQ resistance *in vitro* (Fidock *et al.* 2000b) and *in vivo* (Djmede *et al.* 2001, 2001b). Earlier on the phenomena of reduced drug accumulation and resistance reversibility shared between multi-drug resistant cancer cells and CQR *P. falciparum* (Karcz and Cowman 1991) prompted the search and subsequent discovery of *P. falciparum* mdr-like (*Pfmdr1*) gene in chromosome 5 which was linked to CQ resistance (Foote *et al.* 1990; Cowman and Karcz 1991). The *Pfcrt* encodes for the chloroqunie resistance transporter (PfCRT) protein and the *Pfmdr* a P-glycoprotein homologue 1 (Pgh1) protein. Both PfCRT (Fidock *et al.* 200b) and Pgh 1 (Cowman *et al.* 1991) are located on the parasite's DV membrane and currently regarded as the primary mediators of CQ resistance (Fidock *et al.* 2000b; Djimde *et al.* 2001) despite their being on different chromosome.

A model of CQ effect on heme detoxification by wild-type and mutant parasite in the DV was suggested by Warhurst 2001 as shown in Figure 3. In Panel A, in the lysosome of a chloroquine-sensitive parasite, hydrogen ions enter through the proton pump, acidifying the lysosomal environment (pH 5.5). This process is probably regulated by the Pgh1 protein, which releases anions into the lysosome to optimize the difference in the transmembrane charge. During the digestion of hemoglobin (Hb), protonated basic amino acids (AAH$^+$) are released together with toxic ferriprotoporphyrin IX (Fp9). Ferriprotoporphyrin IX is detoxified by polymerization to crystalline hemozoin. The weak base chloroquine, present in the cytoplasm (pH 7.4), dissolves in the lysosomal membrane and enters the acidic environment, undergoing protonation to a form (CQH$^+$) that is insoluble in the membrane and that quickly becomes concentrated. CQH$^+$ binds to ferriprotoporphyrin IX and thus inhibits its polymerization, which leads to the accumulation of ferriprotoporphyrin IX, causing membrane damage. The protonated basic amino acids exit the lysosome by means of the transmembrane protein PfCRT. The PfCRT protein probably has a limited affinity for CQH$^+$ and exports some of the drug from chloroquine-sensitive parasites. Panel B shows the lysosome of a parasite with mutations in *pfcrt* and *pfmdr 1* related to chloroquine resistance. The mutant PfCRT probably has an increased affinity for CQH$^+$ and exports large amounts of the drug, enabling the polymerization of ferriprotoporphyrin IX to proceed normally. Concomitantly, the mutant PfCRT would have a reduced affinity for AAH$^+$, which may reduce the efficiency of the export of AAH$^+$ and, in the absence of chloroquine, result in the accumulation of more protons (H$^+$) in the lysosome. The presence of mutant Pgh1 may partially prevent this accumulation of protons, increasing the fitness of parasites with *pfcrt* and *pfmdr 1* mutations. The mutation in *pfmdr 1* also increases the sensitivity of the parasite to mefloquine and artemisinin, probably as a result of the partial inactivation of the ability of mutant Pgh1 to export these drugs.

Accumulation of mutations in the *Pf*crt at position 72 Ser, 74 Ile, 75 Glu, 76 Thr, 220 Ser, 271 Glu, 326 Ser, 356 Thr and 371 Ile (Fidock *et al.* 2000b) and *Pf*mdr1 at positions 86 Asn/Thr, 184 Phe, 1034 Cys, 1042 Asp and 1246 Tyr (Foote *et al.* 1990) are associated

with increased resistance to CQ. These two genes are believed to interact synergistically (Adagut and Warhurst 2001). The *Pfcrt* 76 mutation is strongly associated with CQ resistance and the GFI calculated using this marker were stable (Djimde *et al.* 2001; Tinto *et al.* 2005). Nonetheless, mutations in *mdr1* only modulate CQ susceptibility of *Pfcrt* mutant parasite but are, by themselves, incapable of conferring CQ resistance (Reed *et al.* 2000; Djimde *et al.* 2001; Babiker *et al.* 2001; Adagut and Warhurst, 2001). The Pfmdr1 86 Tyr is the most important modulator of CQ resistance. Thus *pfcrt* 76 Thr and *pfmdr1* 86 Tyr mutations are recommended for use as markers for *in vivo* CQ resistance (Djimde *et al.* 2001a Djimde *et al.* . 2001b).
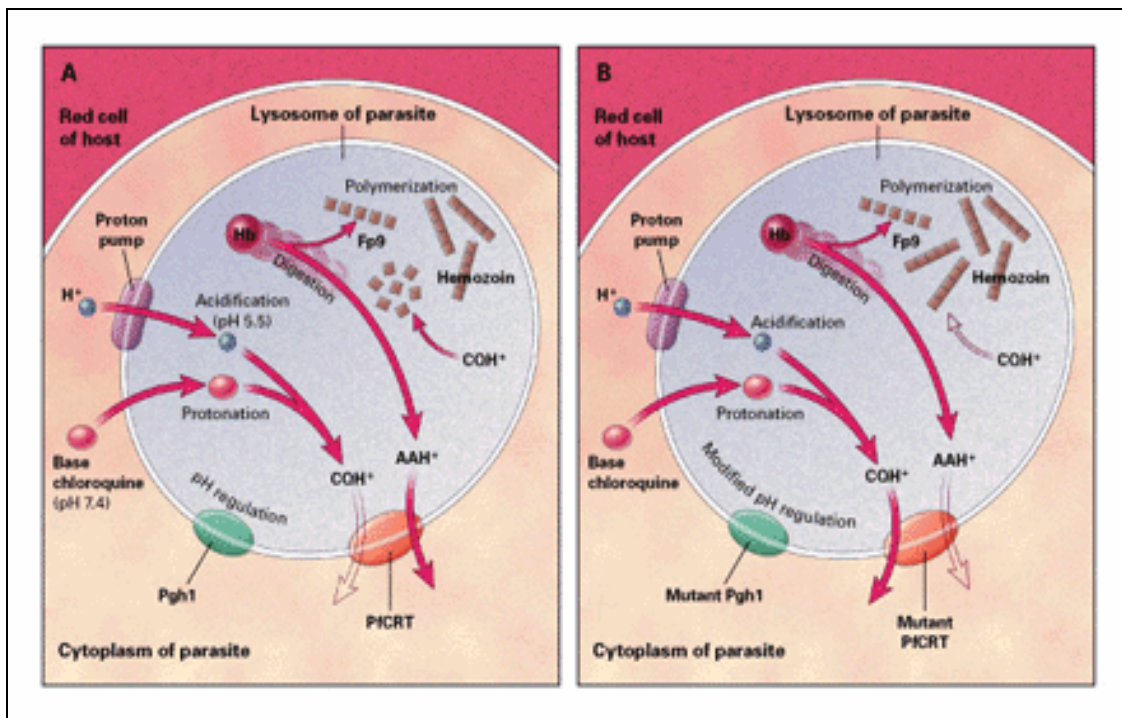
Source: Warhurst, 2001

Figure 3**.** The Effect of Chloroquine on Heme Detoxification in the Lysosome of a Chloroquine-Sensitive *Plasmodium falciparum* Malaria Parasite (Panel A) and a Chloroquine-Resistant Malaria Parasite (Panel B). Reproduced from Warhurst, 2001

Studies have shown also that CQ treatment induces masking of the lipid fraction of the erythrocyte membrane that promotes ferriprotoporphyrin dimerization (Fitch *et al.* 2003a) and reduces the activity of neutral aminopeptidase, an enzyme required for normal

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processing of hemoglobin-laden endocytic vesicles (Fitch *et al.* 2003b). These interferences are also considered to cause heme to accumulate to toxic levels. The detergent-like effect of FP on biological membranes accounts for its lytic toxicity (Fitch *et al.* 1984).

Alternative modes of CQ action have been proposed. Elevation of vacuolar pH above the optimal values for aspartic and cysteine proteases activity in the food vacuole may lead to inhibition of hemoglobin degradation, consequently inhibiting parasite growth. It has been postulated that CQ may interfere with glutathione mediated detoxification of toxic superoxide ($^{\cdot}O^{2-}$) and hydroxyl ($^{\cdot}OH$) radicals liberated as a result of reaction of Fe (II) reacts with dioxygen. Over 30 years ago it was shown that CQ intercalates with DNA (O'Brien *et al* 1966; Meshnick, 1990; Yin *et al.* 2003) leading to inhibition of DNA replication as observed in many prokaryotic and eukaryotic cells. However, this hypothesis received little attention until recently when various studies provided clues that the nucleus may be the key site of CQ action and resistance arises due to alteration of targeting proteins or certain mechanism which prevents CQ from entering its targeting proteins in the nucleus (Li, 2006). Phospholipid is another potential target for CQ. Although CQ binds with low affinity to phospholipid, CQ-heme complex is believed to bind with high affinity to phospholipids (via the FP bridge) and mediate cytotoxic effect to the parasite (Fitch, 2004). The possibilities of nucleus and phospholipids being targets of CQ action need to be further explored.

2.4.3 Quinoline-4-methanols

This class includes quinine (QN), mefloquine (MQ), lumefantrine and halofantrine, etc. Similarly, the precise mode of these quinolines is not known. However, there is evidence of binding of these drugs to targets other than the ferriprotoporphyrin IX. Exposure of CQR parasites to quinine and mefloquine does not lead to accumulation and aggregation hemoglobin-laden endocytic vesicles (Jacobs *et al.* 1987; Oliaro *et al.* 1989), increased masking of the linoleic acid (Chou and Fitch 1993; Fitch and Chou 1997; Fitch *et al.* 2003a) or excess accumulation of undimerized ferriprotoporphyrin. Mefloquine, quinine

and other quinoline-4-methanol subclass bind with high affinity to phospholipid targets in malaria parasites (Porcar *et al.* 2003). The drugs also inhibit and reverse vesicular docking in the endolysosomal system, either by impairing membrane function directly or indirectly by inhibiting calcium release from an acid stores (Fitch, 2004). Mefloquine and quinine antagonize CQ-induced abnormalities in malaria parasites, primarily by inhibition of hemoglobin ingestion (Famin and Ginsburg 2002) and secondarily they inhibit membrane recycling, leading to killing of the parasite. However, the binding of CQ-FP complex to phospholipids is an agonist for vesicular docking in malaria parasites (Fitch, 2004).

The inverse effects of 4-aminoquinolines and quinoline-4-methanol on parasite morphological abnormalities are consistent with observations made on the role of the two membrane transport genes on resistance to these antimalarial drugs. Similar to CQ resistance to mefloquine and/or quinine is also influenced by *Pfcrt* (Mu *et al.* 2003, Bray *et al.* 2005) and *Pfmdr1* genotype, and/or copy number /over expression of the *Pfmdr1* gene (Foote *et al.* 1990; Duraisingh *et al.* 2000; Price *et al.* 2004; Duraisingh and Cowman, 2005). However, in contrast to CQ, mutations in *Pfcrt* have been associated with increased susceptibility to mefloquine and quinine (Sidhu *et al.* 2002) and the wild type *Pfmdr1* allele further augments resistance to mefloquine (Cowman *et al.* 2002; Duraisingh *et al.* 2000; Price *et al.* 1999) and artemether+lumefantrine (coartem®) (Sisowath *et al.* 2005). In another study (Mu *et al.* 2003 and Bray *et al.* 2005), the mutants *Pfcrt* were associated with QN resistance. This ability of *Pfmdr1* to influence sensitivity of parasites to aminoquinolines, arylaminoalcohols and artemisinins provides evidence of contribution of *mdr1* to multi-drug resistance (Duraisingh and Cowman 2005; Price *et al.* 1999). Multi-drug resistance is said to have occurred when cells selected for resistance to one agent, are rendered resistant to a number of structurally unrelated drug (Juliano and Ling, 1976 in Duraisingh and Cowman 2005). In addition to *Pfmdr1* and *Pfcrt,* 9 more genes have been found to be associated with *P. falciparum* sensitivity to CQ and QN. However, in this study mutations in *Pfcrt* were insufficient to confer QN resistance suggesting that overlapping non identical sets of genes explain why parasites can be resistance to CQ but highly sensitive to QN (Mu *et al.* 2003). In general,

these observations show that drug resistance is a complex phenotype involving interaction of many different genes.

2.4.4 Artemisinins

Originally the mode of action of artemisinins was considered to be similar to CQ; inhibition of heme polymerization (Pandey *et al.* 1999). However, it was later shown that artemisinins kill parasites by heme-depended activation of the endoperoxide bridge (Meshnick, 1994). The cleavage of the bridge activates a series of reactions culminating into the formation of an oxygen-centered free radical, carbon–centered free radical and finally an epoxide. Carbon-centered radicals and epoxide are highly active alkylating agents and may kill parasites by alkylating some, unidentified targets (Olliaro, 2001; Jefford *et al.* 2001; Olliaro *et al.* 2001). However, this theory was not universally accepted. It was proposed that the antimalarial activity of artemisinins is conferred by the 1,2,4 trioxane pharmacophore within artemisinins (Olliaro *et al.* 2001). The trioxane structure is now being exploited in developing synthetic peroxide antimalarials (Vennerstrom *et al.* 2004). However, the theory of heme dependent activation of the endoperoxide bridge contrast the mode of action of most bioactive molecules where activity is mediated by binding to an active site. The observations that artemisinins (i) localize to parasite and not food vacuole membranes (Ellis *et al.* 1985) (ii) are capable of killing tiny rings lacking hemozoin (ter Kuile *et al.* 1993) and (iii) do not inhibit hemozoin formation (Haynes *et al.* 2003) have ruled out food vacuole as the site for artemisinin action.

Thapsigargin, a plant-derived sesquiterpene lactone, is a highly specific inhibitor of sarco/endoplasmic reticulum Ca^{2+} (SERCA). Since thapsigargin and artemisinins show structural similarities, it was hypothesized and later proven that artemisinins can specifically and selectively inhibit *PfATPase6*, the only SERCA-type Ca^{2+}-ATPase in *P. falciparum* genome, after activation by iron (Eckstein-Ludwig *et al.* 2003). The interaction of artemisinins with thapsigargin-binding cleft of susceptible SERCAs was confirmed (Uhlemann *et al.* 2005), pointing out that mutations which modulate its

sensitivity to artemisinins may mark emergence of resistance. Subsequent *in vitro* studies showed that *P. falciparum* with elevated IC$_{50}$ values for artemisinins share particular mutations i.e *PfATPase6* 769, 623 and 431 (Jambou *et al.* 2005). However, the development of stable artemisinin resistant *P. chabaudi chabaudi* lacking mutations or amplification of the *ATPase6* gene failed to establish the role of this gene in resistance to artemisinin (Afonso *et al.* 2006). *P. falciparum* sensitivity to artemisinins is also considered to be influenced the *Pfmdr1* genotype and amplification (Price *et al.* 2004; Duraisingh *et al.* 2000). While the mode of action of artemisinins is still controversial and unclear, mutations in the *PfATPase6* and *Pfmdr1* are the only currently available markers that may be used as warning signals for emergence of *in vivo* resistance to artemisinins. Uncontrolled use of artemisinins monotherapies such as that reported in Urban Dar-es salaam, Tanzania (Kachur *et al.* 2006) or in combination with ineffective partners might lead to faster selection of resistance resulting into reduction of presumed long useful therapeutic life (UTL) of the ACTs (Duffy and Sibley, 2005).

2.5 Methods used in the detection of SNPs in resistance-associated genes

SNPs in resistance-associated genes can be identified by sequencing the DNA fragment of interest. This method is not commonly used in field studies. It is suitable for detection of new mutations and confirmation ambiguous cases. In 1990s PCR-based methods for SNP detection in malaria resistance-associated genes were developed and applied in the filed. These include mutation-specific PCR (MS-PCR) (Plowe *et al.* 1995) and PCR-restriction fragment length polymorphism (PCR-RFLP) (Duraisingh *et al.* 1998). These methods are labor and time intensive, therefore, less attractive for large-scale studies. In attempts to increase high throughput in SNP analysis, dot-blot hybridization methods were established in early 2000s. These methods are more or less similar to PCR-RFLP but instead of developing the restriction map by RFLP, the PCR products are spotted onto membrane and hybridized to either radio-labeled (Abdel-Muhsin *et al.* 2002) or digoxigenin-labeled (Pearce *et al.* 2003) sequence-specific oligonucleotide probes (SSOP). Comparison of these methods showed high sensitivity to MS-PCR but the

method was non-specific. On the other hand PCR-RFLP was specific but less sensitive. The radio-labeled dot blot method was as specific and sensitive as PCR-RFLP (Ranford-Cartwright *et al.* 2002). The use of PCR, followed by SSOP enzyme-linked immunosorbent assay (ELISA) (Alifrangis *et al.* 2006) and real-time PCR (Purfield *et al.* 1994; Farcas *et al.* 2006) in the detection of *P falciparum* resistance alleles was recently demonstrated with advantage of increased specificity over the old methods.

2.6 Emergence and Spread of *P. falciparum* Resistance

The *Pfdhr* and *Pfdhps* mutations occur in a progressive, stepwise fashion occur in a progressive stepwise fashion starting with single 108 mutation generation replaced by 108 Asn + 51 Ile followed by 108 Asn + 51 Ile + 59Arg and finally 164 Leu is added and higher levels of resistance are observed in multiple mutant parasites (Plowe *et al.* 1997). Parasites carrying the 108 mutation spreads because they tolerate higher levels of SP and can invade human approximately 12 days post SP treatment compared to 50 days in wild-type (Hastings *et al.* 2002). Addition of 51 appears to restore the lost DHFR enzymatic activity than increasing the tolerance of 108+51 mutant parasites to SP. This genotype is associated with increases parasite clearance time and presence of gametocyte, probably as a result of prolonged parasite survival under drug pressure (Méndez *et al.* 2002). The subsequent *Pfdhr* triple and quadruple mutations are driven by drug pressure and provide no information on natural selection (Hastings *et al.* 2005).

Analysis of microsatellite DNA flanking the *dhfr* and *dhps* loci showed a few common origins of resistant alleles in South America Amazon (Cortese *et al.* 2002), Southern Africa (Roper *et al.* 2003) and Southeast Asia (Nair *et al.* 2003). Comparison of *dhfr* and *crt* resistant alleles of parasites from Southern Africa and Southeast Asian also showed common ancestral origin. Thus it has been proposed that (i) there are a few origins of resistant alleles (ii) *de novo* mutations are less important than migration for introducing resistance alleles into parasite population and (iii) selective sweep or gene flow is a primary mode of spread of resistance (Roper *et al.* 2004; Anderson and Roper, 2005).

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The reasons for few origins of resistant alleles are detailed in Anderson and Roper, (2005). In summary, for a resistant allele to spread it must be found in parasite lineages that are committed to become gametocytes. Less than 1% of asexual parasites are committed to become gametocytes (Taylor and Read 1997). In addition, parasites expressing a predominant *var* gene are targeted for clearance by the immune system. Only mutations borne by proliferating parasites expressing newly switched *var* genes will escape clearance and achieve transmission. As a result, the "effective population" in terms of transmission and spread of resistant alleles is usually lower than the actual population of parasites infecting humans. Secondly, resistance involves multiple mutations. Since mutations are accumulated progressively, it is unlikely that multiple mutations occur in the same allele during a single replication. This process presents sequential bottlenecks in the population of resistant alleles. Thirdly, drug resistance and compensatory mutations, that reduces fitness cost, rarely arise simultaneously in the same parasite genome. Indeed, after 5 - 10 years of SP use in parts of Africa, the *Pfdhfr* 164 mutation has not been reported consistently. It is hypothesized that the African parasites lack the genetic traits that would confer the ability to bear the dhfr 164 mutation (Nzila *et al.* 2005).

Reduction of CQ drug pressure following its discontinuation resulted in decreased prevalence of crt 76 mutation in Malawi (Kublin *et al.* 2003; Mita *et al.* 2003). Concurrently there was recovery of CQ sensitivity with 100% clearance of asymptomatic infections and lack of *in vitro* resistant isolates (Kublin *et al.* 2003). The recovery of sensitivity following suspension of CQ use is attributable to expansion of wild-type allele rather than back mutations (Mita *et al.* 2004). Similar observations have been recorded following suspension of CQ use in China (Liu *et al.* 1995) and Vietnam (Nguyen *et al.* 2001; Nguyen *et al.* 2003; Thahn *et al.* 2001). Decline in drug resistance after removal of drug pressure could provide a new paradigm for antimalarial treatment policies in Africa (Laufer and Plowe, 2004).

The magnitude of loss of fitness cost incurred by *Pfmdr1* mutations *in vitro* has been estimated to be 25% and it was shown that there is greater selective pressure on the

mutant *Pcfrt* than on the mutant *Pfmdr1* (Hayward *et al.* 2005). This observation is consistent with the negligible change in *Pfmdr1* 86 Asn compared to *Pfcrt* 76 mutant allele observed in Malawi after CQ withdrawal (Mita *et al.* 2003). The data on the reversal of resistance in Malawi (Kublin *et al.* 2003 and Mita *et al.* 2003) suggested that the *Pfcrt* 76 mutation is 5% less fit than wild-type, under the assumption of 5 generations of malaria per year and no parasite immigration was occurring (Hastings and Donnelly, 2005). However, reversal of SP resistance has not been observed but there has been reports on persistence of *Pfdhfr* mutations after discontinuation of SP use in South-east Asia (Nair *et al.* 2002) which was incriminated to mutations that fully or partially compensate for metabolic defect on the original *Pfdhfr* mutation (Nair *et al.* 2003). Decline in drug resistance after removal of drug pressure could provide a new paradigm for antimalarial treatment policies in Africa (Laufer and Plowe, 2004).

2.7 Discrimination of recrudescents from new infections by molecular genotyping.

Many *Plasmodium falciparum* genes show extensive genetic polymorphism which can be used for genetic finger printing. High polymorphism has been shown in *msp1*, *msp2* and *glurp* genes in different geographical locations in malaria endemic areas (Felger *et al.* 1994; Babiker *et al.* 1997; Snounou *et al.* 1999; Peyerl-Hoffmann *et al.* 2001; Magesa *et al.* 2001; Aubouy *et al.* 2003). Therefore, these loci have been used in many trials to distinguish recrudescence from new infections. Because of their extensive polymorphism, it is highly unlikely for a patient in areas of intense transmission to become newly infected with a parasite possessing an identical genotype during follow-up because this probability is the product of individual allele frequencies of each allele of the three genes (Snounou and Beck 1998). Therefore, by comparing the genotypes of these three loci together at baseline and at the time of parasite recurrence, recrudescent can be distinguished reliably from new infections (Snounou and Beck, 1998; Viriyakosol *et al.* 1995; Beck, 1999; Greenwood, 2002). However there is variation not only in the sample analysis but also in interpretation of genotyping data, limiting comparison of data from various sites. Recurrent parasites can be potentially classified into four categories based

on the degree of allelic matching: (i) all alleles in the baseline and recurrent parasites are identical, (ii) some alleles are missing in the recurrent parasites (iii) recurrent parasites contain alleles identical to those at baseline with additional/new ones not observed at baseline (iv) alleles in the baseline and recurrent parasite samples are different. It is generally accepted that categories (i - iii) represent recrudescent and (iv) new infection (Magesa *et al.* 2001; Snounou and Beck, 1998; Basco and Ringwald, 2000; Brockman *et al.* 1999; Basco *et al.* 2002; Ranford-Cartwright *et al.* 1997; Happi *et al.* 2004). Some investigators (Cattamanchi *et al.* 2003; Kyabayinze *et al.* 2003) consider that category (iii) represents a new infection because of the appearance of new alleles. It is believed that, resolution of symptoms and parasite clearance are regarded as the most accurate measures of the intrinsic resistance of the parasites to a drug (Sibley and Hunt, 2003). The controversy surrounding category (iii) has not been resolved and the need for standardized genotyping protocol has been recognized.

Interpretation of genotyping data may be complicated by (i) re-infections with new parasites possessing identical genotypes to those present on Day 0 may lead to an erroneous diagnosis of recrudescence, (ii) inability of PCR to detect all clones present on Day 0 whose reappearance may therefore be regarded as a new infection and (iii) micro-epidemics in which the same parasite(s) circulates over and over again in the same small population e.g. a household. However, the first possibility is negligibly low when two or more discriminatory markers are being used (Snonou and Beck, 1998). The second possibility is also low because it has been shown that symptomatic infections are less complex than asymptomatic ones (Irion *et al.* 1998; Farnert and Bjorkman, 2005). Therefore, single time-point samples may reliably represent all subpopulations present on Day 0. Nonetheless, these weaknesses point to the need for some caution in interpreting PCR-adjusted treatment outcomes.

3 Chapter 3

3.1 Rationale of the project

3.1.1 SP efficacy testing

CQ was used as first line drug in Tanzania for over 45 years. By 1999 the average country-wide CQ resistance was 50% (ranging from 45 to 70%). In 2001 Tanzania adopted SP as an interim first line drug for the treatment of uncomplicated malaria. Following the experience that SP resistance developed shortly after widespread use of SP in Southeast Asia (low transmission area), predictions were made that resistance to SP would spread faster following its widespread use in areas of intense malaria transmission areas. Therefore, it was important to monitor SP efficacy to provide policy makers with evidences on which they can make rational policy changes. Around 2001 little was known about the role of resistance markers in SP resistance in Tanzania. Using the clinical setting the study sought to establish SP baseline clinical and molecular marker profile and assess the association to treatment failure rates.

3.1.2 Community approach for monitoring *P. falciparum* resistance to antimalarial drugs

The mode of action and mechanism of resistance to antifolate and quinolines has been studied but remains to be partially characterized. Nonetheless, some genes have been associated with resistance to quinoline (*Pfcrt* and *Pfmdr1*), antifolates (*Pfdhfr* and *Pfdhps*) and artemisinins (PfATPase6). Mutations in resistance-associated genes alter the respective protein structural conformation, hence reduced binding or molecular transport system leading to parasite surviving the drug effect. These mutations are considered as markers for resistance and are useful as early warning signals for increasing resistance. Most of the current reports on the role of mutant alleles in antimalarial drug resistance are based on data collected in relatively small samples sizes of children < 5 years of age. The parasite population in the untreated asymptomatic infections (parasite reservoir) has been

largely ignored but might play an important role in the development and spread of resistance. In addition, because of technical and time limitations only a few mutations are usually analyzed. Hence there is a need for high throughput methods capable of handling many samples and detecting many SNPs in several genes (genogram) at a time. Such systems should allow for community-based monitoring of dynamics of resistance to withdrawn, "in-use" and undeployed antimalarial drugs.

3.1.3 Distinction of recrudescent from new infections

The assessment of antimalarial drug efficacy post-treatment follow up should be long enough to detect recrudescent infections after initial parasite clearance. Longer follow-up periods pose difficulties in interpreting drug efficacy outcomes, particularly in high transmission areas, because new infections occurring during follow up may be interpreted as treatment failures. Thus molecular genotyping of *P. falciparum msp1*, *msp2* and *glurp* genes is recommended to distinguish between new and recrudescent infections and adjust treatment outcomes accordingly (WHO, 2002; WHO, 2006). This approach has been used in many trials to distinguish recrudescence from new infections (Basco and Ringwald, 2000; Brockman *et al.* 1999; Basco *et al.* 2002; Ranford-Cartwright *et al.* 1997; Happi *et al.* 2004; Cattamanchi *et al.* 2003; Kyabayinze *et al.* 2003; Irion *et al.* 1998) but its use-effectiveness, particularly in large clinical trials has not been fully evaluated.

3.2 Broad Objective

The study aimed at assessing the dynamics of *in vivo P. falciparum* resistance to SP, profiles and dynamics of drug resistance molecular markers and use-effectiveness of molecular genotyping in adjusting antimalarial treatment failure rates

3.2.1 Specific Objectives

1. To assess the baseline SP efficacy and resistance marker profile prior to its adoption as first line drug in Tanzania and determine the potential of the latter in monitoring resistance

2. To assess SP efficacy 2 -3 years after its widespread use as first line antimalarial drug in Tanzania.

3. To evaluate a novel high throughput DNA microarray SNPs detection technique as a tool for community-based approach for simultaneously monitoring dynamics of resistance to "in-use" and withdrawn drugs.

4. To assess the use-effectiveness of molecular genotyping in adjusting antimalarial treatment outcomes.

In the following Chapters each of the specific objectives is presented as a working paper. Chapter 4 and 5 summarizes the baseline SP efficacy and resistance marker profile prior to and after its adoption of SP as first line drug in Tanzania whereas chapter 6 assesses the usefulness of a community-based approaches for monitoring antimalarial drug resistance. Finally, in the framework of WHO combination therapy trials conducted in Sub-Saharan Africa, chapter 7 and 8 shows the use-effectiveness of molecular genotyping in distinguishing recrudescents from new infection. Chapter 9 brings together chapters 4 – 8 in a synoptic general discussion and conclusions.

4 Chapter 4.

4.1 Therapeutic efficacy of sulfadoxine-pyrimethamine and prevalence of resistance markers in Tanzania prior to revision of malaria treatment policy: *Plasmodium falciparum dihydrofolate reductase and dihydropteroate synthase* mutations in monitoring in vivo resistance

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THERAPEUTIC EFFICACY OF SULFADOXINE-PYRIMETHAMINE AND PREVALENCE OF RESISTANCE MARKERS IN TANZANIA PRIOR TO REVISION OF MALARIA TREATMENT POLICY: *PLASMODIUM FALCIPARUM* DIHYDROFOLATE REDUCTASE AND DIHYDROPTEROATE SYNTHASE MUTATIONS IN MONITORING *IN VIVO* RESISTANCE

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Abstract. Prior to the 2001 malarial treatment policy change in Tanzania, we conducted trials to assess the efficacy of sulfadoxine-pyrimethamine (SP) and the usefulness of molecular markers in monitoring resistance. A total of 383 uncomplicated *Plasmodium falciparum* malaria patients (between 6 and 59 months old) were treated with SP and their responses were assessed. Mutations in the *P. falciparum* dihydrofolate reductase (*pfdhfr*) and dihydropteroate synthase (*pfdhps*) genes in admission day blood samples were analyzed. Results indicated that 85.6% of the patients showed an adequate clinical response, 9.7% an early treatment failure, and 4.7% a late treatment failure. The quintuple mutant genotype (*pfdhfr* 51 Ile, 59 Arg, and 108 Asn and *pfdhps* 437 Gly and 540 Glu) showed an association with treatment outcome (odds ratio = 2.1; 95% confidence interval = 0.94–4.48, $P = 0.045$). The prevalence of the triple *pfdhfr* mutant genotype (51 Ile, 59 Arg, and 108 Asn) at a site of high SP resistance (23.6%) was four times higher compared with that observed at sites of moderate SP resistance (6.8–14.4%) ($P = 0.000001$). The genotype failure index calculated by using this marker was invariable (1.96–2.1) at sites with moderate SP resistance, but varied (3.4) at a site of high SP resistance. In conclusion, our clinical and molecular findings suggest that SP may have a short useful therapeutic life in Tanzania; thus, its adoption as an interim first-line antimalarial drug. The findings also point to the potential of the triple *pfdhfr* mutant genotype as an early warning tool for increasing SP resistance. These data form the baseline SP efficacy and molecular markers profile in Tanzania prior to the policy change.

INTRODUCTION

In 2001, the Tanzania mainland adopted sulfadoxine-pyrimethamine (SP) and amodiaquine (AQ) as first- and second-line antimalarial drugs, respectively, following increased chloroquine (CQ) resistance (45–70%). The use of SP for first-line purposes is an interim measure while different antimalarial combinations are being evaluated for long-term use. Before this change, SP was used as a second-line antimalarial drug.[1] Several other countries in southern Africa including Kenya, Burundi, Rwanda, the Tanzania islands (Zanzibar), and Malawi have switched to SP, AQ, or artesunate (AS) monotherapies or combination therapies, whereas Uganda opted for the SP/CQ combination following widespread CQ resistance.[2] Sulfadoxine-pyrimethamine is one of the few, cheap, and relatively safe antimalarial drugs that is still effective against CQ-resistant malaria in Africa. Recent studies in southern Africa have recorded high efficacies, ranging from 82% to 92%.[2–8] However, the fact that *Plasmodium falciparum* rapidly develops resistance to SP following wide use of the drug poses a serious threat to malarial control efforts in endemic countries.[9] High levels of SP resistance have been recorded in a highly endemic northeastern part of Tanzania where pyrimethamine[10,11] and sulfadoxine[12] were used at different periods between 1950 and 1994 for prophylactic and therapeutic trials, respectively. In a recent study conducted in this area, 45% of the patients treated with SP failed to clear their parasitemias to below patency levels on day 7.[13] This failure rate is substantially higher compared with 25% in Ifakara (southeastern Tanzania)[14] and 26% in Kigoma (western Tanzania),[5] both of which are also highly endemic areas

in Tanzania, but in which SP had not been widely used. Therefore, it is obvious that following wide use of SP in Tanzania, resistance is likely to increase rapidly. Given appropriate tools, the National Malaria Control Program (NMCP) framework provides a better platform for regularly updating information on antimalarial drug resistance situation in Tanzania. Currently, the *in vivo* efficacy test is the gold standard method for monitoring antimalarial drug resistance in countries endemic for malaria. However, the method is expensive and complex in terms of interpreting outcomes, especially in high transmission areas where chances of re-infection are high. Thus, the need for a cheap, rapid, and reliable epidemiologic tool for SP surveillance has been recognized.

Molecular markers of SP resistance are considered to be a cheap and less complex candidate tool for *in vivo* SP resistance surveillance. There is a large body of data showing that a combination of mutations in the *P. falciparum* dihydrofolate reductase (*pfdhfr*) (51 Ile, 59 Arg, and 108 Asn) and dihydropteroate synthase (*pfdhps*) (437 Gly and 540 Glu) genes might constitute a useful marker for field surveillance of SP resistance in Africa.[15–24] However, the usefulness of these markers remains controversial because other investigators[25,26] did not establish an association with treatment outcome. Furthermore, some new mutations in the *pfdhfr* gene have been discovered[27]; thus, their roles in *vivo* resistance must be assessed. New approaches for understanding the relationship between mutations and antimalarial drug resistance have been suggested. The genotype resistance index (GRI) and the genotype failure index (GFI) concepts[28] and the ratio of mutant to wild-type *pfcrt* alleles[29] have been pointed out as practical models using a *pfcrt* 76 Thr mutation

in the surveillance of CQ resistance. There is a need to verify such models (by using the *pfdhfr* and *pfdhps* gene markers) in areas where SP is used as the first-line antimalarial drug.

As a preparation for the policy change, we conducted studies to determine SP efficacy and prevalence of SP resistance molecular markers (*pfdhfr* and *pfdhps* gene mutations) in Tanzania. We also assessed the applicability of these markers in monitoring SP resistance. The findings presented here form the baseline SP efficacy and molecular markers profile for Tanzania and support the decision made by the Ministry of Health to adopt SP as an interim first-line antimalarial drug. Our findings also present evidence of association between treatment failure and quintuple mutant genotype. The prevalence of mutant genotypes and GFI values in high versus moderate resistance sites point to the potential of the triple *pfdhfr* mutant genotype as an early warning tool for increasing SP resistance in Tanzania. Nonetheless, we recommend further studies, at both community and health facility levels, to verify the usefulness of *pfdhfr* and *pfdhps* genotypes in estimating SP resistance.

MATERIALS AND METHODS

Study sites. These trials were carried out in Butimba, Kyela, Masasi, Mkuzi, and Mlimba Rural Health Centers in Tanzania. These areas are antimalarial drug resistance surveillance sites of the NMCP, classified epidemiologically as mesoendemic (Kyela and Butimba) or holoendemic (Mkuzi, Mlimba, and Masasi), and are located in different geographic areas in the country (Figure 1). The catchment areas for these health facilities are rural-based communities of similar socioeconomic background.

Recruitment of study subjects. All patients between 6 and 59 months old who reported to the health centers were evaluated and considered for recruitment by the study team. Detailed medical histories were obtained and clinical examinations were conducted. Thick and thin smears were made from finger prick blood and stained with Giesmsa for parasite detection and identification by microscopy. Patients were eventually recruited for study if they had an axillary temperature ≥ 37.5°C, microscopically confirmed *P. falciparum* monoin-
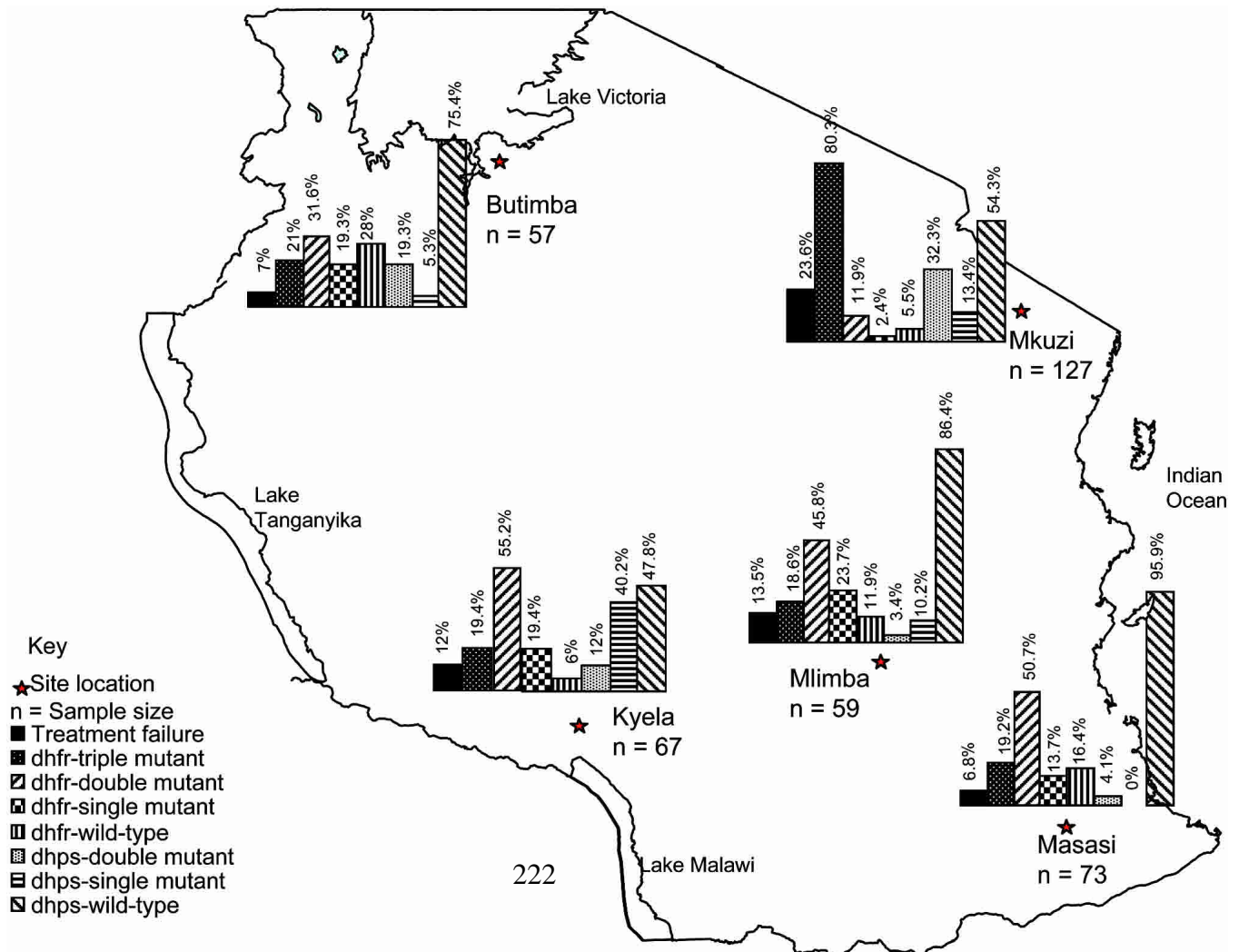
FIGURE 1. Map of Tanzania showing the geographic location of the study sites, resistance to sulfadoxine-pyrimethamine (S), and the prevalence of *Plasmodium falciparum* dihydrofolate reductase (*pfdhfr*) and dihydropteroate synthase (*pfdhps*) genotypes. The triple *pfdhfr* (51 Ile, 59 Arg, and 108 Asn) and double *pfdhps* (437 Gly and 540 Glu) mutant genotypes are highly prevalent in Mkuzi, an area with the highest level of resistance to SP. The rest of the sites (Butimba, Kyela Masasi, and Mlimba) have moderate levels of resistance to SP and prevalences of this mutant genotype, but high prevalences of wild-type genotype. This figure appears in color at www.ajtmh.org.

fections (parasitemia between 2,000 and 100,000 asexual stage parasites/μL), no history of antimalarial use in the last 14 days prior to the episode, an absence of co-infection with other diseases, and consent from parents or guardians. Patients who had mixed *Plasmodium spp.* infections, severe malaria or danger sings, history of allergy to sulfa drugs, or other chronic infections were not recruited for study but, respectively, were given appropriate treatment by the study team.

Treatment of patients. Recruited patients were treated with SP (Fansidar®, 500 mg of sulfadoxine and 25 mg of pyrimethamine; Roche, Basel, Switzerland) in a single oral dose of 1.25 mg/kg of pyrimethamine and 25 mg/kg of sulfadoxine and observed for 30 minutes. If vomiting occurred within this period, a replacement dose was administered and again observed for an additional 30 minutes. Further vomiting led to exclusion of the patient from the study. These patients were rescued by parenterally administered quinine (nine doses of 30 mg/kg). Parents or guardians of recruited children were asked to return to the health centers for response evaluation on days 2, 3, 7, and 14 post-treatment. In addition, they were advised to return at any other (unscheduled) day if temperature or sickness persisted or relapsed. Patients who did not turn up for scheduled follow-ups were visited at home by a member of the study team. Clinical and parasitologic examinations were conducted on each follow-up day. A patient was withdrawn from the study if any of the following occurred during the follow-up period: development of a concurrent infection, treatment with another antimalarial drug, the patient could not be traced at a home visit on a scheduled day or the day after, or the parent/guardian requested that the patient be withdrawn from the study. Treatment responses were classified as an adequate clinical response (ACR), an early treatment failure (TF), and a late treatment failure (LTF) as described in the 1996 World Health Organization (WHO) *in vivo* efficacy testing protocol for areas of intense transmission.[30] Patients who failed to respond were treated with amodiaquine (10 mg/kg for dose 1 and 2 and 5 mg/kg for dose 3). At the end of the study, 414 patients were recruited (67 in Butimba, 70 in Kyela, 78 in Masasi, 133 in Mkuzi, and 66 in Mlimba). Thirty-one cases were either lost to follow-up or excluded from the study during follow-up. Thus, 383 patients completed the study or were followed-up to day of failure. The study was reviewed and approved by the both institutional (Ifakara Health Research and Development Centre [IHRDC] Ethics Committee) and national (Medical Research Coordinating Committee) authorities and consent was obtained from parents or guardians prior to recruitment of each patient.

Blood sample collection, extraction of DNA, and mutation analysis. Before treatment of recruited patients, finger prick blood was spotted onto filter paper (3MM; Whatman International Ltd., Maidstone, United Kingdom), air-dried, transported to the IHRDC laboratory, and stored dry in self-sealing plastic bags at room temperature until required for extraction of DNA. The DNA was extracted from the filter paper using the Chelex extraction method previously described.[31] Polymorphisms in *pfdhfr* codons 51, 59, 108, and 164 and *pfdhps* codons 436, 437, 540, 581, and 613 were determined by performing primary and nested polymerase chain reaction (PCR) amplifications with subsequent restriction fragment length polymorphism (RFLP) analysis of the nested PCR products as described in detail elsewhere.[32] The RFLP

products were resolved by electrophoresis on 10% polyacrylamide gels, stained with ethidium bromide, photographed, and scored. A 2 × 2 chi-square table was used to analyze associations between clinical and molecular data and Epitable in Epi-Info (Centers for Disease Control and Prevention, Atlanta, GA and World Health Organization, Geneva, Switzerland) and was used to compare differences in the prevalence of SP resistance and molecular markers in the study sites. P values < 0.05 (and confidence intervals [CIs] > 1 for odds ratio [OR]) were considered significant. The GFI was calculated as the ratio of the prevalence of resistant genotype to the prevalence of drug failure,[28] and the variability of the values among study sites was assessed by linear regression.

RESULTS

Treatment outcome for SP and association with the quintuple mutant genotype. Of 383 SP treated patients, 328 (85.6%) showed ACR with highest level of efficacy (93%) being recorded in Butimba and the lowest (76.4%) in Mkuzi. Fifty-five (14.4%) cases did not respond to SP treatment of which 37 (9.7%) and 18 (4.7%) were ETF and LTF cases, respectively (Table 1). There was no significant difference in the prevalence of SP treatment failure in Butimba, Kyela, Masasi, and Mlimba ($\chi^2 = 2.52$, degree of freedom [df] = 3, $P = 0.4723$), but significant difference was observed ($\chi^2 = 15.06$, df = 4, $P = 0.0046$) when the Mkuzi Health Center was included in the analysis. Table 2 relates the clinical and molecular data for the SP-treated patients. Of 55 treatment failure cases 12 (22%) and 43 (78%) carried parasites with quintuple and non-quintuple genotypes, respectively. Of the 328 patients who showed ACR, 39 (12%) and 289 (88%) individuals harbored the quintuple and non-quintuple (any other combination of genotypes apart from quintuple) genotypes, respectively. Statistical analysis showed association between the quintuple mutant genotype and SP treatment failure (OR = 2.1, 95% CI = 0.94–4.48, $P = 0.045$). Although the lower 95% CI was slightly less than 1, a Pearson chi-square test ($\chi^2 = 4.0$) indicated that this represented a statistically significant association (Table 2). In a separate analysis, the triple *pfdhfr* mutant and the double *pfdhps* mutant genotype did not show a predictive value for SP treatment failure.

Prevalence of multiple *pfdhfr* and *pfdhps* mutant genotypes and estimated GFI values. The prevalence of SP resistance and *pfdhfr* and *pfdhps* genotypes is summarized and shown in Figure 1. Mkuzi showed highest prevalence of triple *pfdhfr* (80.3%) and double *pfdhps* (32.3%) mutant genotypes, while

TABLE 1
Summary of sulfadoxine-pyrimethamine treatment outcome in the five sentinel sites in Tanzania*

Site	No.	ETF	LTF	Overall TF	ACR
Butimba	57	4	0	4 (7%)	53 (93%)
Kyela	67	5	3	8 (12%)	59 (88%)
Masasi	73	4	1	5 (6.8%)	68 (92.9%)
Mkuzi	127	21	9	30 (23.6%)	97 (76.4%)
Mlimba	59	3	5	8 (13.5%)	51 (86.5%)
Total	383	37 (9.7%)	18 (4.7%)	55 (14.4%)	328 (85.6%)

* ETF = early treatment failure; LTF = late treatment failure; TF = treatment failure; ACR = adequate clinical response.

TABLE 2

Assessment of association between *pfdhfr* and *pfdhps* genotypes and treatment outcome*

Genotype	TF	ACR	OR (95% CI)	Chi-square	*P*
Quintuple mutants	12	39			
Non-quintuple	43	289	2.1 (0.94–4.48)	4.0	0.045
Total	55	328			

* *pfdhfr* = *Plasmodium falciparum* dihydrofolate reductase; *pfdhps* = *P. faciparum* dihydropteroate synthase. TF = Treatment failure; ACR = adequate clinical response; OR = odds ratio; CI = confidence interval, quintuple = *pfdhfr* 108 Asn, 51 Ile, 59 Arg and *pfdhps* 437 Gly and 540 Glu.

Mlimba showed the lowest prevalences of 18.6% and 3.4%, respectively. There was no difference in the prevalence of the triple *pfdhfr* mutant genotype ($\chi^2 = 0.12$, df = 3, $P = 0.9893$) at the Butimba, Kyela, and Masasi, and Mlimba sites. However, a significant difference ($\chi^2 = 131$, df = 4, $P = 0.000001$) are observed when the Mkuzi site was included in the analysis. Conversely, the prevalence of the double *pfdhps* mutant genotype was significantly different ($\chi^2 = 12$, df = 3, $P = 0.0074$) at the Butimba, Kyela Masasi, and Mlimba sites and more so ($\chi^2 = 39$, df = 4, $P = 0.00012$) when Mkuzi was included in the analysis. Similarly, the prevalence of pure wild *pfdhfr* and *pfdhps* genotypes was different among the low resistance sites ($\chi^2 = 12.3$, df = 3, $P = 0.006345$ and $\chi^2 = 49.4$, df = 3, $P = 0.00011$, respectively). Using the prevalence of different combinations of mutations in *pfdhfr and pfdhps* as a marker for SP resistance, we calculated the GFI and observed that only the triple *pfdhfr* mutant genotype generated invariable indices (ranging from 1.96 to 2.1) in moderate resistance areas (Butimba, Kyela, Masasi, and Mlimba), suggesting a relationship between the marker and SP treatment failure. The GFI observed in Mkuzi (a high resistance area) was 3.4, which was different from that observed in other sites. Indices derived by other markers (combination of triple and double *pfdhfr* or double *pfdhps* mutant genotypes) are highly variable (Table 3) and do not suggest any relationship with treatment failure. We did not detect *pfdhps* 436 Ala/Phe, 581 Gly, and 613 Thr/Ser and *pfdhfr* 164 Leu mutations in any of our study sites.

DISCUSSION

In 2001, Tanzania-mainland adopted SP as an interim, first-line antimalarial drug. As a preparation for this policy change, we conducted studies to establish the baseline SP efficacy and prevalence of SP resistance molecular markers (*pfdhfr* and *pfdhps* mutations) in this country. We have established that SP was effective against uncomplicated malaria when the mainland of Tanzania revised its malaria treatment policy.

TABLE 3

GFIs calculated by using different combinations of mutations in *pfdhfr* and *pfdhps* as markers of SP resistance in five sentinel sites in Tanzania*

Site	Overall TF (%)	GFI~Triple dhfr~	GFI~Triple + Double dhfr~	GFI~Double dhps~
Butimba	7	2.1	9.64	2.76
Kyela	12	1.99	5.3	1
Masasi	6.8	1.98	5.7	0.6
Mkuzi	23.6	3.4	4.4	1.37
Mlimba	13.5	1.96	6.4	0.25

* TF = Treatment failure; GFI = genotype failure index (subscripts are markers used to calculate the GFI). For definitions of other abbreviations, see Tables 1 and 2.

The ACR ranged from 76.4% to 93% (average efficacy = 85.6%), which was similar to efficacies reported in other southern and eastern Africa countries (82–98%) between 1997 and 2002.[2–8] In our trials, Mkuzi showed highest levels of overall SP resistance (23.6%) followed by Mlimba (13.5%), Kyela (12%), Butimba (7%), and Masasi (6.8%), with average resistance being 14.4%. According to the criteria for changing malaria treatment policy,[33,34] by 2000 resistance in all sentinel sites had gone beyond the grace period (combined ETF and LTF < 5%) and all except Mkuzi were within the alert period (combined ETF and LTF between 6% and 15%). It is interesting to note that before the policy change, SP resistance in Mkuzi was already in the action period (combined ETF and LTF between 16% and 24%). In 2001, the SP parasitological failure rate by day 28 in the Muheza District (in which the Mkuzi site is located) was 45%.[13] So far this is the area with the highest SP resistance in Tanzania. The prevalent SP resistance observed in these studies is attributable to the country-wide use of SP in the last several years as a second-line antimalarial drug. However, the higher prevalence in Mkuzi is also due to the use of pyrimethamine for prophylactic and/or therapeutic trials at different periods from 1950s to 1994[10–12] and to the use of SP since 1984 for first-line treatment in children less than five years of age at the Muheza District Hospital.[13] Thus, with the deployment of SP for country-wide use, it is obvious that resistance will increase rapidly. Therefore, our findings support the decision to adopt SP as an interim, first-line, antimalarial drug, while some combination antimalarials are being evaluated for long-term use. High levels of SP resistance have also been observed in the neighboring countries of Burundi and Rwanda, with failure rates beyond the critical 25% value in most sentinel sites. These countries have already switched to SP/AQ and AQ/AS, respectively, as their first-line antimalarial drugs.[2]

In this study, the quintuple mutant genotype was associated (OR = 2.1) with SP treatment failure by day 14. In previous studies in Uganda[23] and Malawi,[24] stronger associations (OR > 10) between treatment failure and *pfdhfr* 59 Arg and *pfdhps* 540 Glu mutations (the quintuple mutant predictors) were observed. The smaller OR value observed in our study is partly attributable to a shorter (14 days) follow-up period used in this study. The majority of the SP treatment failure cases are known to occur beyond day 14. Therefore, extended follow-ups with subsequent distinction of recrudescence by genotyping would have provided more reliable interpretation of treatment response[35] and improved the association.[23] In addition, inclusion of *in vitro* data would have been of paramount importance in elucidating the reason for the smaller OR value and providing a wider SP efficacy baseline data for Tanzania.

Our study has established that the prevalence of the triple *pfdhfr* mutant genotype was four times higher in an area of high SP resistance compared with areas of moderate SP resistance. This observation clearly suggests a relationship between the marker and SP resistance, and points to the potential of this genotype in the development of a reliable early warning tool for escalating SP resistance in Tanzania. The GFI calculated by using this marker also varied between high (3.4) and moderate SP resistance (1.96–2.1) sites. Nonetheless, values observed in the later sites are invariable and comparable with those observed using *pfdhfr* 59 Arg and

pfdhps 540 Glu genotypes as markers for SP resistance in Uganda (1.9)[23] and Malawi (2.2).[24] The GFIs observed in our study imply that the prevalence of the triple *pfdhfr* mutant genotype was 3.4 and 2 times higher than treatment failure rates in high and moderate SP resistance sites, respectively. It should be noted that the deviation observed in the former site might limit the applicability of GFI-based models as tools for monitoring SP resistance. This deviation may partly imply that during the genesis of SP resistance, a plateau/saturation point may be reached beyond which a further increase in the prevalence of the triple *pfdhfr* mutant genotype produces only a marginal decrease in *in vivo* SP treatment failure. It has also been argued that differences in prevalence of the triple *pfdhfr* mutant genotype among sites might only reflect the duration and magnitude of SP use and not differences in SP resistance.[36] Therefore, to better understand the relationship between mutations and treatment failure, we recommend multi-sites community and health facility longitudinal studies to be carried out. These studies should focus on exploring the use of mutant genotypes in estimating resistance, rather than predicting individual treatment failures. In addition, they should be designed so as to allow controlling for confounders such as age and parasite densities. The GFIs generated by using other combinations of *pfdhfr* and *pfdhps* mutations were highly variable. Thus, the role of these markers in monitoring SP resistance (in the GFI context) needs further investigation. In this study, the moderate resistance sites showed no significant difference in the prevalence of the triple *pfdhfr* mutant genotype, contrary to a previous report in which sites with small differences in SP resistance showed major differences in *pfdhfr* genotypes.[36] Instead, these sites showed marked differences in the prevalence of double *pfdhps* mutant genotypes. A high prevalence of triple *pfdhfr* and double *pfdhps* point mutations haplotypes has also been observed in northern Tanzania with significant interregional heterogeneity in allele frequency.[37]

The *pfdhfr* 164 Leu mutation has been detected in the Muheza District in Tanzania, an area with high SP resistance, by using yeast expression assays.[38] However, this mutation was not detected by the standard PCR-RFLP and/or sequencing methods in the present and other studies conducted in the same[39] or other parts of Africa.[40,41] Therefore, it has been suggested that the *pfdhfr* 164 Leu mutant allele detected in Tanzania represents only the normal baseline and nonfunctional mutations of the *pfdhfr* gene that occur naturally during DNA replication.[39,40] Similar to another report from Malawi,[41] we did not detect the *pfdhps* 436 Ala/Phe, 581 Gly, or 613 Thr/Ser mutations at any of our study sites. All of these mutations that have not been detected in Africa are prevalent in southeast Asia and South America.[42] Interestingly, however, the *pfdhps* 436 mutation was detected in Kibaha, Tanzania.[37] Studies on polymorphic microsatellite repeats in the flanking regions of the *pfdhfr* and *pfdhps* genes in southern Africa and southeast Asia suggest gene flow/selective sweep rather than new mutations as the most likely means by which SP resistance spreads.[43,44] Therefore, the absence of these alleles in areas such as Muheza, where antifolate antimalarials have been used for a long time and resistance is high, suggests that the southern African and southeast Asian parasite populations may have different evolutionary origins. This hypothesis is yet to be verified. However, there will always be a necessity to constantly monitor for parasites carrying the *pfdhfr* 164 Leu alleles in sub-Saharan Africa because its appearance, through importation or otherwise, and subsequent spread would compromise the useful therapeutic life (UTL) of other alternative antimalarial drugs such as chlorproguanil-dapsone.

These findings constitute the baseline data on SP efficacy and prevalence of *pfdhfr* and *pfdhps* genotypes in Tanzania. The clinical and molecular information gained from these studies signal that SP may have a short UTL in Tanzania, the basis for adoption of SP as an interim, first-line antimalarial drug. Thus, there is a need to advocate for rational use of the drug and conduct regular surveillance to monitor resistance concurrent with accelerated evaluation of different alternative treatments, especially combination antimalarial therapies. These data provide preliminary evidence suggesting that the triple *pfdhfr* mutant genotype may form a suitable early warning tool for increasing SP resistance in Tanzania. Further studies need to be done, at both community and health facility levels, to verify the usefulness of *pfdhfr* and *pfdhps* genotypes in estimating SP resistance.

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5 Chapter 5

5.1 Efficacy of sulfadoxine-pyrimethamine in Tanzania after two years as first-line drug for uncomplicated malaria: assessment protocol and implication for treatment policy strategies

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Research

Efficacy of sulfadoxine-pyrimethamine in Tanzania after two years as first-line drug for uncomplicated malaria: assessment protocol and implication for treatment policy strategies

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Abstract

Background: Systematic surveillance for resistant malaria shows high level of resistance of *Plasmodium falciparum* to sulfadoxine-pyrimethamine (SP) across eastern and southern parts of Africa. This study assessed *in vivo* SP efficacy after two years of use as an interim first-line drug in Tanzania, and determined the rates of treatment failures obtained after 14 and 28 days of follow-up.

Methods: The study was conducted in the Ipinda, Mlimba and Mkuranga health facilities in Tanzania. Children aged 6–59 months presenting with raised temperature associated exclusively with *P. falciparum* (1,000–100,000 parasites per μl) were treated with standard dose of SP. Treatment responses were classified according to the World Health Organization (WHO) definition as Adequate Clinical and Parasitological Response (ACPR), Early Treatment Failure (ETF), Late Clinical Failure (LCF) and Late Parasitological Failure (LPF) on day 14 and day 28.

Results: Overall 196 (85.2%) of 230 patients had ACPR on day 14 but only 116 (50.9%) on day 28 (57.7% after excluding new infections by parasite genotyping). Altogether 21 (9.1%) and 13 (5.7%) of the 230 patients assessed up to day 14 and 39 (17.1%) and 55 (24.1%) of the 228 followed up to day 28 had clinical and parasitological failure, respectively.

Conclusion: These findings indicate that SP has low therapeutic value in Tanzania. The recommendation of changing first line treatment to artemether + lumefantrine combination therapy from early next year is, therefore, highly justified. These findings further stress that, for long half-life drugs such as SP, establishment of cut-off points for policy change in high transmission areas should consider both clinical and parasitological responses beyond day 14.

Background

There is controversy over the therapeutic life of sulfadoxine-pyrimethamine (SP) when used alone for the treatment of uncomplicated malaria in Africa. Experts do not all agree on which drug efficacy measurements more accurately predict usefulness of a drug in a community. Some

Table 1: Mean age, temperature, haemoglobin and parasite density on admission day

Site	Means				
	Weight in kg(SD)[1]	Age in years (SD)[1]	Temperature in °C (SD)[1]	Hb[1] in g/dl (SD)[1]	parasites/μl (SD)
Ipinda (n = 73)	10.7(2.9)	1.7(1.3)	38.4(1.0)	9.4(1.6)	31'499 (30,260)
Mlimba (n = 75)	11.0(2.9)	1.7(1.4)	38.3(0.9)	9.0(1.6)	53,206(30,782)
Mkuranga (n = 93)	10.9(3.7)	1.3(1.0)	38.7(0.8)	8.6(1.9)	44,877(38,572)

Hb = haemoglobin; SD = standard deviation

consider clearance of symptoms alone [1] or plus parasites by day 14, as advised by the World Health Organization [Regional Office for Africa (WHO/AFRO) Consultative Meeting on Antimalarial Policy in the Africa Region, 14th–15th August 2003, Harare, Zimbabwe]. Others regard clearance of both symptoms and parasites over a much longer period as the most accurate measure of drug effectiveness [2-4].

The assessment methodology has profound implications in terms of treatment policy strategies. Attempts have been made to define the cut-off points for changing first-line malaria treatment. Using the old treatment response classification criteria, the action period was due when a combined Early Treatment Failure (ETF) and Late Treatment Failure (LTF) were between 16 – 24% [4,5]. A couple of years ago, WHO/AFRO recommended 15% clinical and 25% parasitological treatment failure rates at day 14 as cut-off points for implementation of policy change in intense transmission areas (WHO/AFRO consultative meeting on antimalarial policy in the Africa region, 14th–15th August 2003, Harare, Zimbabwe).

Systematic surveillance for efficacy of antimalarial drugs shows increasing levels of *Plasmodium falciparum* resistance to SP across eastern and southern parts of Africa [6,7]. In 2001, Tanzania adopted SP as interim first-line treatment for uncomplicated malaria while awaiting for the results of different combination therapies trials. As part of the National Malaria Control Programme (NMCP), this study assessed *in vivo* SP efficacy after two years of widespread use in Tanzania.

Methodology

The study was conducted from July to November 2003 in the Ipinda (south-west), Mlimba (south-east) and Mkuranga (east) health facilities in Tanzania. Malaria transmission in these areas is perennial with peaks between May and July. A slightly modified WHO antimalarial drug efficacy testing protocol [8] was used, so as to conform with another study that was being conducted at the same time under the same project framework in Papua New Guinea, in areas with lower levels of endemicity.

Children aged 6–59 months presenting with raised temperature (37.5°C–39.5°C) associated with *P. falciparum* parasitaemia between 1,000–100,000 parasites per μl were recruited. Exclusion criteria and other procedures were as detailed in the protocol [8]. Patients were treated (under observation) with a standard dose of SP (Fansidar® Roche), i.e 1.25 mg/kg of pyrimethamine and 25 mg/kg of sulfadoxine. The responses were classified according to the new WHO definition as ACPR, ETF, LCF and LPF at day 14 and day 28 [8].

Treatment failures rates were corrected after genotyping the *msp2* locus to detect new infections. Extensive diversity in this locus has been observed with over 84 allelic variants in south-eastern Tanzania [9,10] and other investigators observed high genotype complexity with an average of 4.9 genotypes per asymptomatic individual in eastern Tanzania [11]. These observations are suggestive that *msp2* alone may sufficiently discriminate recrudescence from reinfection in Tanzania. It has previously been shown that analysis of *msp2* locus alone can effectively distinguish recrudescence from reinfection in Uganda [12]. The clinical and molecular data were combined and analysed using Stata version 8.0 (Stata Corporation Inc, Texas, USA).

Results

A total of 241 patients were recruited, of which 13 were lost to follow-up. Table 1 summarizes patient age and clinical parameters recorded on admission day by site. Table 2 provides details of treatment outcome by site. On day 28, only 116 (50.9%) of the 228 patients showed ACPR. Molecular genotyping showed that 27/112 (24.5%) recurrent infections were due to re-infections, therefore were excluded from the analysis and recorded as withdrawn. Hence, PCR-corrected ACPR was 116/201 (57.7%). 196 (85.2%) of the 230 patients had ACPR by day 14.

The total clinical failure by day 14 and 28 was observed in 21 (9.1%) out of 230 and 39 (17.1%) out of 228 patients, respectively. 13 (5.7%) and 55 (24.1%) of the patients had LPF by day 14 and 28, respectively. Thus 34 (14.9%)

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Table 2: Sulfadoxine-pyrimethamine treatment outcomes

Results	no. of included patients	LF and corr.	Evaluable patients	ACPR n (%)	ETF n (%)	LCF n (%)	Total CF n (%)	LPF n (%)	Overall TF n (%)
At D14									
Ipinda	73	1	72	62(86.1)	7(9.7)	2(2.8)	9(12.5)	1(1.4)	10(13.9)
Mlimba	75	3	72	63(87.5)	3(4.3)	4(5.7)	7(9.1)	2(2.9)	9(12.9)
Mkuranga	93	7	86	71(82.6)	3(3.5)	2(2.3)	5(6.9)	10(11.6)	15(17.4)
Total	241	11	230	196(85.2)	13(5.7)	8(3.5)	21(9.1)	13(5.7)	34(14.9)
At D28									
Ipinda	72	0	72	44(61.1)	7(9.7)	5(6.9)	12(16.7)	16(22.2)	28(38.9)
Mlimba	72	2	70	34(48.6)	3(4.3)	9(12.9)	12(17.1)	24(34.3)	36(51.4)
Mkuranga	86	0	86	38(44.2)	3(3.5)	12(13.9)	15(17.4)	33(38.4)	48(55.8)
Total	230	2	228	116(50.9)	13(5.7)	26(11.4)	39(17.1)	55(24.1)	112(49.1)
At D28 PCR-corrected									
Ipinda	72	6	66	44(66.7)	7(9.7)	5(6.9)	12(16.7)	10(13.9)	22(33.3)
Mlimba	70	10	60	34(56.7)	3(4.3)	5(7.1)	8(11.4)	18(25.7)	26(43.3)
Mkuranga	86	11	75	38(50.7)	3(3.5)	8(9.3)	11(12.8)	26(30.2)	37(49.3)
Total	228	27	201	116(57.7)	13(5.7)	18(7.9)	31(13.6)	54(23.7)	85(42.3)

ACPR = Adequate Clinical and Parasitological Response; TF = Treatment failure, ETF = Early Treatment Failure; LCF = Late Clinical Failure; LPF = Late Parasitological Failure; CF = Clinical failure, n = Sample Size; TF = Treatment failure, FP = Lost to Follow-up, corr. = corrected

out of 230 and 112 (49.1%) out of 228 patients had overall treatment failure by day 14 and 28, respectively. After genotyping recurrent infections day-28 treatment failures decreased to 85 (42.3%). In this study Mkuranga recorded the highest rates of both PCR-adjusted and unadjusted treatment failures followed by Mlimba and Ipinda.

Discussion

In 2001 Tanzania replaced chloroquine with SP as interim first-line antimalarial drug. Prior to this change, baseline clinical trials with SP had been conducted throughout the country using the 14 day follow-up protocol, and indicated an average efficacy of 86% on day 14. These findings paved the way for the malaria treatment policy change [13] and SP is still used as first line antimalarial drug in Tanzania. The present study assessed SP efficacy (using a 28 day follow-up) in three sites in Tanzania after two years of use as first-line antimalarial drug. With this extended period of follow-up, half of the patients (49.1%) failed treatment. Even when new infections were taken into account by genotyping, the overall treatment failure rate (42.3%) was still high. This level of resistance is close to that observed in Muheza (45%), an area of high SP resistance in Tanzania [14].

Restricting our analysis to outcomes at day 14 would have led to misleadingly low clinical (9.1%) and parasitological (5.7%) treatment failure rates with the overall treatment failure (14.9%) being equal to that recorded at baseline prior to policy change. Using a shorter follow-up period, another study in parts of Tanzania also recorded an overall SP treatment failure of only 9.2% [15]. According to WHO/AFRO proposed thresholds for policy change (i.e 15% and 25%, respectively), these failure levels would

still be considered acceptable. Retention of SP clinical efficacy at day 14 after 10 years of its use as first-line drug has been demonstrated in Malawi [1]. However, extending the follow-up to day 28 the total failure was as high as 66%. Even at day 14 the clinical and total failure rates were far above 15% and 25%, respectively. As in Malawi, the majority of the recurrent infections in our study were LPFs observed between day 14 and 28. The new WHO efficacy testing protocol [8] recommends follow up for 28 days for drug with long half-life such as SP, if genotyping can be done to distinguish recrudescence from re-infections. When efficacy assessment is based only on clearance of symptoms in the first 14 days, the level of parasite resistance can be grossly underestimated. It is widely accepted that clearance of both parasitaemia and symptoms is the most accurate measure of the intrinsic resistance of the parasite to a drug [2-4]. The resistant parasite that is apparently causing asymptomatic infection in LPF is likely to lead in the short-term to a new clinical episode [14] and/or to anaemia, depending on the immunity of the subject.

Our observations show that SP efficacy in Tanzania is compromised and fully justify the recent decision to review the current malaria treatment policy from early next year in favour of artemether + lumefantrine combination therapy. This recommendation should be implemented at a large scale as soon as possible. Such a change would be welcome to protect the use of SP in its indication for the intermittent preventive treatment of pregnant women (IPTp). Indeed, it is at present the only drug that can be used for IPTp purpose because of its good safety profile. As far as methodology is concerned, the findings stress that cut-off points for malaria treatment policy

change in high transmission areas should consider both clinical and parasitological responses beyond day 14, coupled with distinction of recrudescence from re-infection using molecular genotyping.

Authors' contributions

B. Genton, H-P. Beck and K. Mugittu designed the study. S. Abdulla and K. Mugittu organised the clinical work. H. Mshinda was the supervisor in Tanzania. N. Falk, I. Felger, H-P. Beck performed molecular genotyping. H. Masanja carried out the statistical analysis. K. Mugittu and B. Genton wrote the article and all others contributed to it.

Ethical approval

The study was approved by the Tanzanian national and institutional ethics bodies

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6 Chapter 6

6.1 Community-based assessment of antimalarial drug resistance by generating a resistance genogram by microarray technique

This paper has been prepared for submission in *American Journal of Tropical Medicine and Hygiene*.

Community-based assessment of antimalarial drug resistance by generating a resistance genogram by microarray technique

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Abstract

Background

Single nucleotide polymorphisms (SNPs) in 5 *P falciparum* genes has been associated with resistance of the parasite to antimalarial drugs. These SNPs are considered as molecular markers of drug resistance that may be useful for assessing the dynamics of antimalarial drug resistance. However, their role in *in vivo* resistance remains unclear and controversial. We evaluated the usefulness of a novel high throughput DNA microarray in a community-based approach for monitoring resistance.

Methodology

In 2003 and 2004, we assessed SP efficacy against uncomplicated pediatric *P. falciparum* malaria in Ipinda, Mlimba and Mkuranga health facilities in Tanzania using the WHO 2002 protocol. Concurrently, we assessed asymptomatic infections in 1202 individuals of all age groups in the health facilities catchment areas. Using a newly developed DNA

microarray technique, we determined SNPs in *P. falciparum* resistance-associated genes (*dhfr* and *dhps, mdr1, crt* and ATPase6), and estimated the frequencies of mutant alleles, taking into account the multiplicity of infection (MOI) in each person. We further assessed whether the frequency of these molecular markers in communities can be used to monitor the dynamics of resistance.

Results

The overall Day 28 PCR-adjusted SP treatment failure in 2003 (42.3%) and 2004 (41%) were high and not significantly different (p > 0.05) with and The frequencies of individual *Pfdhfr* mutant alleles were high and varied in the communities and did not reflect SP failure rates at health facilities. The prevalence of triple-*Pfdhfr* genotype was also not significantly different between 2003 and 2004. Ratios of this genotype and treatment failure were relatively stable. The frequency of *Pfcrt* 76 Thr mutant allele decreased in Ipinda and Mkuranga, but increased significantly in Mlimba (0.47 to 0.88), which is coherent with a significant increase in the frequency of *Pfmdr1* 86 Tyr allele also observed in Mlimba. No mutations in at position 769 and 623 in the ATPase6 gene were detected.

Conclusion

The triple-*Pfdhfr* mutant genotype may be useful in community based monitoring of dynamics of resistance to SP but requires further assessment including areas with different treatment failure rates. The frequencies of *Pfcrt* 76 Thr and *Pfmdr1* 86 Tyr alleles has decreased following suspension of Chloroquine (CQ) use but seems to be influenced by the pattern of quinine (QN) or amodiaquine (AQ) usage in the sites. The lack of ATPase6 mutant alleles suggest that artemisinin resistance is yet to be selected in Tanzania. The DNA microarray technology may provide a suitable system for simultaneously assessing, on a large scale, the spread of resistance to newly adopted artemisinin-based combination therapies (ACTs) and reversal of CQ and SP resistance after their suspension in Sub-Saharan Africa. However, the interval between assessments must be long enough in order to appreciate parasite genotypic dynamics following alteration of drug pressure.

Introduction

Emergence and subsequent spread of *P. falciparum* to resistance to the cheap and safe antimalarials such as sulfadoxine-pyrimethamine (SP) and chloroquine (CQ) hinders effective malaria control in Sub-Saharan Africa. Hence ACTs including artemether-lumefantrine, artesunate + amodiaquine and artesunate + mefloquine are now advocated to replace these monotherapies in the region (WHO, 2006). Currently, the deployment of ACT is underway in 15 Sub-Saharan countries out of 43 listed by WHO-AFRO whereas 15 countries have already adopted the policy of AS-AQ as first-line (WHO, 2005). Tanzania is also in the process of revising malaria treatment policies to adopt ACTs (NMCP, unpublished data). Thus there is a need for regional-wide, standardized high throughput methods that can simultaneously monitor emergence and spread of resistance to the newly adopted ACTs and decreasing resistance to withdrawn drugs, with a view to assess the possibility of recycling the limited number of safe and cheap drugs as combination partners with artemisinins (Laufer and Plowe, 2004).

Antimalarial drug efficacy is classically assessed by using the standard WHO *in vivo* and *in vitro* tests. These methods can be supplemented by assessment of molecular markers of antimalarial drug resistance. Molecular biological research has advanced our understanding of modes of drug action and mechanism of *P. falciparum* resistance to antiumalarials. It is now known that antifolates (pyrimethamine, sulfadoxine. Proguanil, chlorproguanil and trimethoprim) act by inhibiting either the DHFR or DHPS enzymes in the folate biosynthesis pathway and resistance to these drugs is mediated by SNPs in the *Pfdhfr* codons Ala 16 Val, Asn 51 Ile, Cys 59 Arg, Ser 108 Asn/Thr and Ile 164 Leu (Cowman *et al.* 1988; Reeder *et al.* 1996) and *Pfdhps* codons Ser 436 Phe, Ala 437 Gly, Lys 540 Glu, Ala 581 Gly and Ala 613 Thr/Ser (Triglia *et al.* 1997).

On the other hand, the modes of action and mechanisms of resistance to quinolines and artemisinins are still largely unclear. A detailed review of quinoline drug action (Fitch, 2004) shows that 4-aminoquinolines (e.g. CQ) act by interfering with heme detoxification in the parasites digestive vacuoles whereas quinoline-4-methanols [quinine (QN) and

mefloquine (MQ) and lumefantrine (LM)] antagonizes the CQ-induced morphological changes in susceptible parasites by inhibiting and reversing vesicular docking in endolysosomal system, therefore, kill parasites by secondarily inhibiting membrane recycling. QN and MQ are also considered to inhibit hemoglobin ingestion (Fitch 2004). Two membrane transport proteins *P. falciparum* chloroquine resistance transporter (PfCRT) and Glycoprotein homologue 1 (Pgh1) encoded by *Pfcrt* and *Pfmdr1* genes, alters drug accumulation and pH in the digestive vacuole. Both proteins influence quinolines' antimalarial activity in a variety of ways. Therefore, SNPs in the *Pfcrt* codons Cys 72 Ser, Met 74 Ile, Asn 75 Glu, Lys 76 Thr, 152, 163 Ala 220 Ser, Gln 271 Glu and Asn 326 Ser, Ile 356 Thr and Arg 371 Ile (Fidock *et al.* 2000) and *Pfmdr1* codons Ser 86 Asn/Thr, Tyr 184 Phe, Ser 1034 Cys, Asn 1042 Asp and Asp 1246 Tyr (Foote *et al.* 1990) mediate resistance to CQ whereas *Pfmdr1* copy number amplification confers resistance to mefloquine and probably atemisinins (Cowman *et al.* 2002; Duraisingh *et al.* 2000; Price *et al.* 1999; Price *et al.* 2004). In contrary, mutations in the *Pfcrt* (Sidhu *et al.* 2002) and *Pfmdr1* (Bray *et al.* 2005; Sisowath *et al.* 2005) are associated with increased susceptibility to QN and MQ. On the other hand, artemisinins interact and inhibit malarial parasite's calcium ATPase (sarcoplasmic endoplasmic reticulum calcium ATPase (SERCA) (Eckstein-Ludwig *et al.* 2003; Uhlemann *et al.* 2005). *In vitro* studies showed that resistant *P. falciparum* share the ATPase6 Ser 769 Asn, Ala 623 Glu and Glu 431 Lys (Jambou *et al.* 2005).

Therefore, the mutations in *Pfdhfr*, *Pfdhps*, *Pfcrt*, *Pfmdr1* and *PfATPase6* are recommended for use as early warning signals for emergence and/or spread of resistance to antimalarial drugs. However, reports on their roles in *in vivo* resistance have been inconsistent. Some studies (Kublin *et al.* 2002; Kyabayinze *et al.* 2003) established strong association while others (Francis *et al.* 2006; Aubouy *et al.* 2003) didn't. This inconsistence could partly be brought about by restricting studies to small numbers of symptomatic children < 5 years attending health facilities for treatment. The asymptomatic infections circulating in the community (parasite reservoir) has frequently been ignored but may play an important role in the spread of antimalarial drug resistance. More importantly, assessment of the role of mutations in resistance is complicated by the

fact that most malaria infections in high transmission areas are multiclonal. Thus in a mixed (wild-type and mutant) infection, it is difficult to determine the frequency of mutant allele or whether the observed multiple mutations are linked.

In this study we used a novel high throughput DNA microarray technique to detect many reported SNPs in *Pfdhfr, Pfdhps*, *Pfmdr1*, *Pfcrt* and *PfATPase6* (resistance genogram) in the asymptomatic infections and estimate the frequency of mutant alleles considering the multiplicity of infection (MOI). Finally we assessed whether the profile of *Pfdhfr* and *Pfdhps* mutant genotype in the community (catchment area) may reflect SP treatment failure rates in respective health facilities. In addition, we compared the current levels of *Pfcrt* and *Pfmdr1* mutations to those recorded in our previous studies prior to CQ suspension in Tanzania to assess whether suspension of CQ has resulted into decline of CQ resistance markers.

Methodology

Study areas and design

The study was conducted between the end of May and November 2003 and 2004 in the Ipinda (south-west), Mlimba (south-east) and Mkuranga (east) health facilities in Tanzania. According to the Tanzania national census conducted in 2002 the populations of Ipinda, Mlimba and Mkuranga were 16,756, 32875 and 26,551 people, respectively, of more or less similar socioeconomic status. Malaria transmission in these areas is perennial with peaks between May and July. The study comprised of SP efficacy testing in the NMCP framework in the three the health facilities in parallel with assessment of the prevalence of drug resistance markers in parasite populations circulating as asymptomatic infection in catchment areas.

Assessment of SP efficacy

We used a slightly modified (minimum parasitaemia 1000 instead of 2000 parasites per µl) WHO antimalarial drug efficacy testing protocol for areas of intense transmission. This modification was made to conform to, and allow data comparison with, another

study that was being conducted at the same time under the same project framework in Papua New Guinea, in lower transmission areas. Children aged 6-59 months presenting with raised temperature (37.5°C - 39.5°C) associated with *P. falciparum* parasitaemia between 1,000 - 100,000 parasites per µl were recruited. Exclusion criteria and other procedures were as detailed in the WHO protocol. Patients were treated (under observation) with a standard dose of SP (Fansidar®, Roche), i.e. 1.25mg/kg of pyrimethamine and 25mg/kg of sulfadoxine. Patients were followed up for 28 days to assess response. The responses were classified according to the new WHO definition as Adequate Clinical and Parasitological Response (ACPR), Early Treatment Failure (ETF), Late Clinical Failure (LCF) and late Parasitological Failure (LPF) at day 14 and day 28 (WHO 2002). Treatment failures rates were corrected after genotyping the *msp2* locus in Day 0 and recurrent Day samples using a genescan-based technique as described (Falk *et al.* 2006). These studies obtained ethical clearance from both institutional and national bodies. Prior to recruitment of a study subject in clinical study informed consent was obtained from parent or guardian of each child.

Assessment of antimalarial resistance markers in the catchment areas

Due to logistic reasons community surveys in 2003 were delayed and conducted between August and mid October, outside the peak transmission. These community-based cross-sectional surveys were conducted in health-facilities' catchment areas. A total of 1202 individuals from randomly selected households were recruited.

From finger prick blood, thick and thin smears were made and approximately 500µl of blood was collected into an EDTA microtainer®. Smears were examined under the microscopy and parasitaemia reported as number of parasite per 200 WBC or gametocyte per 500 WBC. All 1202 individuals were also screened for *P. falciparum* infection by msp2 PCR amplification. The blood in the EDTA microtainer® tubes was further allowed to settle for one hour. Plasma was discarded and the remaining pellets dissolved into 2 volumes of 6M GCT (GuHCl in Tris pH 8, 2 mM EDTA) and SDS added to a final conc. of 0.5%. The tubes were mix well by gentle shaking and stored at room temperature

for up to 2 days or at -20ºC until DNA extraction. Before recruitment of the subjects, consent was obtained from community leaders, heads of household as well as individual participants or guardians (in case of younger children).

Extraction of DNA, PCR amplification and Detection of SNPs by microarray

200 µl of the GCT-treated blood was used for DNA extraction by the conventional phenol-chloroform method. The DNA pellet was dissolved in 50 µl TE and kept at -20oC until required. P. falciparum DNA from all msp2 positive samples was amplified separately using a set of ten resistance gene-specific primers. Both primary and nested PCR amplifications were done in 96-well plates in 50 and 100µl reaction volumes, respectively. 10µl of each the 10 nested PCR amplifications were pooled into one new 96 well plate (pool 1), briefly vortexed and centrifuged. 10µl of each of the 96 wells in pool 1 were transferred into a new 96 well plate and diluted 1:10 with sterile distilled water (pool 2), briefly vortexed and centrifuged. 5µl of each of the wells in pool 2 were transferred in duplicate into a new 96-well plate (each plate then contained 48 patients) and SAP digested in 12µl reaction volumes, containing 1x shrimp alkaline phosphatase (SAP) buffer, 2 units of SAP and the 5 µl of the diluted PCR product by incubating the mixture at 37 ^{o}C. After 1 hour SAP was heat inactivated by heating at 90 ^{o}C for 15 minutes.

Single base primer extension reactions were carried out using 20-30bp locus-specific extension primers, thermo sequenase and cy3- or cy5-labeled ddNTPs. The extension primers are designed such that the last bases on their 3'end preceeds a polymorphic site. The extended primers (whose last bases on their 3' ends are now labeled with a fluorochrome) were denatured by heating at 96ºC for 5 minutes and hybridized at 52ºC for 90 minutes to specific probe anchored onto aldehyde-coated microscopic slide. The slides were washed thrice in saline-sodium citrate (SSC) and sodium dodecyl sulfate (SDS), dried, scanned by GMS 418 scanner and arrays analyzed by Genepix to detect SNPs. The PCR amplification and detection of SNPs by DNA microarray technique are described in details elsewhere (Crameri *et al.* unpublished data).

Table 1 SP treatment Outcome in 2004 *in vivo* studies

Results	n	LF & corr	Evaluable patients	Treatment outcome					
				ACPR (n%)	ETF (n%)	LCF (n%)	Total CF (n%)	LPF (n%)	Overall TF (n%)
At Day 14									
Ipinda	75	1	74	68(91.9)	4(5.4)	0(o)	4(5.4)	2(2.7)	6(8.1)
Mlimba	81	3	78	63(80.8)	2(2.6)	7(9)	9(11.5)	6(7.7)	15(19.2)
Mkuranga	75	0	75	65(88.7)	1(1.3)	4(5.3)	5(6.7)	5(6.7)	10(13.3)
Total	231	4	227	196(86.3)	7(3.1)	11(4.8)	18(7.9)	13(5.7)	31(13.7)
At Day 28: uncorrected									
Ipinda	74	0	74	50(67.6)	4(5.4)	9(12.2)	13(17.6)	11(14.9)	24(32.4)
Mlimba	78	0	78	39(50)	2(2.7)	17(21.8)	19(24.4)	20(25.6)	39(50)
Mkuranga	75	0	75	39(52)	1(1.3)	21(28)	22(29.3)	14(18.7)	36(42)
Total	227	0	227	128(56.4)	7(3.1)	47(20.7)	54(23.8)	45(19.8)	99(43.6)
At Day 28: PCR corrected									
Ipinda	74	1	73	50(68.5)	4(5.5)	8(11)	12(16.4)	11(15.1)	23(31.5)
Mlimba	78	4	74	39(52.7)	2(2.7)	14(18.9)	16(21.6)	19(25.7)	35(47.3)
Mkuranga	75	5	70	39(55.7)	1(1.4)	19(27.1)	20(28.6)	11(15.7)	31(44.3)
Total	227	10	217	128(58.9)	7(3.2)	41(18.9)	48(22.1)	41(18.9)	89(41)

ACPR = Adequate Clinical and Parasitoloical Response; TF = Treatment failure, ETF = Early Treatment Failure, LCF = Late Clinical Failure; LPF = Late Parasitoloical Failure; CF = Clinical failure, n = Sample Size; TF = Treatment Failure, LF = Lost to Follow-up, corr. = corrected

Data analysis

The clinical and community data were analyzed using Stata v 8.0 (Stata Corporation Inc, Texas, USA). A non-linear statistical model was used to estimate the frequency of mutant alleles. The model assumes that resistant and sensitive parasite clones are transmitted independently. Therefore, the likelihood of a sample to contain no resistant clone is $(1 - p)^n$, where p is the frequency for the mutant allele and n is the multiplicity of infection of the sample. Similarly, the likelihood for the sample to contain no wild-type clone is $1 - p - (1 - p)^n$. The likelihood over the whole data set for p is computed as the product of these likelihood over all samples, using values of n derived from the msp2 typing. A Markov Chain Monte Carlo algorithm (Program Winbugs 1.3) was used to obtain estimates and credible intervals (Bayesian confidence intervals (CI) for p, making use of these likelihood, and assuming a uniform (0.1) prior distribution for p (Schneider *et al.* 2002).

Results

SP treatment outcome

The findings of the *in vivo* study conducted in 2003 are detailed elsewhere (Mugittu *et al.* 2005). Briefly, SP treatment failure rates were 34/230 (14.9%) and 112/228 (49.1%) [or 85/201 (42.3%) after PCR-adjustment] at day 14 and 28, respectively. Only 116/201 (57.7%) had ACPR at day 28. The clinical results of the second study conducted in 2004 are detailed in Table 1. Overall SP treatment failure at day 14, and 28 was observed in 31/227 (13.7%) and 99/227 (43.6%) patients. Molecular genotyping of the *msp2* locus distinguished 10 (10%) new infections out of 99 recurrent infections. Thus the overall (Day 28) PCR-corrected treatment failure decreased to 89/217 (41%) and only 128/217 (58.9%) of patients had ACPR. There was no significant intra- or inter-sites difference in crude or PCR-adjusted treatment failure rates observed in 2003 and 2004 (p > 0.05)

Assessment of antimalarial drug use pattern showed that 68/402 (17%), 121/398 (30%) and 24/402 (6%) individual in Ipinda, Mlimba and Mkuranga, respectively, had used either QN or AQ under prescription or self medication for treatment of malaria within 12 months prior to recruitment into the study.

Frequency and pattern of mutant alleles in catchment areas

Table 2 shows that the overall mean age, temperature and MOI was similar but there was a significant difference between the prevalence of asymptomatic infections by msp2 amplification (*p = 0.002*) between 2003 and 2004. 146 samples collected in 2003 were excluded from analysis due to technical problems. These samples were randomly found within the whole data set and are unlikely to be source of biasness in the study. High frequencies of the *Pfdhfr* mutant alleles were found when compared to *Pfdhps* mutant alleles in both years and in all sites (Table 3). The frequencies of *Pfdhfr* 51 Ile, 59 Arg and 108 Asn in Mlimba and Mkuranga and *Pfdhfr* 51 Ile and 108 Asn in Ipinda were significantly higher (confidence intervals do not overlap) in 2004 than 2003. In Ipinda the frequencies of *Pfdhfr* 59 Arg in the 2 years were not significantly different. In contrast the frequencies of *Pfdhps* 437 Gly and 540 Glu mutant alleles were statistically different in Ipinda but not in Mlimba and Mkuranga. The *Pfcrt* 76 Thr mutant allele in 2003 and 2004 significantly decreased in Ipinda (0.71 to 0.49) and Mkuranga (0.62 to 0.49). However, it increased significantly in Mlimba (0.47 to 0.88) coherent with significant increase in *Pfmdr1* 86 Tyr mutant allele (0.43 to 0.72) that did not show any change in the other sites. No clear trend was depicted by the rest of the CQ resistance markers, but their frequencies, especially the *Pfmdr1* alleles were rather low. The *Pfdhfr* 164, *Pfdhfr* 613 and *PfATPase6* 769 Asn, 623 Glu mutant alleles were not observed. SNPs in *Pfdhps* 436, 581, 640 and 645 are not reported because their post-PCR hybridization parameters were not optimsed. SNPs at these positions have not been associated with in vivo drug resistance.

Table 2. Mean age, axilary temperature and parasitological characteristics collected community surveys

Site	2003					2004				
	N	mean age in years (SD)	Temp (SD)	msp2 +ve (%)	MOI (CI)	n	mean age in yeras (SD)	Temp (SD)	msp2 +ve (%)	MOI (CI)
Ipinda	173	21.3 (19.1)	36.6 (0.32)	58 (33.5)	2.49 (2.14,2,84)	200	20 (17.8)	36.3 (0.58	98 (49)	3.25 (2.88, 3.62)
Mlimba	142	16.0 (14.5)	36.6 (0.33)	49 (34.5%)	2.33 (2.02, 2.65)	198	18 (17.3)	36.6 (0.78)	88 (44.4)	2.64 (2.29, 2.98)
Mkuranga	157	21.2 (19.1)	36.6 (0.19)	45 (28.7%)	3.07 (2.69, 3.45)	200	20.7 (20.6)	36.8 (0.47)	98 (49)	2.73 (2.38, 3.07)
Total	472	19.5 (17.9)	36.6 (0.5)	152 (32.2%)	2.65 (2.44, 2.85)	598	19.7 (18.6)	36.6 (0.6)	284 (47.5)	2.88 (2.68, 3.09)

Note: n = sample sizeSD = standard deviation, temp = Temperature, msp2 +ve samples positive by msp2, MOI = multiplicity of infection, CI = confidence interval.

Since most of the infections were multiclonal, we could not establish whether the observed SNPs in *Pfdhfr, Pfdhps, Pfmdr1* and *Pfcrt* were linked. Nonetheless, we have assessed the co-occurrence of mutant alleles per individual samples to give an impression on the magnitude of resistance markers in Tanzania. Figure 1 shows that most individuals in all 3 communities were predominantly (54 – 74%) harboring parasites with either the *Pfdhfr* 51 Ile, 59 Arg and 108 Asn (triple-*Pfdhfr*) alone or in combination with both *Pfdhps* 437 Gly and 540 Glu (quintuple mutant genotype) or *Pfdhps* 437 Gly and 540 Glu, separately. The increase in the prevalence of quintuple mutant genotype, was significant in Ipinda (X^2 = 12.2, p = 0.0005) and Mkuranga (X^2 = 4.6, p = 0.033) but did not seem to influence, at the same level, the SP treatment failure rates observed in respective health facilities. In addition, the ratios of prevalence of quintuple genotype and treatment failure rates varied markedly from 0.4 – 1.4 (results not shown). However, considering the triple-*Pfdhfr* mutant genotype alone, whose prevalence is summarized in Table 4, we did not observe a significant difference between 2003 and 2004 in any site (p > 0.05). This is consistent with the insignificant intra-site differences in SP treatment failure rates. The intra-site ratios of triple-*pfdhfr* to SP treatment failure rates were more stable than the inter-site ones (Table 4). The pure *dhfr/dhps* wild-type genotype observed at low levels (4.7 to 15%) in 2003 was almost absent (1% in Mkuranga only) in 2004.

Discussion

The 2004 *in vivo* study further reaffirms the high SP treatment failure rate already observed in 2003 (Mugittu *et al.* 2005) in Tanzania. The failure rates established in these two years were not significantly different, an observation which was not reflected by the prevalence of individual *Pfdhfr* or *Pfdhps* mutant alleles that increased drastically (by 0.2 to 0.47) in their respective communities. Dramatic increase in the frequency of dhfr 108 Asn allele with the advent of SP usage was also recorded in symptomatic patients in eastern Sudan. In addition, higher frequencies of mutant alleles were seen during the dry season than during the wet season, most likely reflecting seasonal variation in drug pressure and differences in the fitness of resistant and sensitive parasites (Abdel-Muhsin *et al.* 2004).

Table 3. Frequency of mutant alleles estimated by Markov Chain Monte Carlo algorithm by taking into account the multiplicity of infection in each individual person.

Mut allele	Ipinda				Mlimba				Mkuranga			
	2003		2004		2003		2004		2003		2004	
	N	freq (CI)	n	freq (CI)	n	freq (CI)	n	freq (CI)	n	freq (CI)	n	freq (CI)
ATPase6 623	35	0	72	0	31	0	62	0	28	0	60	0
ATPase6 769	22	0	52	0	15	0	62	0	19	0	38	0
Pfcrt 152	29	0.03 (0, 0.07)	66	0	29	0.09 (0.04, 0.18)	51	0	29	0	62	0
Pfcrt 163	29	0.29 (0.19, 0.39)	64	0.17 (0.12, 0.22)	28	0.2 (0.11, 0.31)	60	0.04 (0.01, 0.07)	26	0.24 (0.16, 0.34)	58	0.46 (0.46, 0.47)
Pfcrt 220	25	0.13 (0.06, 0.23)	61	0.21 (0.16, 0.27)	31	0.43 (0.33, 0.5)	60	0.49 (0.46, 0.5)	23	0.12 (0.06, 0.21)	61	0.34 (0.32, 0.37)
Pfcrt 271	28	0.43 (0.32, 0.54)	68	0.31 (0.24, 0.37)	31	0.46 (0.39, 0.5)	62	0.74 (0.67, 0.8)	26	0.31 (0.21, 0.41)	63	0.36 (0.29, 0.43)
Pfcrt 326	25	0.16 (0.08, 0.26)	63	0.15 (0.11, 0.2)	21	0.26 (0.15, 0.38)	62	0.09 (0.05, 0.13)	26	0.2 (0.12, 0.29)	54	0.29 (0.22, 0.37)
Pfcrt 356	33	0.06 (0.04, 0.08)	65	0.01 (0, 0.03)	16	0	62	0	26	0	62	0
Pfcrt 371	27	0.36 (0.24, 0.49)	61	0.28 (0.21, 0.36)	23	0.54 (0.4, 0.69)	59	0.74 (0.66, 0.8)	23	0.35 (0.23, 0.48)	49	0.44 (0.36, 0.5)
Pfcrt 76	31	0.71 (0.61, 0.8)	63	0.49 (0.48, 0.5)	31	0.47 (0.47, 0.48)	61	0.88 (0.82, 0.92)	30	0.62 (0.52, 0.72)	63	0.49 (0.46, 0.5)
Pfdhfr 108	47	0.49 (0.47, 0.5)	88	0.96 (0.93, 0.98)	33	0.49 (0.46, 0.5)	66	0.89 (0.84, 0.93)	37	0.79 (0.71, 0.87)	77	0.93 (0.89, 0.97)
Pfdhfr 164	41	0	71	0	29	0	69	0	35	0	66	0
Pfdhfr 16	51	0.2 (0.14, 0.27)	88	0.12 (0.08, 0.16)	38	0.17 (0.16, 0.17)	69	0.08 (0.05, 0.12)	40	0.16 (0.09, 0.23)	80	0.12 (0.08, 0.17)
Pfdhfr 51	43	0.49 (0.46, 0.5)	71	0.82 (0.76, 0.87)	28	0.47 (0.41, 0.5)	66	0.8 (0.74, 0.86)	36	0.54 (0.43, 0.65)	69	0.75 (0.68, 0.81)
Pfdhfr 59	48	0.65 (0.59, 0.70)	77	0.68 (0.63, 0.76)	33	0.56 (0.50, 0.65)	66	0.75 (0.70, 0.83)	37	0.45 (0.34, 0.56)	68	0.65 (0.57, 0.72)
Pfdhps 437	49	0.24 (0.17, 0.27)	93	0.38 (0.31, 0.44)	32	0.36 (0.25, 0.45)	69	0.37 (0.33, 0.43)	38	0.31 (0.22, 0.4)	79	0.34 (0.27, 0.42)
Pfdhps 540	51	0.16 (0.1, 0.23)	92	0.42 (0.36, 0.48)	31	0.45 (0.35, 0.5)	71	0.45 (0.38, 0.5)	38	0.06 (0.02, 0.11)	86	0.18 (0.16, 0.2)
Pfdhps 613	33	0	73	0	21	0	58	0	27	0	54	0
Pfmdr1 1034	47	0.31 (0.25, 0.35)	79	0.22 (0.17, 0.28)	37	0.41 (0.3, 0.49)	68	0.09 (0.05, 0.13)	32	0.3 (0.21, 0.39)	72	0.36 (0.3, 0.43)
Pfmdr1 1042	52	0 (0, 0.01)	93	0.01 (0, 0.03)	36	0.02 (0.02, 0.02)	68	0.02 (0, 0.04)	38	0.02 (0, 0.05)	80	0
Pfmdr1 1246	41	0.03 (0.01, 0.07)	87	0.03 (0.01, 0.05)	32	0.12 (0.11, 0.12)	67	0.03 (0.01, 0.06)	36	0.05 (0.02, 0.1)	81	0.04 (0.02, 0.06)
Pfmdr1 184	39	0.15 (0.09, 0.23)	75	0.24 (0.18, 0.3)	32	0.16 (0.08, 0.26)	69	0.13 (0.08, 0.18)	36	0.09 (0.04, 0.15)	73	0.06 (0.03, 0.1)
Pfmdr1 86	41	0.41 (0.38, 0.44)	78	0.42 (0.35, 0.49)	34	0.43 (0.43, 0.43)	70	0.72 (0.65, 0.78)	38	0.46 (0.37, 0.5)	73	0.43 (0.36, 0.49)

Note: Mut = mutant, n samples, Freq = frequency, CI = confidence Interval, Yellow = significant increase in frequency from 2003, Blue = significant decrease in frequency.

In our study the differences in some frequencies of mutant alleles were big and unlikely to have occurred in one year. These differences may partly be attributable to slightly different sampling time-frames with different climatic conditions. This could be explained by the higher prevalence of asymptomatic infections in the communities in 2004 than 2003. In addition, some of these differences may be due to some possible technical limitations of this new microarray technology. However, we believe that the difference in the frequencies of mutant alleles largely represent high selection pressure after widespread use of SP in Tanzania.

We observed higher, almost fixed frequencies of Pfdhfr 108 Asn and 51 Ile and comparatively lower frequencies of 59 Arg as in Uganda (Francis *et al.* 2006). This observation can be explained by the theory of progressive stepwise occurrence of mutations (Plowe *et al.* 1997; Plowe *et al.* 1998).

Table 4. Ratio of prevalence of triple-*Pfdhfr* in community /SP failure rate in health facility

Site	2003			2004		
	Triple-*Pfdhfr* (%)	TF (%)	*Pfdhfr*/TF ratio	Triple-*Pfdhfr* (%)	TF (%)	*Pfdhfr*/TF ratio
Ipinda	60.0	33.3	1.8	72.0	31.5	2.3
Mlimba	71.5	43.3	1.7	74.3	47.3	1.6
Mkuranga	62.9	49.3	1.2	51.6	44.3	1.2
Total	63.6	42.2	1.5	72.8	41	1.8

Note**:** Triple-Pfdhfr = Pfdhfr 51 Ile, 59 Arg and 108 Asn mutations, TF = treatment failure rate

The highly resistant 108 Asn genotype appear first followed by 51 Ile that increases the fitness of the double mutant parasite under high drug pressure, by restoration of enzymatic activity (Hastings IM, *et al.* 2002). Subsequently *Pfdhfr* 59 Arg and 164 mutations are driven by drug pressure (Hastings *et al.* 2005). The *Pfdhfr* 59Arg seemed to modulate the prevalence of triple-*Pfdhfr* mutation, a genotype that reflected SP failure

rates better than any other one in our study. This allele can predict the triple-*Pfdhfr* mutant genotype whereas together with *Pfdhps* 540 Glu they predict quintuple mutant genotype (Kublin *et al.* 2002). Despite the high SP treatment failure rate and high frequencies of other *Pfdhfr* mutant alleles, the *Pfdhfr* 164 Leu allele, which was previously reported at low levels in Tanzania (Hastings MD, *et al.* 2002) and Uganda (Francis *et al.* 2006), was not detected in our study. This mutation has consistently not been found in Africa (Ochong *et al.* 2003; Alifrangis *et al.* 2003; Pearce *et al.* 2003; Mugittu *et al.* 2004; Wichmann *et al.* 2003; Bwijo *et al.* 2003), suggesting that the African parasites lack the genetic traits that would conferthe ability to bear the *Pfdhfr* 164 mutation (Nzila *et al.* 2005).

Although we could not assess linkage disequilibrium of the various *Pfdhr* or *Pfdhps* alleles in our study, prevalence recorded in 2004 in the 3 communities (51.6% - 74%) are more or less similar to estimated community frequencies of 0.6 and 0.8 reported in northeastern part of Tanzania (Pearce *et al.* 2003) but are higher than 19.4% and 18.6% recorded in 1999 in children < 5 years at health facilities prior to SP adoption (Mugittu *et al.* 2004). Similarly, the prevalence of double-*Pfdhps* in our study (31% - 45%) are more or less similar to 0.43 – 0.64 recorded in northeastern Tanzania (Pearce *et al.* 2003).

The overall ratio of triple-*Pfdhfr* mutant allele in communities to treatment failure rates in health facilities in 2003 (1.5) and 2004 (1.8) were stable compared to ratios observed between sites (1.2 – 2.3). However, the ratios are lower than genotype failure index (GFI) reported previously in health facility-based studies in Tanzania (1.96 – 2.1) (Mugittu *et al.* 2004), Uganda (1.9) (Kyabayinze *et al.* 2003) and Malawi (2.2) (Kublin *et al.* 2002), when triple-*Pfdhfr* or quintuple mutant genotypes were used as markers of SP resistance. On the other hand, the ratios calculated using the quintuple mutant genotype were much more variable, indicating lack of correlation between the prevalence of this genotype in communities and SP failure rates in their respective health facilities.

These observations support previous reports on the inability of the *Pfdhps* genotype to account for *P. falciparum in vivo* SP (Mugittu *et al.* 2004; Alifrangis *et al.* 2003; Mutabingwa *et al.* 2001; Mockenhaupt *et al.* 2005) or *in vitro* sulfadoxine or dapsone resistance in (Mberu *et al.* 2002), but contrast another report from Uganda (Dorsey *et al.* 2004) and one of our studies in Papua New Guinea (Marfurt *et al.* , In preparation) that showed the principal role of *Pfdhps* in mediating SP resistance. In general our study did not establish a clear relationship between the pattern and profile of molecular markers in the community and treatment failure rates in their respective health facilities. However, we believe that wider intervals between surveys and inclusion of sites with different SP failure rates may help to understand the relationship between the two parameters if triple-*Pfdhfr* is used as a marker of *P falciparum* resistance to SP.

The *Pfmdr1* 86 Tyr and *Pfcrt* 76 Thr, the most important determinant of CQ failure rate (Djimde *et al.* 2001), recorded highest frequencies in comparison to other mutant alleles in these genes. Their frequencies in 2004 in Ipinda, Mlimba and Mkuranga were much lower than >76% and >90%, respectively, recorded in 1999, just prior to CQ withdrawal in Tanzania (Schnieder *et al.* 2002; Ndejembi *et al.* unpublished data). On the other hand, the increase in Mlimba can be partly attributable to the observed rampant and uncontrolled use of QN and AQ, drugs whose efficacy is considered to be influenced by mutations in *Pfmdr* (Sidhu *et al.* 2005) and *Pcfrt* (Bray *et al.* 2005) genes. Decline of *Pfmdr1* 86Tyr and *Pfcrt* 76 Thr alleles has been observed in Malawi (Mita *et al.* 2003; Kublin *et al.* 2003), China (Liu *et al.* 1995) and Vietinam (Nguyen *et al.* 2001; Nguyen *et al.* 2003; Thahn *et al.* 2001) following withdrawal of CQ use. The recovery of CQ sensitivity is attributable to expansion of wild-type allele rather than reversal of mutations (Mita *et al.* 2004). These observations point to the possibility of recycling the limited number of safe and cheap drugs as combination partners with artemisinins (Laufer and Plowe 2004).

Figure 1. SP resistance molecular marker patterns in the catchment areas of health facilities where SP efficacy was conducted. Patterns occuring only once are not included
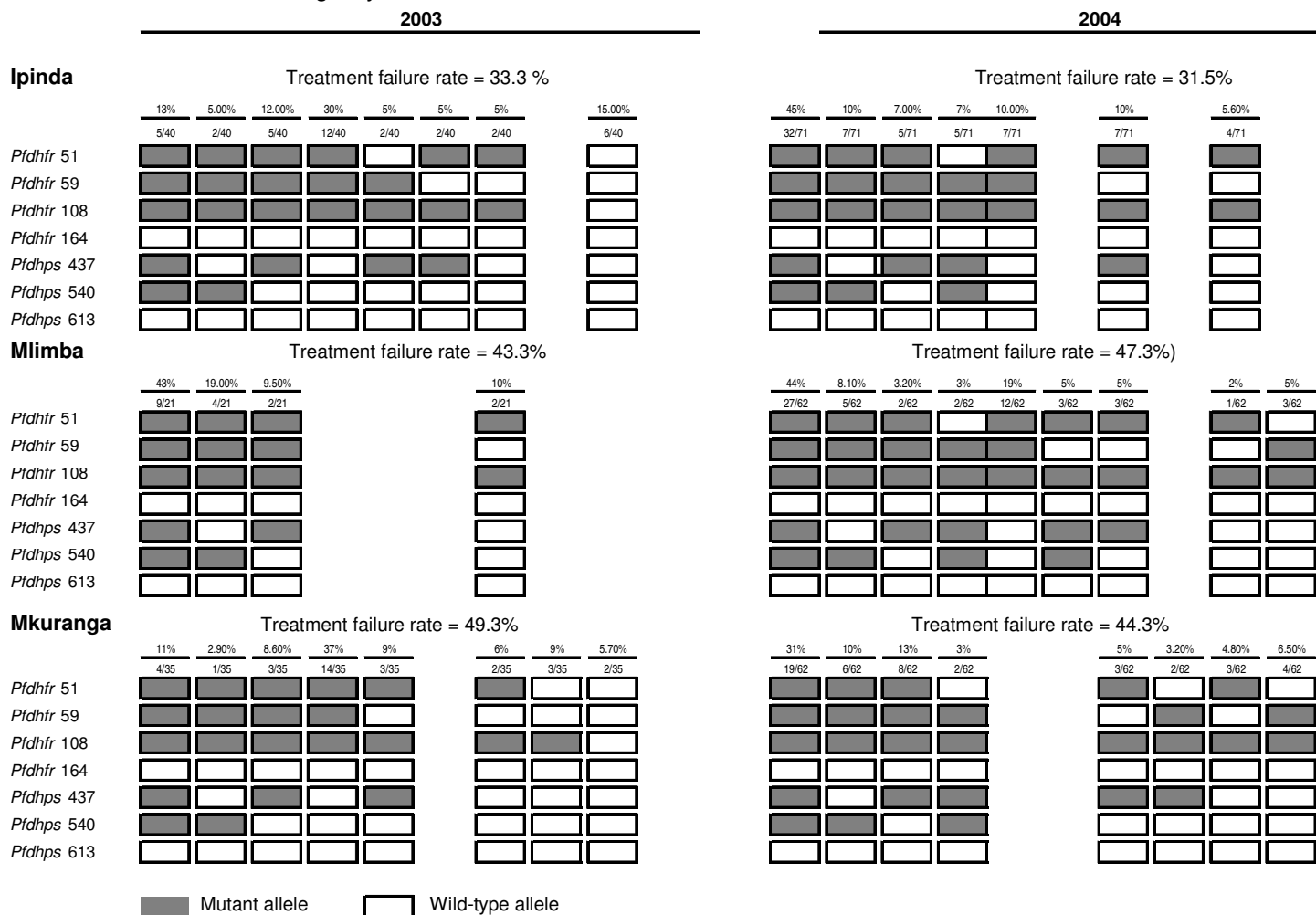
The absence of ATPase6 769 Asn and 623 Glu alleles in Tanzania suggests that artemisinin resistance has not been selected and is in line with a high artemether-lumefantrine efficacy reported in parts of Tanzania in multi-country studies (Falade *et al.* 2005). These observations point to the potential of these markers in monitoring emergence of resistance to ACTs after their widespread use in Sub-saharan Africa. Apart from one study (Jambou *et al.* 2005), it has not been widely proven that these mutations actually reflect resistance. Thus their value in monitoring *P falciparum* resistance to artemisinin remains to be seen.

Our findings show high frequencies of individual *Pfdhfr* 51 Ile, 59Arg and 108 Asn and prevalence of triple-*Pfdhfr* and quintuple mutant genotype in the community without a clear correlation to SP failure rate in health facilities. However, further studies need to be done, preferably in areas with different SP failure rates in order to assess the role of triple-*Pfdhfr* as a community marker for SP resistance. The frequencies of *Pfcrt* 76 Thr and *Pfmdr1* 86 Tyr have decreased following CQ withdrawal in Tanzania, but seem to be influenced by the pattern of QN and AQ use. The lack of ATPase mutant alleles suggest that artemisinin resistance has not been yet selected in Tanzania. As the majority of Sub-Saharan African countries are revising their malaria treatment policy to switch to ACTs, DNA microarray technology may provide a suitable tool for simultaneously assessing, on a large scale, the emergence and spread of resistance to ACTs and reversal of CQ and SP resistance after being withdrawn. However, in order to appreciate parasite genotypic dynamics following alteration of drug pressure, the interval between assessments should be long enough and consider seasonal genotype dynamics.

Authors' contribution

HP. Beck, B. Genton, and K. Mugittu designed the study. K. Mugittu S. Abdulla and H. Mshinda organised the clinical work. H. Mshinda was the supervisor in Tanzania. K. Mugittu and A. Crameri developed and performed DNA microarray analysis. K Mugittu, H. Masanja and Tom Smith carried out the statistical analysis. K. Mugittu, B. Genton and HP. Beck wrote the article and all others contributed to it.

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7 Chapter 7

7.1 Molecular genotyping to distinguish between recrudescents and new infections in treatment trials of *Plasmodium falciparum* malaria conducted in Sub-Saharan Africa: adjustment of parasitological outcomes and assessment of genotyping effectiveness.

This paper has been accepted for publication in *Tropical Medicine and International Health*.

Molecular genotyping to distinguish between recrudescents and new infections in treatment trials of *Plasmodium falciparum* malaria conducted in Sub-Saharan Africa: adjustment of parasitological outcomes and assessment of genotyping effectiveness.

Running title: PCR-adjustment of antimalarial treatment outcomes.

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Summary

Molecular genotyping of baseline and post-treatment recurrent *Plasmodium falciparum* is recommended to distinguish recrudescent from new infections. However, genotyping performance and adjustment of treatment outcomes have not been evaluated in large field trials.

Parasitological outcomes were assessed in 9 double-blinded trials of uncomplicated *P. falciparum* malaria in African children treated with artesunate/placebo plus standard monotherapies. Day 28 failure rates were adjusted by stepwise genotyping the *P. falciparum glutamate rich protein* (*glurp*), *merozoite surface protein* 1 (*msp1*) and *2* (*msp2*). We calculated overall and laboratory genotyping performance and compared unadjusted (crude) and PCR-adjusted outcomes. 3,455 (93.6%) of 3,691 enrolled patients were evaluable by Day 28. 767 (22%) had post-Day 14 recurrent parasitemias of which 686 could be genotyped: 246 were recrudescence, 286 new infections, and 154 unresolved. The overall and laboratory genotyping performance were 69 (12-100)% and 78 (50-100)%, respectively. The mean Day 28 crude parasitological failure rate was 44 (range 3-87)%. PCR-adjusted rates were 36 (2-86)% if unresolved infections were counted as failures or 33 (2-86)% if excluded from analysis. The Overall differences between crude Day 28 and Day 14 failure rates was 22% (95% CI 20.3, 24.6) but decreased to 14% (12.1, 16.3) (if unresolved infections are counted as failures) or 11% (9.8, 16.3) if excluded from the analysis. Genotyping refined treatment outcomes but diligence is needed in sample collection and analysis to improve its performance. Our findings support the WHO recommendation of PCR genotyping in malaria clinical trials and suggest that stepwise genotyping of only two loci (*msp2* and *msp1* or *glurp*) can reliably discriminate recrudescents from new infections.

Keywords: drug trials, molecular genotyping, PCR-adjusted antimalarial treatment outcomes.

Introduction

In malaria clinical trials post-treatment follow up should be long enough to detect recrudescent infections after initial parasite clearance. However, conducting such trials in areas of intense malaria transmission poses difficulties in interpreting drug efficacy outcomes because re-infections occurring during follow up may be interpreted as treatment failures. The optimal length of follow up for high transmission areas has not yet been established. With good evidence that the previously recommended 14 days protocol underestimates treatment failure rates, an extended period of follow up is needed (WHO, 2003; White 2002; Stepniewska *et al.* 2004). The World Health Organization (WHO) now recommends follow up for 28 (for chloroquine and amodiaquine) 42 (for sulfadoxine-pyrimethamine and artemether-lumefantrine) and 63 days (for

mefloquine), provided polymerase chain reaction (PCR) is available to reliably distinguish between new and recrudescent infections (WHO, 2003).

Many *Plasmodium falciparum* genes show extensive genetic polymorphism which can be used for genetic finger printing. High polymorphism has been shown in *msp1*, *msp2* and *glurp* genes in different geographical locations in malaria endemic areas (Felger *et al.* 1994; Babiker *et al.* 1997; Snounou *et al.* 1999; Peyerl-Hoffmann *et al.* 2001; Magesa *et al.* 2001; Aubouy *et al.* 2003). Therefore, these loci have been used in many trials to distinguish recrudescence from new infections. Because of their extensive polymorphism, it is highly unlikely for a patient in areas of intense transmission to become newly infected with a parasite possessing an identical genotype during follow up because this probability is the product of individual allele frequencies of each allele of the three genes (Snounou and Beck, 1998). Therefore, by comparing the genotypes of these three loci together at baseline and at the time of parasite recurrence, recrudescent can be distinguished reliably from new infections (Snounou and Beck, 1998; Viriyakosol *et al.* 1995; Beck, 1999; Greenwood, 2002).

Recurrent parasites can be potentially classified into four categories based on the degree of allelic matching: (i) all alleles in the baseline and recurrent parasites are identical, (ii) some alleles are missing in the recurrent parasites (iii) recurrent parasites contain alleles identical to those at baseline with additional/new ones not observed at baseline (iv) alleles in the baseline and recurrent parasite samples are different. It is generally accepted that categories (i - iii) and (iv) represent recrudescent and new infection, respectively (Magesa *et al.* 2001; Snounou and Beck 1998; Basco and Ringwald 2000; Brockman *et al.* 1999; Basco *et al.* 2002; Ranford-Cartwright *et al.* 1997; Happi *et al.* 2004). However, some investigators (Cattamanchi *et al.* 2003; Kyabayinze *et al.* 2003) consider that category (iii) represents a new infection because of the appearance of new alleles.

Despite the widespread use of molecular genotyping in clinical trials, questions remain regarding its effectiveness and usefulness in determining/adjusting drug failure rates in large field trials. Here we assess these parameters using the *P. falciparum* loci *glurp*, *msp2* and *msp1* to differentiate recurrent parasites in a series of randomized, clinical trials in Sub-Saharan Africa

(Adjuik *et al.* 2002; Adjuik *et al.* 2004; Priotto *et al.* 2003; von Seidlein *et al.* 2000; Sirima *et al.* 2003; Obonyo *et al.* 2003; Gil *et al.* 2003).

Methods

Study Areas and Design

Randomized, double blind, placebo controlled, efficacy trials were conducted in Burkina Faso, Gabon, The Gambia, Sao Tomé, Senegal, Uganda, Malawi and Kenya [(at Kenya Medical Research Institute (KEMRI) and African Medical Research Foundation (AMREF)]. Children with acute, uncomplicated falciparum malaria received active artesunate (4mg/kg/d x 3d) or placebo plus one of the following: amodiaquine [AQ (30 mg/kg total dose)], chloroquine [CQ (25 mg/kg total dose)], or sulfadoxine [(25 mg/kg total dose)/pyrimethamine (S/P)]. In the S/P trials, an additional arm consisted of one day of artesunate/placebo (Tables 1 and 2). Treated patients were followed up for 28 days. End points were the crude (unadjusted) and PCR-adjusted Day 28 parasitological failure rates. Recurrent parasitemias occurring before or on Day 14 were considered as treatment failures without genotyping, whereas those occurring beyond Day 14 were genotyped to distinguish recrudescence from new infections. Patients who were randomized wrongly or lost to follow up were excluded from the analysis. Study site characteristics and further methodological details are described elsewhere (references cited above). Ethical approvals were obtained from the relevant local ethics committees and from the WHO. Prior to recruitment of children informed consents were obtained from their parents or guardians.

Collection of blood samples and preparation of blood smears

Finger prick blood samples were collected on Days 0, 7, 14 21 and 28 onto Isocode stixs® (Schleicher & Schull, Dassel, Germany). The Isocode stix® were air-dried, placed in self sealing plastic bags with desiccant, and stored under ambient conditions until used. Concurrently, thick and thin films were prepared, Giemsa–stained, examined and reported as the number of parasites per microlitre, assuming a total white cell count of 8,000 per µl.

DNA extraction and molecular genotyping

Sample analysis was centralized at two laboratories in order to limit inter-laboratory variability (Farnert *et al.* 2001) and to speed-up analysis. Blood samples from Burkina Faso, The Gambia, Sao Tomé, Senegal, Uganda, Malawi and Kenya-KEMRI were processed and analyzed at Ifakara Health Research and Development Centre (IHRDC) laboratory in Tanzania whereas those from Kenya-AMREF and Gabon were analyzed at Tübingen University laboratory in Germany. At both sites template DNA was extracted from Isocode stix® according to the manufacturer's protocol. Paired samples (Day 0 and Day of recurrence after Day 14) were genotyped by analyzing region II of *glurp,* block 3 of *msp2* (3D7 and FC27 allelic families), and block 2 of *msp1* (K1, MAD20 AND RO33 allelic families), according to previously published methods (Felger *et al.* 1994; Viriyakosol *et al.* 1995).

At IHRDC PCR amplifications were done using an MJ Thermal Controller PTC-100™ (MJ Research Inc. Watertown, USA). A Biometra Uno II thermal cycle (Biometra, Göttingen, Germany) was used at Tübingen. Primary PCR reactions were multiplexed with *glurp, msp1* and *msp2* whereas nested PCR amplifications were done separately for each locus. The primary and nested amplifications were carried out in 20μl and 30μl reaction volumes using 5μl of template DNA and 2μl of primary PCR product, respectively. For *glurp* and *msp2* one nested PCR amplification reaction for each locus was done whereas three nested reactions were done for *msp1* for the detection of K1, MAD20 and RO33 allelic families. Each reaction contained 1x PCR buffer (Gibco BRL®), 0.125mM of each dNTP, 0.4 units of Taq DNA polymerase (Gibco BRL®) and 0.25mM of each primer. All Primers and reference DNA were obtained from Malaria Research and Reference Reagents Resource Centre (MR4). The oligonucleotide primers have been described elsewhere (Snounou *et al.* 1999; Foley *et al.* 1992). Temperature cycling parameters were: initial denaturation at 94ºC for 5 minutes followed by 30 cycles of denaturation at 94ºC for 1 minute, annealing at 55ºC for 2 minutes (for primary PCR) or 58ºC for 2 minutes (for nested PCR) and extension at 72ºC for 2 minutes. The last extension cycle was prolonged for 10 more minutes. 10μl of *msp2* PCR product were digested with 3 units of *Hin*f III for 2.5 hrs at 37ºC and the resulting fragments were resolved on 10% polyacrylamide gels. The *glurp* and *msp1* PCR products were directly resolved on 2% agarose gel. For better comparison of fragments paired samples were loaded side by side. Gels were stained with ethidium bromide,

visualized under UV illumination and photographed. Gels photographs were scored (distinction of recrudescence from re-infections) by visual comparison of DNA fragments on baseline and recurrent samples.

Definitions of recrudescent, new and unresolved infections

Recurrent infections were genotyped in a stepwise approach. At IHRDC laboratory sample pairs were sequentially genotyped by initially amplifying *glurp* (step 1) followed by *msp2* (step 2) and lastly by *msp1* (step3). The sequence of analysis at Tübingen University laboratory was *msp*2 (step 1) then *msp*1 (step 2), and finally *glurp* (step 3). At both laboratories, only the samples provisionally classed as recrudescence were further analyzed for the next locus; new and unresolved recurrent infections were not analyzed further (Figure 1). A recurrent parasitaemia was considered as a recrudescence whenever pre-treatment alleles were also found in recurrent samples, whether the two allelic patterns were completely identical, or recurrent sample had missing or additional alleles with respect to baseline. A recurrent parasitaemia was considered as a new infection if the allelic pattern for any one of the loci differed completely between the pre-treatment and the recurrent sample. An "unresolved" result was recorded when we could not amplify and therefore failed to genotype any one locus (Snounou and Beck 1998).

Definitions of genotyping performances

Overall genotyping performance for differentiating post-Day 14 recurrent parasitemias = number of post-Day 14 recurrent parasitemias with a PCR result / total number of post-Day 14 recurrent cases. Laboratory genotyping performance = number of post-Day 14 recurrent parasitemias with a PCR result / total number of recurrent paired samples analyzed.

Data analysis

Data were analyzed using Stata v 8.0 (Stata Corporation Inc, Texas, USA). Day 28 parasitological failure rates are reported as crude (without PCR-adjustment) or PCR-adjusted treatment failure rates. The latter have been reported in two ways by (i) classifying all unresolved recurrent infections as failures or (ii) excluding them from the analysis. In the calculation of the adjusted failure rates, all new infections were classified as 'non failures' and counted in the denominator with those remaining aparasitaemic throughout follow up.

Figure 1. Stepwise genotyping of *P. falciparum* in paired [Day 0 and Day of recurrent (Day 15 - 28)] samples. Only paired samples provisionally classified as recrudescence by one locus were further analyzed for the next locus. The rest were definitively classified as either new infections or unresolved and excluded from further analysis.

	IHRDC laboratory	Results	Tübingen University laboratory	
	564 pairs of recurrent infections	Results	122 pairs of recurrent infections	
Step 1	**glurp** → 182(32.3%) / 105(18.6%) / 277(49.1%)	new infections / unresolved / recrudescences	49(40.2%) / 11(9.0%) / 62(50.8%) ← **msp2**	
Step 2	**msp2** → 41(14.8%) / 46 (16.6%) / 190(68.6%)	new infections / unresolved / recrudescences	18(29.0%) / 2(3.3%) / 42(67.7%) ← **msp1**	
Step 3	**msp1** → 12(6.3%) / 4(2.1%) / 174(91.6%)	new infections / unresolved / recrudescences	0(0%) / 0(0%) / 42(100%) ← **glurp**	

Multinomial logistic regression was used to assess the effect of parasitemia on the outcome of resolved (successful) or unresolved (unsuccessful) genotyping at different PCR-end points. For the purpose of summarizing the results we have pooled proportions across the study sites. Differences between unadjusted and adjusted treatment failure rates were calculated with 95% confidence intervals.

Results

Treatment outcomes, genotyping performance and adjustment of treatment failure rates.

A total of 3,691 patients were recruited of which 236 (6.4%) were lost to follow up or withdrawn from study. Thus 3,455 (94%) patients were evaluable patients on Day 28; 1,937 (56%) showed treatment successes and 1,518 (44%) were failures of which 767 occurred after Day 14. 81 (11%) pairs of post-Day14 recurrences were not genotyped because they had one or both samples missing. Hence a total of 686 paired samples were genotyped: 246 (36%) were classed as recrudescence, 286 (42%) as new infections, and 154 (22%) [8 (5.2%) baseline and 146 (94.2%) recurrent samples] could not be resolved (Figure 2) because of failure to either extract or amplify DNA. The overall genotyping performance ranged from 12 - 100% across the sites (average 69%) and the laboratory performance from 50-100% (average 78%) (Table 1).

Table 2 summarises the Day 14 (crude) and 28 (crude and PCR-adjusted) failure rates and their differences. The pooled crude Day 28 parasitological failure rate was 44% (95% CI: 43.2, 44.8). The PCR-adjusted failure rates including unresolved as failures or excluding them from analysis rates were 36% (95% CI: 35.2, 36.8) or 33% (95% CI: 32.2, 33.8), respectively. The two pooled PCR-adjusted failure rates differed by 3% (95% CI: 1.8, 3.8). The pooled Day 14 failure rate was 22% (95% CI: 21.3, 22.7). The crude and two PCR-adjusted Day 28 failure rates were 22% (95% CI: 20.3, 24.6), 14% (95% CI: 12, 16.3), and 11% (95% CI: 12.1, 16.3) respectively higher, than the pooled Day 14 failure rate.

Figure 2. Clinical trial and molecular genotyping profiles.
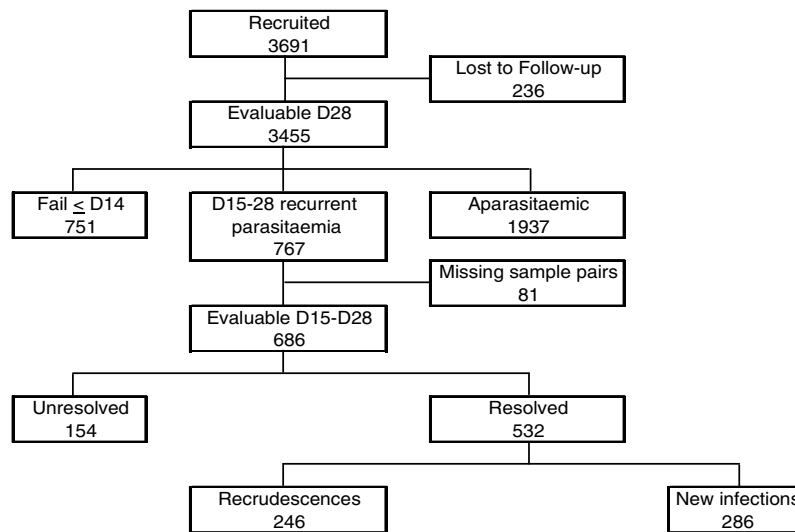
Differentiating recurrent parasitemia by stepwise genotyping of loci

At total of 564 and 122 paired samples were sequentially genotyped at IHRDC and Tübingen University laboratory, respectively. Similar proportions of recrudescences were identified at the two sites in all three steps. However, twice as much (29%) reinfection were identified by *msp1* at step 2 at Tübingen University than at IHRDC (14.8%). A total of 141/564 (25.2%) and 13/122 (10.7%) recurrent infections were unresolved at IHRDC and Tübingen University laboratories, respectively. The geometric means parasitaemia of the resolved infections were 23,940 (95% CI: 21,783, 26,312) at baseline (day 0), 5,135 (95% CI: 3972, 6637) at day 21 and 5,256 (95% CI: 3,734, 7,399) at day 28 whereas the corresponding means for unresolved infection were 21,624 (95% CI: 17,944, 26,060), 976 (95% CI: 586, 1,625) and 957 (95% CI: 490, 1,867) (Figure 3). Logistic regression analysis indicated that for every unit increase in the log of the parasite count, the odds of a resolved infection to that of unresolved (as baseline) increased by 29% (OR 1.29, 95% CI: 1.20 1.39, $p < 0.001$), pointing to a relationship between higher parasite count and success in amplifying DNA, hence resolving the recurrent infection.

Table1. Treatment outcomes and performance of molecular genotyping in ACT trials conducted in Sub-Saharan Africa

Country	Regimen	n	D28 CR(%)	Failure rate		>D14 recurrences		Genotyping results			Genotyping Performance	
				≤D14 FR	>D14 FR	Complete pairs (%)	Incomplete Pairs (%)	Rc	Ni	Unres	Overall	Laboratory
Burkina Faso	CQ	142	27(19)	90(63)	25(18)	24(96)	1(4)	6(25)	7(29)	11(46)	13/25(52)	13/24(54)
Burkina Faso	CQ+AS	145	71(49)	27(18)	47(32)	47(100)	0(0)	7(15)	25(53)	15(32)	32/47(68)	32/47(68)
Gabon	AQ	98	70(71)	10(10)	18(18)	17(94)	1(6)	12(71)	4(24)	1(6)	16/18(89)	16/17(94)
Gabon	AQ+AS	94	82(87)	2(2)	10(11)	10(100)	0(0)	4(40)	4(40)	2(20)	8/10(80)	8/10(80)
Kenya-Amref	AQ	183	75(41)	48(26)	60(33)	57(95)	3(5)	32(56)	22(39)	3(5)	54/60(90)	54/57(95)
Kenya-Amref	AQ+AS	180	123(68)	17(9)	40(22)	38(95)	2(5)	24(63)	10(26)	4(11)	34/40(85)	34/38(89)
Kenya-Kemri	SP	189	68(36)	49(26)	72(38)	67(93)	5(7)	23(34)	36(54)	8(12)	59/72(82)	59/67(88)
Kenya-Kemri	SP+1AS	191	77(40)	32(17)	82(43)	77(94)	5(6)	21(27)	42(55)	14(18)	63/82(77)	63/77(82)
Kenya-Kemri	SP+3AS	192	100(52)	18(9)	74(39)	66(89)	8(11)	13(20)	40(61)	13(20)	53/74(72)	53/66(80)
Malawi	SP	129	36(28)	61(47)	32(25)	26(81)	6(19)	8(31)	6(23)	12(46)	14/32(44)	14/26(54)
Malawi	SP+1AS	138	48(35)	40(29)	50(36)	45(90)	5(10)	21(47)	11(24)	13(29)	32/50(64)	32/45(71)
Malawi	SP+3AS	134	94(70)	7(5)	33(25)	30(91)	3(9)	10(33)	9(30)	11(37)	19/33(58)	19/30(63)
Sao Tomé	CQ	191	24(13)	153(80)	14(7)	13(93)	1(7)	8(62)	3(23)	2(15)	11/14(79)	11/13(85)
Sao Tomé	CQ+AS	194	109(56)	32(17)	53(27)	44(83)	9(17)	20(45)	13(30)	11(25)	33/53(62)	33/44(75)
Senegal	AQ	156	123(79)	10(6)	23(15)	12(52)	11(48)	4(33)	4(33)	4(33)	8/23(35)	8/12(67)
Senegal	AQ+AS	159	130(82)	12(8)	17(11)	4(24)	13(76)	1(25)	1(25)	2(50)	2/17(12)	2/4(50)
The Gambia	SP	193	173(90)	10(5)	10(5)	10(100)	0(0)	5(50)	5(50)	0(0)	10/10(100)	10/10(100)
The Gambia	SP+1AS	187	171(91)	7(4)	9(5)	5(56)	4(44)	3(60)	2(40)	0(0)	5/9(56)	5/5(100)
The Gambia	SP+3AS	187	181(97)	2(1)	4(2)	3(75)	1(25)	1(33)	2(67)	0(0)	3/4(75)	3/3(100)
Uganda	SP	144	55(38)	62(42)	27(18)	27(100)	0(0)	9(33)	5(19)	13(48)	14/27(52)	14/27(52)
Uganda	SP+1AS	113	32(28)	45(38)	36(31)	35(97)	1(3)	9(26)	17(49)	9(26)	26/36(72)	26/35(74)
Uganda	SP+3AS	116	68(59)	17(15)	31(26)	29(94)	2(6)	5(17)	18(62)	6(21)	23/31(74)	23/29(79)
Total		3455	1937(56)	751(22)	767(22)	686(89)	81(11)	246(36)	286(42)	154(22)	532/767(69)	532/686(78)

Note: n = sample size; FR = Failure rate; D = Day; Rc = recrudescence; Ni = new infection; Unres = unresolved; 1As = one day artesunate; 3AS = three days daily artesunate; AQ = amodiaquine; CQ = chloroquine; SP = sulfadoxine-pyrimethamine; KEMRI = Kenya Medical Research Institute; AMREF = African Medical Research Foundation.; Performance - Overall = number of post-Day 14 recurrent parasitemias with a PCR result / total number of post-Day 14 recurrent cases; Performance - Laboratory = number of post-Day 14 recurrent parasitemias with a PCR result / total number of paired samples analyzed

Discussion

In the clinical trials reported previously (Adjuik *et al.* 2002; Adjuik *et al.* 2004; Priotto *et al.* 2003; von Seidlein *et al.* 2000; Sirima *et al.* 2003; Obonyo *et al.* 2003; Gil *et al.* 2003), a 28 day follow up was used to assess drug efficacy. In these studies we genotyped post-Day 14 recurrent parasites to distinguish recrudescent from new infections, and adjusted treatment outcomes accordingly. To limit inter-laboratory variations (Farnert *et al.* 2001) sample processing and analysis was done at only two laboratories.

Our findings show that the overall effect of molecular genotyping as a tool for differentiating recurrent parasitaemia was reasonable (69%) and the laboratory performance better (78%). In most sites the differences between crude and the two PCR-adjusted treatment failure rates were appreciably high (> 10%). The mean difference between the two Day 28 PCR-adjusted failure rates was small (3%), suggesting that the inability to resolved some recurrent parasitaemias in these trials did not have a significant effect on the adjusted efficacy rates. Nevertheless, study denominators became progressively smaller as data were lost: 3,691 (recruited), 3,455 (evaluated at Day 28), 3,374 (PCR-adjusted with unresolved infections counted as failures), 3,220 (PCR-adjusted with unresolved infections excluded from the analysis). This represents an overall loss of about 13% of data, comprising of 6% lost to follow up and 7% lost during PCR genotyping. In general, there was wide inter-study variance in genotyping performance and differences between crude and adjusted treatment failure rates. These observations highlight the need for diligence in field sample collection, laboratory analysis, and for vigorous quality control measures throughout the data generation process.

Table 2: PCR-adjustment of parasitological failure rates in ACT trials conducted in Sub-Saharan Africa

Country	Regimen	D14 FR (CI)	D28 FR Crude (CI)	Adjusted 1 (CI)	Adjusted 2 (CI)	Failure Rate Differences D28 Crude - D14	D28 Adjusted 1 - D14	D28 Adjusted 2 - D14	D28 Adjusted 1 – D28 Crude (Diff1)	D28 Adjusted 2 – D28 Crude (Diff2)	Diff-1 – Diff-2
Burkina-Faso	CQ	63(59.7,66.3)	81(77,85)	76(72.3,79.7)	74(70.2,77.8)	18(7.9,28.1)	13(2.9,23.5)	11(2.1,23.9)	5(-4.4,14.4)	7(-2.9,16.9)	2(-1.9,5.9)
Burkina-Faso	CQ+AS	18(14.7,21.3)	51(47,55)	34(30.3,37.7)	26(22.1,29.9)	33(22.6,43.4)	16(5.6,26)	8(6.2,25.8)	17(5.7,28.3)	25(13.9,36.1)	8(1.3,14.7)
Gabon	AQ	10(5.9,14.1)	29(24.1,33.9)	26(21.5,30.5)	26(21.5,30.5)	19(8.2,29.8)	16(5.2,26.6)	16(5.4,26.6)	3(-9.5,15.5)	3(-9.5,15.5)	0(-3.4,3.4)
Gabon	AQ+AS	2(-2.1,6.1)	13(8,18)	9(4.5,13.5)	7(2.4,11.6)	11(3.6,18.4)	7(-0.4,13.4)	5(1.1,12.9)	4(-4.9,12.9)	6(-2.6,14.6)	2(-2.4,6.4)
Kenya-Amref	AQ	26(23.1,28.9)	59(55.4,62.6)	47(43.7,50.3)	46(42.7,49.3)	33(23.5,42.5)	21(11.5,30.6)	20(11.4,30.6)	12(1.8,22.2)	13(2.8,23.2)	1(-3.8,5.8)
Kenya –Amref	AQ+AS	9(6.1,11.9)	32(28.4,35.6)	26(22.7,29.3)	24(20.7,27.3)	23(15.1,30.9)	17(9.1,24.6)	15(9.5,24.5)	6(-3.4,15.4)	8(-1.3,17.3)	2(-1.7,5.7)
Kenya-Kemri	SP	26(23.1,28.9)	64(60.5,67.5)	45(41.8,48.2)	43(39.7,46.3)	38(28.8,47.2)	19(9.8,28.4)	17(9.5,28.5)	19(9.2,28.8)	21(11.1,30.9)	2(-3.7,7.7)
Kenya-Kemri	SP+1AS	17(14.1,19.9)	60(56.5,63.5)	38(34.8,41.2)	33(29.7,36.3)	43(34.2,51.8)	21(12.2,29.7)	16(12.3,29.7)	22(12.2,31.8)	27(17.2,36.8)	5(-1.2,11.2)
Kenya-Kemri	SP+3AS	9(6.1,11.9)	48(44.5,51.5)	27(23.8,30.2)	22(18.7,25.3)	39(30.8,47.2)	18(9.8,25.5)	13(10.7,25.3)	21(11.5,30.5)	26(16.6,35.4)	5(-1.1,11.1)
Malawi	SP	47(43.5,50.5)	72(67.8,76.2)	67(63.1,70.9)	64(59.9,68.1)	25(13.7,36.3)	20(8.7,31.5)	17(8.1,31.9)	5(-6,16)	8(-3.4,19.4)	3(-1.2,7.2)
Malawi	SP+1AS	29(25.6,32.4)	65(60.9,69.1)	57(53.2,60.8)	53(49.1,56.9)	36(24.7,47.3)	28(16.7,39.6)	24(16.1,39.9)	8(-3.9,19.9)	12(-0.2,24.2)	4(-1.2,9.2)
Malawi	SP+3AS	5(1.6,8.4)	30(25.8,34.2)	23(19.2,26.8)	16(12,20)	25(16.5,33.5)	18(9.5,25.9)	11(10.6,25.4)	7(-3.4,17.4)	14(4,24)	7(1.8,12.2)
Sao Tomé	AQ+AS	17(14.1,19.9)	44(40.5,47.5)	37(33.8,40.2)	33(29.8,36.2)	27(18.2,35.8)	20(11.2,28.7)	16(11.4,28.6)	7(-2.8,16.8)	11(1.3,20.7)	4(-0.1,8.1)
Sao Tomé	CQ	80(77.1,82.9)	87(83.5,90.5)	86(82.8,89.2)	86(82.8,89.2)	7(-0.4,14.4)	6(-1.4,13.5)	6(-1.6,13.6)	1(-5.8,7.8)	1(-5.9,7.9)	0(-1.4,1.4)
Sénégal	AQ	6(2.8,9.2)	21(17.1,24.9)	19(15.5,22.5)	16(12.4,19.6)	15(7.6,22.4)	13(5.6,20.2)	10(6.1,19.9)	2(-6.9,10.9)	5(-3.6,13.6)	3(0.1,5.9)
Sénégal	AQ+AS	8(4.8,11.2)	18(14.2,21.8)	18(14.5,21.5)	17(13.5,20.5)	10(2.7,17.3)	10(2.7,17.3)	9(2.8,17.2)	0(-8.4,8.4)	1(-7.4,9.4)	1(-0.1,2.1)
The Gambia	SP	5(2.1,7.9)	10(6.5,13.5)	8(4.8,11.2)	8(4.8,11.2)	5(-0.3,10.3)	3(-2.3,8)	3(-2,8)	2(-3.8,7.8)	2(-3.8,7.8)	0(-2,2)
The Gambia	SP+1AS	4(1.1,6.9)	9(5.5,12.5)	7(3.8,10.2)	7(3.8,10.2)	5(0.1,9.9)	3(-1.9,7.5)	3(-1.5,7.5)	2(-3.4,7.4)	2(-3.4,7.4)	0(-2,2)
The Gambia	SP+3AS	1(-1.9,3.9)	3(-0.5,6.5)	2(-1.2,5.2)	2(-1.2,5.2)	2(-0.8,4.8)	1(-1.8,3.5)	1(-1.5,3.5)	1(-2.2,4.2)	1(-2.2,4.2)	0(-1.4,1.4)
Uganda	SP	42(38.7,45.3)	62(58,66)	55(51.3,58.7)	51(47.2,54.8)	20(7.4,32.6)	13(0.4,25.8)	9(0,26)	7(-5.6,19.6)	11(-1.9,23.9)	4(-1.2,9.2)
Uganda	SP+1AS	38(34.2,41.8)	72(67.5,76.5)	56(51.9,60.1)	52(47.7,56.3)	34(23.2,44.8)	18(7.2,29.3)	14(6.3,29.7)	16(5.1,26.9)	20(8.7,31.3)	4(-2.3,10.3)
Uganda	SP+3AS	15(11.3,18.7)	41(36.5,45.5)	26(21.9,30.1)	22(17.8,26.2)	26(14.8,37.2)	11(-0.2,21.4)	7(0.7,21.3)	15(2.8,27.2)	19(6.9,31.1)	4(-3,11)
Mean		**22(21.3,22.7)**	**44(43.2,44.8)**	**36(35.2,36.8)**	**33(32.2,33.8)**	**22(20.3,24.6)**	**14(12,16.3)**	**11(9.8.1,16.3)**	**8(6,10.6)**	**11(8.7,13.4)**	**3(1.8,3.8)**

FR = Failure rate; CI = Confidence Interval; Adjusted 1 = failure rate with PCR-unresolved recurrences counted as failure; Adjusted 2 = failure rate with PCR unresolved recurrences excluded from the analysis; D = Day; Diff = Difference; 1As = one day artesunate; 3AS = three days daily artesunate; AQ = amodiaquine; CQ = chloroquine; SP = sulfadoxine-pyrimethamine; KEMRI=Kenya Medical Research Institute; AMREF = African Medical Research Foundation.

The mean difference in failure rate between the Day 14 and Day 28 was 11% if unresolved are excluded from analysis or 14% if classified as failures. These broad differences might reflect the underlying levels of parasite resistance to the drug given in combination with of artesunate. They tended to be smaller with drug regimens that were either highly effective (e.g. S/P in the Gambia) or ineffective (e.g. chloroquine in Sao Tomé) and broader with regimens of intermediate efficacy (e.g. S/P in Malawi); while such regimens appear effective at Day 14, recrudescence is detected by Day 28.

Figure3. Geometric mean parasitaemias at baseline (day 0) and recurrence (day 21 and 28) in resolved and unresolved infections
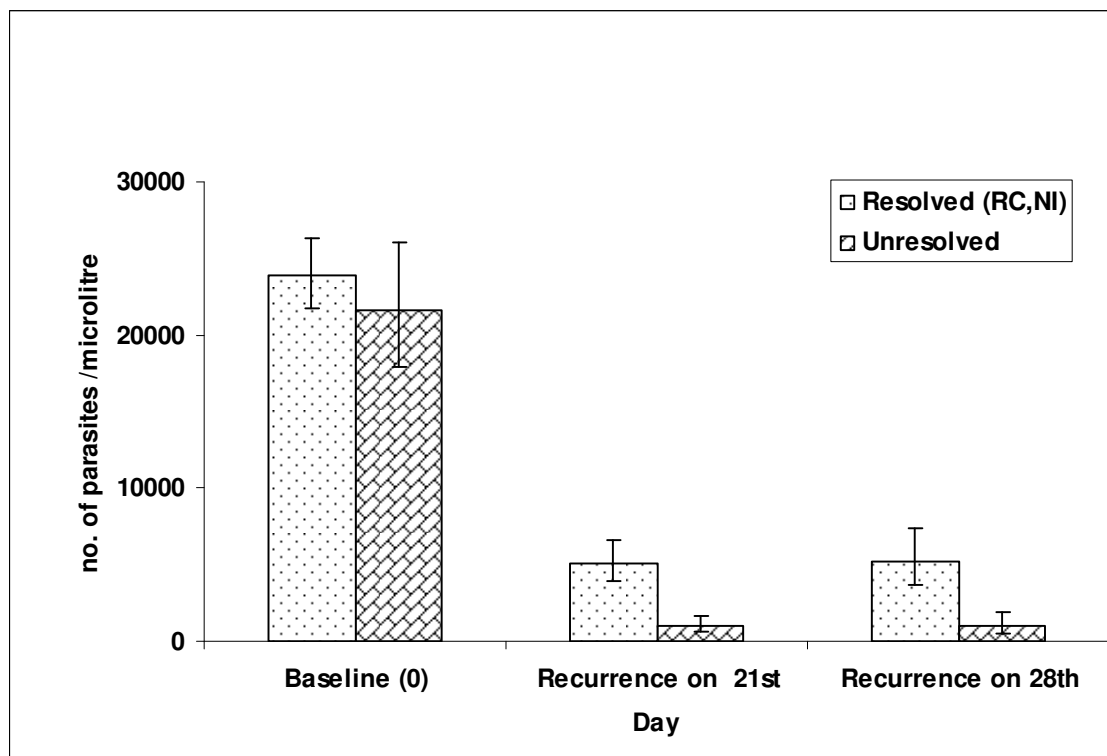
These data reemphasize the importance of an extended follow up so that policy makers are reliably informed of drug efficacy. Similarly, PCR-adjusted clinical data are essential to avoid discontinuing a drug that appears to have low efficacy on Day 28 because of a high rate of new infections. Admittedly, we might have underestimated the rate of new infections by regarding all recurrent parasiatemias observed on or before day 14 as

treatment failure. The pre-patent period of *P. falciparum* is known to be between 6-12 days. Hence, within 14 days of treatment, there would be time for parasites either inoculated after treatment or sequestered in the liver at the time of treatment to emerge. Without genotyping such infections may wrongly be regarded as being due to treatment failure. A detailed assessment of pre- and post-Day 14 rates of genotypically new infections and its implication in assessment of drug efficacy in the Ugandan study has been done and will be published separately.

In our study, laboratory failure (22%) was a more important cause of loss of genotyping data than field sample collection (11%). Nearly all unresolved infections (94.8%) were due to failure to extract or amplify DNA in recurrent samples, whose parasitaemias were on average 5-folds lower than those of the resolved recurrent infection samples. This, together with the simple DNA extraction method adopted (simply boiling the Isocode stix®), may have resulted in low DNA yield and/or persistence of PCR inhibitors which compromised PCR performance. A higher yield might have been obtained using an advanced method of extracting DNA. Even if DNA is successfully extracted, PCR may fail to detect all genotypes present in a mixed-clones infection both at baseline and at the time of parasite recurrence because some might be present below PCR detection level (Jafari *et al.* 2004) or might be sequestered. However, some evidence indicates that symptomatic infections are less complex than asymptomatic ones. A study by Irion *et al.* (1998) found that less than 2% of recrudescent genotypes were absent on Day 0 but were detected on Day 3. Similarly, Farnert and Bjorkman (2005) detected the same genotypes in consecutive samples obtained every 12 hours for at least three days post treatment. These observations suggest that single time-point samples may reliably represent all subpopulations present in asymptomatic infections prior to treatment.

A further complication in the interpretation of paired genotyping data arises from infections with new parasites possessing identical genotypes to those present on Day 0, leading to an erroneous diagnosis of recrudescence. However, in endemic areas the probability of this occurrence is rather low and negligible when two or more discriminatory markers are being used. We believe that this theoretical possibility does

not affect the interpretation of our findings because we observed a high overall rate (42%) of new infections (different genotypes). Nonetheless, all these weaknesses point to the need for some caution in interpreting PCR-adjusted treatment outcomes.

Molecular genotyping of *P. falciparum msp1, msp2* and *glurp* has been used to differentiate recurrent parasites in both longitudinal surveys and randomized trials in Africa and Asia (Basco and Ringwald 2000; Brockman *et al.* 1999; Basco *et al.* 2002; Ranford-Cartwright *et al.* 1997; Happi *et al.* 2004; Cattamanchi *et al.* 2003; Kyabayinze *et al.* 2003; Irion *et al.* 1998). However, the interpretation of genotyping data varies. In longitudinal studies examining the dynamics of malaria infections (i.e. the acquisition of new or loss of old parasites), new infections are always recorded if a new allelic pattern is detected even when alleles from prior infection/s persist. This contrasts with drug efficacy studies in which this finding indicates a failure of the drug to clear an infection present before treatment. Such cases are classified as recrudescence.

Our stepwise approach shows that genotyping a third marker (*msp1* at IHRDC or *glurp* at Tübingen University) added limited discriminatory power over the two markers. Thus in resource-constrained malaria endemic countries only two markers may be enough to discriminate recrudescents from re-infections in malaria clinical trials. Cattamanchi *et al.* (2003) suggest even the use of *msp2* alone based on reported 89% concordance with all three makers. Admittedly, stepwise genotyping may result in considerable proportions of unresolved infections if robust methods for DNA extraction are not used. On the other hand, genotyping all three loci is expensive and may not be feasible in large-scale trials. Thus based on our experience, stepwise genotyping of only two loci (*msp2* and *msp1* or *glurp*) coupled with use of more advanced DNA extraction methods seems a reasonable compromise.

In summary, genotyping improved the accuracy of determining the parasitological failure rates. However, the genotyping performance varied markedly between studies, emphasizing the need for diligence in sample collection in the field and processing in the laboratory. Our findings support the use of molecular genotyping in prolonged efficacy

studies and suggest that stepwise genotyping of only two markers can be used reliably in resource constrained countries. Distinction of recrudescence from new infection will provide policy makers with optimal efficacy data for making evidence-based decisions on malaria treatment strategies.

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8 Chapter 8

8.1 Molecular genotyping in a malaria treatment trial in Uganda - unexpected high rate of new infections within two weeks after treatment.

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Molecular genotyping in a malaria treatment trial in Uganda - unexpected high rate of new infections within two weeks after treatment.

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Key words: genotyping, recrudescent, re-infections, *Plasmodium falciparum*, malaria, combination therapy, Uganda

Abstract

Polymerase chain reaction (PCR) genotyping of malaria parasites in drug efficacy trials helps differentiate reinfections from recrudescences. A combination therapy trial of one (n = 115) or three (n = 117) days artesunate (1AS, 3AS 4mg/kg/day) plus sulfadoxine/pyrimethamine (SP) versus SP alone (n=153) was conducted in Mbarara, a mesoendemic area of western Uganda. All paired recurrent *Plasmodium falciparum* parasitaemias on Days 7, 14, 21 and 28 post treatment were genotyped by PCR amplification and analysis of *glutamate rich protein* (*glurp*) and *merozoite surface proteins* (*msp*) *1* and *2* genes to distinguish recrudescent from new infections. 156 (1AS = 61, 3AS = 35, SP alone = 60) of 199 paired recurrent samples were successfully analysed and were resolved as 79 recrudescences (1AS = 32, 3AS = 8, SP = 39) and 77 as new infections (1AS = 29, 3AS = 27, SP = 21). The ratios of proportions of new to recrudescent infections were 0.2, 0.9, 1.4, and 1.9 on Day 7, 14, 21, and 28, respectively (P <0.001, χ^2 test for linear trend). Unexpected high new infection rates were observed early in follow up on Days 7 [5/26 (19.2%)] and 14 [24/51 (47.1%)]. These results impact significantly on resistance monitoring and point to the value of genotyping all recurrent infections in antimalarial trials.

Introduction

The World Health Organisation (WHO) *in vivo* antimalarial efficacy testing protocol is instrumental in assessing and monitoring the emergence and extent of parasite resistance to antimalarial drugs (WHO, 1973). The current WHO *in vivo* protocol for high transmission areas recommends 28 days of follow-up, stipulating that recurrent parasites should be genotyped by polymerase chain reaction (PCR) to distinguish recrudescent from new infections (WHO, 2003; WHO, 2006). The ability to discriminate newly acquired infections by comparison of baseline and recurrent parasite genotypes allows a more accurate estimate of the true levels of treatment failures. However, due to resource constraints in malaria endemic areas, genotyping all recurrent infections, particularly in large trials, is expensive and may not be feasible. To compensate for such limited recourses, correction of treatment outcome could be done by only genotyping post-day 14 recurrences whilst assuming that most recurrent parasites before or on Day 14 are likely

to be due to recrudescences. This strategy was adopted for a series of WHO/TDR coordinated clinical trials assessing artesunate in combination with standard antimalarial drugs for the treatment of paediatric falciparum malaria in several African countries, including Burkina Faso, Gabon, The Gambia, Sao Tomé, Senegal, Uganda, Malawi and Kenya (Adjuik *et al.* 2002; Adjuik *et al.* 2004; Priotto *et al.* 2003; von Seidlein *et al.* 2000; Sirima *et al.* 2003; Obonyo *et al.* 2003; Gil *et al.* 2003). Herein, we report the PCR defined reinfection rates on Days 7, 14, 21 and 28 in the Ugandan efficacy trial (Priotto *et al.* 2003) and assess its effect on the efficacy outcome.

Methodology

Brief description of the study

A 28 days antimalarial combination efficacy trial was conducted in Mbarara District Hospital in Uganda, an area of seasonal, mesoendemic malaria using artesunate (AS 4mg/kg/day)/placebo plus standard dose sulfadoxine/pyrimethamine (S/P) for treating acute uncomplicated, falciparum malaria in children: S/P alone (n=168), S/P+AS 3 days (n=126), S/P+AS 1 day (n=126). The trial profile and clinical findings are detailed in Priotto *et al.* 2003.

PCR amplification

Blood for PCR analysis was collected onto Isocode stix® (Schleicher & Schull, Dassel, Germany) on Days 0, 7, 14, 21, and 28 and DNA extracted following the manufacturer's instructions (i.e. washing and boiling of Isocode stix®). *P. falciparum* in paired samples collected on Days 0 and any day (7, 14, 21 or 28) of recurrent parasitaemia were genotyped by analysing the *glurp*, *msp* 1 and 2 loci. PCR amplifications were done at Ifakara Health Research and Development Centre (IHRDC) laboratory using an MJ Thermal Controller PTC-100™ (MJ Research Inc. Watertown, USA). Primary *glurp, msp1* and *msp2* PCR reactions were multiplexed whereas nested PCR amplifications were done separately for each locus. The primary and nested amplifications were carried out in 20μl and 30μl reaction volumes using 5μl of template DNA and 2μl of primary PCR product, respectively. All oligonucleotide primers and reference DNA were obtained from Malaria Research and Reference Reagents Resource Centre (MR4). The

oligonucleotide primers have been described elsewhere (Felger *et al.* 1994; Irion *et al.* 1998; Snounou *et al.* 1999). Temperature cycling parameters were: initial denaturation at 94ºC for 5 minutes followed by 30 cycles of denaturation at 94ºC for 1 minute, annealing at 55ºC for 2 minutes (for primary PCR) or 58ºC for 2 minutes (for nested PCR) and extension at 72ºC for 2 minutes. The last extension cycle was prolonged for 10 minutes. 10µl of *msp2* PCR product were digested with 3 units of *Hin*f III for 2.5 hrs at 37ºC and the resulting fragments were resolved on 10% polyacrylamide gels. The *glurp* and *msp1* PCR products were directly resolved on 2% agarose gel. For better comparison of fragments paired samples were loaded onto the gels side by side. Gels were stained with ethidium bromide, visualized under UV illumination, photographed and discrimination done as described.

Definitions of recrudescent and new infections and data analysis

A recurrent parasitaemia was classified as a recrudescence (true failure) if the following conditions were met: (i) all alleles of the three loci in the baseline and recurrent parasitaemia were identical, (ii) a sharing of baseline and recurrent alleles but with some missing alleles in the recurrent parasitaemia, and (iii) a sharing of baseline and recurrent alleles but with new alleles in recurrent sample that were not observed at baseline. A recurrent parasitaemia was classified as a new infection or treatment success, if the allelic pattern for any one of the loci differed completely between the baseline and recurrent samples (Snounou and Beck 1998). The clinical and molecular genotyping data were analysed using Stata v 8.0 (Stata Corporation Inc, Texas, USA)

Results

Of the 373 patients who completed the 28 days follow-up 190 had recurrent parasitaemias. All these recurrent parasitaemias were genotyped by PCR amplification to distinguish recrudescence from reinfection. Table 1 provides a summary of genotyping results per treatment arm. PCR was unresolved for 34 patients due to 8 incomplete paired samples and failure to extract/amplify DNA on 26 recurrent samples. The proportions of new infections on Days 7, 14, 21, 28 were 5/26 (19.2%), 24/51 (47.1), 29/50 (58%) and 19/29 (65.5%), respectively. The corresponding values for the recrudescences were 21

(80.8%), 27 (52.9%), 21 (42%), and 10 (34.5%). Figure 1 illustrates proportions of new and recrudescent infections on Days 7, 14, 21 and 28. The ratios of proportions of new to recrudescent infections were 0.2 (19.2:80.8), 0.9 (47.1:52.9), 1.4 (58:42), and 1.9 (65.5:34.5), on Days 7, 14, 21, and 28, respectively. The χ^2 test for linear trend was 12.8 (P <0.001), demonstrating a linear increase in the odds ratios on Days 14, 21 and 28 compared to Day 7. The frequencies of new infections increased over time (i.e. lowest on Day 7, highest on Day 28) but with high rates on Days 7 and 14. The effect of the 29, pre Day 14, new infections is to lower the overall failure rate (all three arms combined) by 7.8% (29/373) by counting unresolved PCR data as failures, or by 8.6% (29/339), if unresolved PCR data are excluded.

Table 1. Distribution of recrudescences, new infections and unresolved recurrent infections in a cohort of Ugandan children treated for *Plasmodium falciparum* malaria.

Genotyping status	SP	SP+1AS	SP+3AS	Total
Recrudescence	39	32	8	79
New infection	21	29	27	77
Unresolved	14	11	9	34
Total	74	72	44	190

Note: SP = sulfadoxine-pyrimethamine, 1AS = one day artesunate, 3AS = three days artesunate.

Discussion

In the artesunate based combination therapy trials conducted by WHO in Sub-Saharan African countries only post-day 14 recurrent parasitaemias were genotyped to distinguish recrudescent from new parasites and treatment failure rates adjusted accordingly. In the present study we genotyped all recurrent parasitaemias observed on Days 7, 14, 21 and 28 from one site, Mbarara, Uganda, in order to assess pre- and post- Day 14 re-infection rates. We observed unexpectedly high frequencies of genotypically new infections on Days 7 (~19%) and 14 (~47%). Although the entomological inoculation rate was not measured in this study, cumulative re-inoculations during follow-up have resulted in these new infections which represent an important fraction of the total number of

recurrent parasitaemias. Given that the prepatent period for *P. falciparum* is 6-12 days, and that pyrimethamine has weak causal prophylactic activity, parasitaemias detected on Days 7 or 14 after initial parasite clearance may represent early new infections. Therefore, the assumption that recurrent parasitaemias on Days 7 and 14 in high transmission areas are due to recrudescence and, therefore, should be excluded from genotyping assays may be erroneous. These findings strongly suggest that genotyping should also be conducted on recurrent parasitaemias recorded before and on Day 14.

Figure 1. Distribution of PCR-determined recrudescence vs. new infections during follow up in Ugandan children with uncomplicated falciparum malaria treated with either artesunate or placebo plus standard dose sulfadoxine/pyrimethamine.
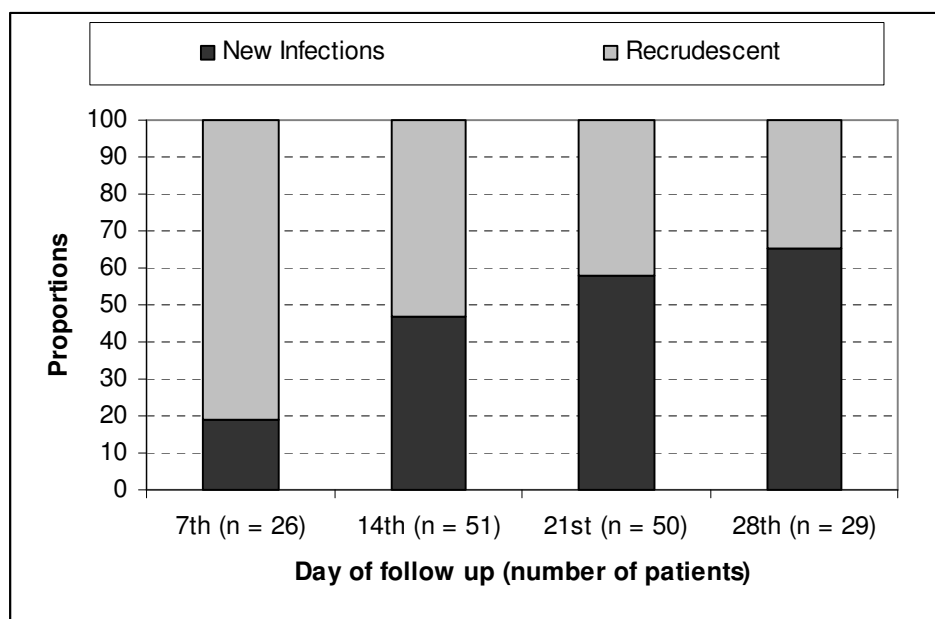
Characterizing these early recurrences may change substantially our appreciation of drug efficacy. By taking these new infections into account, the overall failure rate fell by ~7 - 8%. This change could have a profound effect on deciding drug policy if the efficacy of a given drug is not deemed to have reached a predefined threshold for changing drug policy. The using of PCR-corrected efficacy end points in malaria drug trials has become standard practice but there are variations in the way that the PCR data have been

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interpreted (Basco *et al.* 2002**;** Basco and Ringwald 2000**;** Brockman *et al.* 1999**;** Cattamanchi *et al.* 2003; Magesa *et al.* 2001; Ranford-Cartwright *et al.* 1997; Happi *et al.* 2004). This calls for a standardised genotyping protocol for areas of intense malaria transmission.

It has been speculated that new infections might already be present during the clinical presentation but sequestered and not detected by PCR only to be picked up at the time of recurrence. Although this might indeed occur in few cases ample evidence exists that clinical episodes are less complex. A study by Irion *et al.* (1998), found that less than 2% of recrudescent genotypes were absent on Day 0 but were detected on Day 3. Similarly, Farnert and Bjorkman (2005) detected the same genotypes in consecutive samples obtained every 12 hours for at least three days post treatment in Swedish non immune travelers who acquired falciparum malaria in Africa. These observations suggest that single time-point samples may reliably represent all subpopulations present prior to treatment. A further complication in the interpretation of paired genotype data arises from infections with new parasites possessing identical genotypes to those present on Day 0, leading to an erroneous diagnosis of recrudescence. However, in endemic areas the probability of this occurrence is rather low and negligible when two or more discriminatory markers are being used. We believe this theoretical possibility is not an important consideration in the interpretation of our findings because we observed a high rate (~33%) of new infections (different genotypes) within the first 14 days of follow up.

In our study, laboratory failure to amplifly/detect parasite genetic material in the recurrent samples was the main cause of the loss of genotyping data. This PCR failure is highly attributable to the low parasite densities in the recurrent samples coupled with a less invasive/rigorous DNA extraction method consisting of simple washing and boiling of the Isocode stix®. The performance and use-effectiveness of PCR genotyping will be detailed in a general report on the combination therapy trials that will be published separately.

The definition of drug resistant malaria and the reporting of drug efficacy data are becoming more complicated with our better understanding of *P. falciparum* molecular genetics, seasonal variations in malaria transmission, drug pharmacokinetics (e.g. longer follow up is required for long half life drugs) and the intrinsic parasiticidal effects of antimalarial drugs (White 1998). Further studies are needed to assess the importance of genotyping recurrent parasitaemias observed between Day 7 and 14 and how this might refine the current WHO definitions of late clinical and parasitological failures. More robust definitions will be beneficial to policy makers.

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9   Chapter 9

9.1   General discussion and conclusion

9.1.1   Assessment of SP efficacy and role of SNPs in resistance

Tanzania mainland revised malaria treatment policy in 2001, switching the first line drug from CQ to SP. The baseline studies conducted prior to this change, using the 1996 WHO 14 day, then standard protocol, showed that by the time the change was made, SP failure rate and molecular markers of resistance were already prevalent, probably because SP had been used as second line drug for many year in Tanzania. Therefore, it was predicted that the useful therapeutic life (UTL) of SP in Tanzania would be compromised shortly after widespread use of the drug. These findings provided policy makers with evidence on which SP was adopted as an interim first line antimalarial drug, as a rescue measure while combination therapies were being evaluated for long-term use.

Two to three years later, SP efficacy monitoring studies using the 28 days 2002 WHO protocol recorded unacceptably high failure rates. Restricting the analysis to outcomes at day 14 led indeed to misleadingly high efficacies, 85.2% and 86.3% in 2003 and 2004, respectively. Similarly, using short follow-up period, other studies recorded efficacies as high as 90.8% in Tanzania (Lemnge *et al.* 2005) and 83% in Malawi (Plowe *et al.* 2004). Longer follow-up periods and clearance of both parasitaemia and symptoms are the most accurate determinants of the intrinsic resistance of the parasite to a drug (Sibley and Hunt 2003; Stepniewska *et al.* 2004). Therefore, it follows that the decision to switch to SP may have been based on under-estimated efficacy levels and insufficient evidence. Had the baseline SP efficacy assessment been based on the 28-Day protocol, higher failure rates would have been recorded and probably Tanzania would not have opted for SP. Alternatively, if the baseline data represented the true SP failure rates, then observation of > 40% SP failure rate only 2 -3 years after widespread use of the drug confirms the postulated rapid spread of resistance to SP in high transmission areas. In any case, the clinical and molecular findings in the 2003/2004 studies provided comprehensive evidence of increasing resistance to SP and fully justify the decision of the Ministry of

Health in Tanzania to review the current malaria treatment policy in favor of AL combination therapy.

This study broadly and deeply investigated the potential of SP resistance markers in predicting treatment outcomes (using symptomatic children < 5 years in health facilities) and resistance levels in the health facilities (using asymptomatic infections in the communities). In assessing the relationship between frequency of SP markers of resistance and SP failure rate, we adopted the GFI concept (Djimde *et al.* 2001b), as used in predicting CQ treatment failure. It should be noted that direct comparison of molecular data collected prior to and after adoption of SP can only be done with caution since the study designs and SNP detection methods used in the two studies are different. However, preliminary verification showed a very high agreement between PCR-RFLP and DNA microarray obtained data (Crameri *et al.* unpublished data). Therefore, assuming that the profile of mutant genotypes in symptomatic children in the health facility reflects that in their respective communities (as determined by Marfurt *et al.* unpublished data), these findings show a rapid increase in the levels of *Pfdhfr* and *Pfdhps* mutant genotypes in Tanzania after only a few years of SP use. However, because of the under-estimated SP failure rates in the baseline studies, these changes cannot be regarded as consistent with the observed change in SP failure rate from 14.4% in 1999 to 41% in 2004.

The drastic increase in individual *Pfdhfr* and some *Pfdhps* mutant alleles in the communities between 2003 and 2004 did not reflect the insignificant changes in SP failure rates. Accumulation of mutations in *Pfdhfr* and *Pfdhps* confers increased resistance to SP (Plowe *et al.* 1997). Therefore, the correlation of SP failure rate to molecular markers of resistance is customary assessed by using a combination rather than individual of mutations. The quintuple mutant genotype was shown to be important predictor of SP failure in Malawi (Kublin *et al.* 2002), Uganda (Kyabayinze *et al.* 2003) and Nigeria (Happi *et al.* 2005). However, this genotype did not seem to be correlated to SP treatment failure in both health facility- and community based studies, therefore, may not be useful in the surveillance of dynamics of SP resistance in Tanzania.

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Two previous studies in Tanzania named triple-*Pfdhfr* mutant genotypes as the main predictor of resistance (Mutabingwa *et al.* 2001) or early warning tool (Alifrangis *et al.* 2003). The GFI (1.96 – 2.1) calculated in these studies by using triple-mutant genotype at health facilities closely matched those reported by using quintuple mutant genotype in Uganda (1.9) Kyabayinze *et al.* 2003 and Malawi (2.2) (Kublin *et al.* 2002). However, the ratios of prevalence of the triple-*Pfdhfr* mutant genotype in the catchment areas and their respective SP failure rates are more variable (1.2 – 2.3) than the GFIs obtained exclusively from health facility-based data. The relationship between SP resistance markers and SP treatment failure observed in the two studies is not consistent and unclear. However, the little evidence of relationship gathered pointed to triple-*Pfdhfr* mutant genotype as an important molecular marker for monitoring the dynamics of SP resistance. Its high prevalence in both 2003 and 2004 was consistent with high SP failure rates in these two years.

The observed poor association between molecular markers of resistance and SP treatment failure rate can be attributable to a number of factors. The outcome of antimalarial treatment depends on many factors, such as the host's age, immunity, and nutritional status, and parasite's genotype, its parasite density and drug-related factors. It has been shown that the risk of treatment failure is inversely proportional to transmission intensity and was not explained by differences in molecular markers of antimalarial drug resistance (Francis *et al.* 2006).

Contrary to SP resistance markers, the frequencies of the main mediators of CQ resistance (*Pfcrt* 76 and *Pfmdr1* 86 Asn) recorded in our study are lower than those reported (Schneider *et al.* 2002; Ndejembi *et al.* unpublished data) prior to CQ withdrawal in Tanzania. In addition, between 2003 and 2004 the *Pfcrt* 76 also showed a decreasing trend in Ipinda and Mkuranga communities. However, it increased in Mlimba, consistent with an increase in *Pfmdr1* 86 Try allele whose frequency in the former sites did not change significantly. The increase in Mlimba may be promoted by uncontrolled use (through prescription or self-medication) of QN and AQ for uncomplicated malaria in the area.

The lack of ATPase6 mutant genotype, despite the reported uncontrolled use of artemisinin monotherapy in urban Dar es Salaam (Kachur *et al.* 2006), an area close to Mkuranga (Coast Region) sites, suggest that resistance to artemisinin has not been selected in Tanzania. This is consistent with high (~94%) AL parasitological cure rates recorded in a part of the Coast Region in Tanzania (Falade *et al.* 2005). These observations support the Ministry of Health's decision to adopt this combination as first line treatment for uncomplicated and further encourages rational use of AL and discourages the use of artemisinin monotherapy in order to prevent development of resistance.

The capacity to detect many SNPs in many resistance conferring genes makes DNA microarray technology a potential tool for monitoring dynamics of antimalarial drug resistance. The majority of Sub-Saharan African countries are revising their malaria treatment policy to switch to ACTs. The DNA microarray technology may provide a suitable tool for simultaneously assessing, on a large scale, the spread of resistance to ACTs and reversal of CQ and SP resistance after being withdrawn. However, the interval between assessments should be long enough to better appreciate parasite genotypic dynamics following alteration of drug pressure. Reemergence of sensitivity points to the possibility of recycling the limited number of safe and cheap drugs as combination partners with artemisinins (Laufer and Plowe 2004).

Admittedly, this new promising technology is in its infancy stage. There is still a need to further validate its field applicability, both in high and low transmission areas where MOI differs. Due to time constraints post-PCR detection of SNPs in *Pfcrt* 71, 74, 75 and *Pfdhps* 436, 581, 640 and 645 was not optimized. Therefore, these mutations, which are deemed of less importance in resistance, were not analyzed. There is a need to optimize post-PCR parameters that would enable detection of all *P. falciparum* resistance-associated mutations in order to improve the comprehensiveness of the technique in the community-based surveillance of resistance.

9.1.2 Distinction of recrudescent from new infections

The molecular genotyping of post day 14 recurrent infection from WHO combination therapy trials was centralized at only two places in order to avoid inter-laboratory variations. The stepwise genotyping protocol which excludes new infections detected by any one marker distinguished recrudescence with high precision than would have been using another protocol (Ranford-Cartwright *et al.* 1997; Happi *et al.* 2004; Cattamanchi *et al.* 2003; Magesa *et al.* 2001) in which a new infection must be recorded by all three markers. However, controversy surrounds the interpretation of genotyping data when a recurrent sample contains additional allele(s) not seen in admission sample. In longitudinal studies examining the dynamics of malaria infections (i.e. the acquisition of new or loss of old parasites), new infections are always recorded if a new allelic pattern is detected even when alleles from prior infection/s persist. This contrasts with drug efficacy studies in which this finding indicates a failure of the drug to clear an infection present before treatment. Therefore, in this study such cases were classified as recrudescence.

Molecular genotyping refined treatment outcomes. The performance of the system was better but varied markedly between studies, pointing for diligence both in the field and laboratory data generation processes. In most sites, the differences between crude and the two PCR-adjusted treatment failure rates were appreciably high (> 10%). Additional genotyping of pre-Day 14 recurrences in Uganda site identified many more new infections and further reduced the PCR-adjusted parasitological failure rate by 8%. Thus the assumption that most pre Day 14 recurrences are due to recrudescence may be erroneous. Therefore, the study recommends that all recurrent infections in malaria treatment trials/studies in high transmission areas should be genotyped. Although the mean difference between the two Day 28 PCR-adjusted failure rates (including or excluding unresolved infections) was small (3%), there is a need for further studies to establish the best way of treating unresolved recurrent infections.

9.2   Recommendations

The high SP failure rates fully substantiate the revision of malaria treatment policy in Tanzania. These findings further stress that, for drugs with long half-life such as SP, establishment of cut-off points for policy change in high transmission areas should consider both clinical and parasitological responses beyond day 14.

More studies need to be done to assess the applicability of triple-*Pfdhfr* mutant genotype in community-based approach for monitoring dynamics of SP resistance in Tanzania. However, it should also be born in mind that the majority of the community asymptomatic infections were multiclonal. Therefore, estimation of correct proportions of various genotypes is complex. Unless linkage disequilibrium is assessed, the observed combination genotypes in this study may be slightly exaggerated. Therefore, the relationship needs to be re-assessed after establishing the linkage disequilibrium of the observed alleles. For a fruitful fight against development and spread of resistance, there is need for active involvement in creating awareness to the general community on the rational use of antimalarials and abidance to national policy

Stepwise genotyping may result in considerable proportions of unresolved infections if robust methods for DNA extraction are not used. On the other hand, genotyping all three loci is expensive and may not be feasible in large-scale trials. Hence stepwise genotyping of only two loci (*msp2* and *msp1* or *glurp*) coupled with use of more advanced DNA extraction methods seems a reasonable compromise. This approach need to be validated and considered for adoption as a standard integral part in malaria efficacy studies.

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Curriculum vitae

Biography

First Name: Kefas Nasongelya Mugittu

Date of Birth: 20th November; 1967

Place of Birth: Iramba, Singida, Tanzania.

Marital status: Married

Religion: Christian.

Nationality: Tanzanian

Current address:

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Current position

Research Scientist at Ifakara Health Research and Development Centre (IHRDC)

Academic qualifications

September 1996 – December 1998: Master of Veterinary Medicine (MVM) of Sokoine University of Agriculture (SUA. Major: molecular epidemiology of African Animal Trypanosomosis.

September 1990 - September 1995: Bachelor of Veterinary Medicine (BVM) of Sokoine University of Agriculture (SUA).

Professional duties

February 2003: Attended a WHO/TDR /MR4-organised International training workshop on Microarray Technology for Malaria Parasites National Center for Genetic Engineering and Biotechnology (BIOTEC), held in Bangkok, Thailand, 3th - 7th March 2003.

2000 – To date: Member of WHO/TDR -coordinated International Artemisinin Study Group. Role: molecular genotyping of *P. falciparum* to distinguish recrudescence from new infection and assessment of antimalarials drug resistance markers.

2000 - 2003: Involved in the WHO/TDR Antimalarial Combination Therapy trials that were conducted in 11 countries in Africa by the International Artemisinin Study Group. *P. falciparum* genotyping was centrally done (by myself) at IHRDC laboratories.

August 2002: Attended a WHO/TDR-organised training workshop on *In vitro Plasmodium falciparum* Drug Succeptibility Testing. Held in Cotonou, Benin, 19th -29th August 2002.

September 2001: Attended an IAEA-organised Regional Training Course on Radionuclide and Molecular Techniques in the Detection of Drug-resistant Malaria, held in Kampala, Uganda, 17th -28th September 2001.

August – November 1999: (i) Training in Molecular Biological Methods in Malaria Research in the Department of Parasitology and Infection Biology, Swiss Tropical Institute (STI). Methods learnt include Identification of *pfpfdhfr*, *pfpfdhps*, *pfmdr 1* and *pfpfcrt* variants. (ii). Discrimination of *P. falciparum* recrudescence from re-infections by genotyping *Pfmp1, 2* and *Pfglurp*.

September 1997 - September 1998: Research Fellow in Trypanosomosis Epidemiology project, International Livestock Research Institute (ILRI) - Kenya and attended a training on DNA based parasite detection and identification methods.

September 1995 - September 1996: In-charge Veterinarian; Prima Veterinary Surgery and Pharmacy

Award

December 1998: The Sokoine University of Agriculture Convocation Award to a Student submitting a dissertation within a period of two and a half years and with a recommendable performance in the course work assessment.

Publications

Kefas Mugittu, Andreas Crameri, Nicolas Maire, Honorati Masanja, Salim Abdulla, Ingrid Felger, Hassan Mshinda, Tom Smith, Blaise Genton, Hans-Peter Beck. Community-based approach to assess antimalarial drug resistance: establishment of a resistance genogram by microarray technique. *In preparation*

Kefas Mugittu, Martin Adjuik, Georges Snounou, Bob Taylor, Hassan Mshinda, Piero Olliaro, Hans-Peter Beck. Molecular epidemiology of *Plasmodium falciparum*-malaria in Sub-Saharan Africa: molecular markers of antimalarial drug resistance are risk factors for late treatment failure. *In preparation*

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Kefas Mugittu, Gerardo Priotto, Jean-Paul Guthmann, James Kiguli, Martin Adjuik, Georges Snounou, Hans-Peter Beck, Hassan Mshinda, Piero L Olliaro, Walter RJ Taylor. Molecular genotyping in a malaria treatment trial in Uganda - unexpected high rate of new infections within two weeks after treatment. *Accepted in TM&*IH

Kefas Mugittu, Salim Abdulla, Nicole Falk, Ingrid Felga, Hassan Mshinda, Hans-Peter Beck and Blaise Genton. Efficacy of sulfadoxine-pyrimethamine in Tanzania two years after its use as first line antimalarial drug: assessment protocol and implications for treatment policy strategies. *Malaria Journal* 2005, 4:55.

Kefas Mugittu, Modesta Ndejembi, Allen Malisa, Martha Lemnge, Zurlfikar Premji, Alex Mwita, Watoky Nkya, Johannes Kataraihya, Salim Abdulla, Hans-Peter Beck, and Hassan Mshinda. Therapeutic efficacy of sulfadoxine-pyrimethamine and prevalence of resistance markers in Tanzania prior to revision of malaria treatment policy: *Plasmodium falciparum dihydrofolate reductase* and *dihydropteroate synthase* mutations in monitoring *in vivo* resistance. *American Journal of Tropical Medicine and Hygiene* 2004; 71:696-702.

M Adjuik, P Agnamey, A Babiker, J Baptista, S Borrmann, P Brasseur, P Carnevale, M Cisse, R Collins, U D'Alessandro, N Day, W de Boom, T Doherty, G Dorsey, P Garner, S Gikunda, V Gil, B Greenwood, J P Guthmann, M C Henry, M R Kamya, P G Kremsner, E Konaté, S Krishna, D Lalloo, P Lange, M Loolpapit, G Malenga, W Marquino, K Marsh, P Milligan, M Molyneux, **K Mugittu**, J Niangue, F Nosten, F Ntoumi, C Obonyo, F Ochieng, P Olliaro, A J Oloo, L Osorio, L Pinoges, G Priotto, P J Rosenthal, T Ruebush, J Simpson, S Sirima, E Some, W Taylor, F ter Kuile, A Tiono, L von Seidlein, B Watkins, N White (chair). International Artemisinin Study Group. Artesute combinations for treatment of malaria: meta-analysis. *Lancet* 2004; *363: 9 – 17.*

Charles O. Obonyo, Francis Ochieng, Walter R.J. Taylor, Samuel A. Ochola, **Kefas Mugitu**, Piero Olliaro, Feiko ter Kuile, Aggrey J. Oloo. Artesunate plus Sulfadoxine-Pyrimethamine for uncomplicated malaria in Kenyan children: A randomized, double blind, placebo- controlled trial. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 2003; 97 (5) 585-591.

Sirima SB, Tiono AB, Konate A, Diarra A, Castelli F, Pinoges L, **Mugittu K**, Taylor WR, Olliaros PL. Efficacy of artesunate plus chloroquine for the treatment of

uncomplicated malaria in children in Burkina Faso: a double-blind, randomized, controlled trial. *Transactions of the Royal Society for Tropical Medicine and Hygiene* 2003; 97 (3) 345-349.

Mugittu KN, Silayo RS, Majiwa PAO, Kimbita EK, Mutayoba BM and Maselle R. Application of PCR and DNA probes in the characterization of trypanosomes in the blood of cattle in farms in Morogoro, Tanazania. *Veterinary Parasitology* 2001 94:3, 179 – 191.

Mugittu KN, Mosha RD, and Matovelo JA. Toxicity of *Clotalaria ochroleuca* (marejea) in chickens. *Tanzania Veterinary Journal* 1996 16: Supp. 2, 38 – 46.

Papers

Malisa AL, **Mugittu KN**, Ndejembi M, Mrema E, Mayagaya V, Urassa H and Mshinda H. Biotechnology in the growing momentum of globalisation: Inseparable forces affecting public health at a global scale. Proceedings of the Twenty fist Annual Scientific Conference of the Tanzania Public Health Association held at Arusha International Conference Centre (AICC), Arusha, Tanzania from 4th to 8th November 2002.

Ndejembi MP, **Mugittu KN**, Mutabingwa TK, Nkya W, Senkoro K, Urasa, H, Abdulla S, Drackeley C, Schellenberg D, Plowe C, and Mshinda H. The use of *Plasmodium falciparum* chloroquine resistance transporter *K76T* as a marker for chloroquine resistance in Tanzania. Program and abstracts of the 50th Annual Meeting of the *American Society of Tropical Medicine and Hygiene*.

Mugittu KN, Silayo RS, Kimbita EN, Mutayoba BM and Maselle RM. Employment of DNA-based methods for the detection of bovine trypanosomes in Morogoro, Tanzania. Proceedings of the first University-wide Scientific Conference Held at the Institute of Continuing Education (ICE), SUA from 5th -7th April 2000, pp. 879 – 835.

During my studies, I attended lectures by the following lecturers:

M. Tanner, N. Weiss, R. Brun, G. Pluschke, U. Jenal, T. Smith, P. Vounatsou, J. Utzinger, C. Burri, M. Käser, C. Daubenberger, S. Wittlin, J. Keiser