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Review

Monitoring antimalarial drug resistance: making the most of the tools at hand

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Summary

Most countries in resource-poor, malaria-endemic areas lack current and comprehensive information on antimalarial drug efficacy, resulting in sub-optimal antimalarial treatment policies. Many African countries continue to use chloroquine despite very high rates of resistance, and others have changed policies based on limited data, with mixed success. Methods for measuring antimalarial drug efficacy and resistance include *in vivo* studies of clinical efficacy and parasitological resistance, *in vitro* susceptibility assays and molecular markers for resistance to some drugs. These methods have the potential to be used in an integrated fashion to provide timely information that is useful to policy makers, and the combined use of *in vivo* and molecular surveys could greatly extend the coverage of resistance monitoring. Malawi, the first African country to change from chloroquine to sulfadoxine/pyrimethamine at the national level, serves as a case study for resistance monitoring and evidence-based antimalarial policies. Molecular, *in vitro* and *in vivo* studies demonstrate that chloroquine-sensitive parasites reemerged and now predominate in Malawi after it switched from chloroquine to sulfadoxine/pyrimethamine. This raises the intriguing possibility of rotating antimalarial drugs.

Key words: *Plasmodium falciparum*, drug resistance, PfCRT, dihydrofolate reductase, dihydropteroate synthase, chloroquine, sulfadoxine/pyrimethamine.

Introduction

While we wait for a malaria vaccine, case management with prompt and effective treatment remains the mainstay of malaria control (Winstanley, 2000), and chloroquine has been by far the most important and successful drug used to treat falciparum malaria (Wellems and Plowe, 2001). Chloroquine-resistant falciparum malaria has been associated with large increases in morbidity and mortality due to malaria (Greenberg et al., 1989; Trape et al., 1998).

A consensus has begun to emerge that the development of drug-resistant malaria should be delayed through a strategy of routinely employing combinations of drugs (White et al., 1999; Nosten and Brasseur, 2002), as is done with HIV and tuberculosis. Despite some obstacles to implementing effective combination therapies in Africa (Bloland et al., 2000), programs evaluating the efficacy and effectiveness of new combinations are now underway in Africa (von Seidlein et al., 2000; Doherty et al., 1999; Adjuik et al., 2002). Among the challenges facing combination antimalarial therapy in Africa are cost and safety issues.

Because of their rapid reduction of parasite biomass and the complete absence of documented resistance despite over 2000 years of use, the artemisinin derivatives are components of many candidate combinations. Artemisinin derivatives may also reduce malaria transmission and spread of resistance through their gametocytocidal properties (Price et al., 1996; Nosten et al., 2000). However, some safety concerns persist about this class of drugs, and their eventual cost is uncertain, so it is prudent to consider other drugs suitable for combination therapy.

Chlorproguanil–dapsone (also known as Lapudrine™–dapsone or LapDap) is an antifolate combination similar to sulfadoxine/pyrimethamine (SP) but for two important features: (1) it is rapidly eliminated and therefore exerts less selective pressure for resistance-conferring parasite mutations than does SP (Winstanley et al., 1997; Nzila et al., 2000b) and (2) it is active against the SP-resistant forms of the parasite that are found in Africa (Mutabingwa et al., 2001a,b; Kublin et al., 2002). Moreover, a pediatric course of treatment of LapDap is estimated to cost $0.15 (Mutabingwa et al., 2001b), making it orders of magnitude less expensive than any marketed antimalarial drug other than chloroquine and SP.

However, the present reality is that many African countries with high rates of chloroquine-resistant malaria continue to use chloroquine as their first-line antimalarial, largely due to concerns that alternative drugs are either too expensive or would only provide interim solutions. This prolonged state of

near-paralysis is also exacerbated by a paucity of current and comprehensive information on antimalarial drug efficacy in most endemic areas.

In vivo and *in vitro* methods for measuring drug-resistant malaria

In vivo studies remain the gold standard for monitoring antimalarial drug efficacy and are the primary source of information used by policy makers to shape recommendations for malaria chemotherapy and prophylaxis. *In vivo* methods can be used to measure therapeutic efficacy and/or parasitological resistance, following protocols published by the World Health Organization (1973, 1996). The outcomes of these methods are defined in the Appendix. Briefly, therapeutic efficacy is characterized by early reduction of parasite density followed by lack of fever in the presence of recurrent infection. Recurrent or persistent infection without fever is considered to be an adequate clinical response. Parasitological resistance considers only the presence, absence and level of parasite density and does not take into account clinical factors such as fever or signs of severe malaria. There is a high degree of concordance between the two methods (Plowe et al., 2001, in press), but both probably overestimate the rate of early treatment failure/high-level resistance. Changes to improve the protocols for monitoring antimalarial therapeutic efficacy have been recommended but not yet implemented (World Health Organization, 2002).

These standard methods for measuring antimalarial drug efficacy use a 14-day follow-up period and thus provide information only on short-term efficacy, leading some to advocate longitudinal studies of drug efficacy. In addition to measuring efficacy at 14 days, longitudinal studies measure sustained efficacy with repeated use of the same regimen over time and measure incidence of malaria episodes, reflecting how the treatment would hold up under 'real world' conditions. The incidence of treatment episodes is an outcome that is highly relevant to public health policy makers, as it reflects not only the burden of disease but also the utilization of health resources. If a drug has a moderately impaired ability to eradicate parasites, it may still have high rates of therapeutic efficacy at 14 days but have more late recrudescenses associated with higher rates of recurrent episodes of symptomatic malaria requiring treatment. Longitudinal studies also permit assessment of how pharmacokinetic properties of drugs affect the incidence of treatment episodes.

The first such longitudinal trial of antimalarial drug efficacy was a double-blind, placebo controlled trial of SP and LapDap in Kenya and Malawi that compared the cumulative number of treatment episodes and rates of treatment failure for SP and LapDap when each drug was used to treat sequential episodes of malaria over the course of one year (Sulo et al., 2002). It was hypothesized that children receiving SP might have fewer malaria episodes than those being treated with LapDap because the long half-life of SP provided at least a month of prophylaxis against re-infection. However, this study found that despite the rapid elimination of LapDap, children treated with this drug did not have a higher incidence of malaria episodes than those treated with SP. This is probably because LapDap had higher efficacy and thus fewer late recrudescenses leading to retreatment. In effect, the potential disadvantage of lacking a long prophylactic effect due to LapDap's short half-life was offset by its better efficacy and possibly by less selection for antifolate-resistant parasites. Another recent longitudinal study found fewer treatment episodes in children treated sequentially for one year with artesunate–SP and amodiaquine–SP than in those treated with SP alone (Dorsey et al., 2002).

Although the information gained from *in vivo* studies is exactly what is needed to make rational and evidence-based malaria treatment policies, standard *in vivo* studies remain expensive and time-consuming, and longitudinal clinical efficacy trials are even more so. Most countries in malaria-endemic areas therefore conduct *in vivo* surveys at only a few sites and at infrequent intervals.

In vitro methods for measuring drug resistance (Nguyen-Dinh and Payne, 1980) have proven to be even more limited in scope and suitability for surveillance. They require that venous blood with a high parasite density be quickly frozen or transported cold to a facility for parasite cultivation, the methods are laborious, and failure to establish primary parasite growth is frequent. Micro-test assays of fresh parasite isolates are subject to variation and artifact, and their reliability for different drugs varies, with particularly poor results for SP. While more-rigorous *in vitro* tests of culture-adapted isolates are more reproducible, the processes of freezing, thawing and adaptation to culture also introduce the possibility of selecting sub-populations of parasites, so that the parasites ultimately assayed may be genetically and phenotypically unrepresentative of the original parasite population. Nevertheless, *in vitro* methods are indispensable for confirming and characterizing resistance and for establishing and confirming the molecular mechanisms of resistance.

The limitations of *in vivo* and *in vitro* methods for measuring drug-resistant malaria and the elucidation of molecular mechanisms of resistance to some antimalarial drugs have led to considerable research on molecular markers for resistance. While development of field-friendly molecular assays has been rapid, validation and implementation of these assays has moved at a slower pace.

Molecular basis of chloroquine resistance

Chloroquine resistance in *Plasmodium falciparum* is conferred by mutations in the parasite PfCRT, a putative transporter localized to the digestive vacuole (Fidock et al., 2000b). One mutation, Lys → Thr at codon 76 (76T), was perfectly associated with *in vitro* resistance in all progeny of a genetic cross between chloroquine-sensitive and chloroquine-resistant parental clones and among a set of geographically

Definitions

Genotype-resistance index (GRI) = prevalence of molecular marker/prevalence of parasitological resistance

Genotype-failure index (GFI) = prevalence of molecular marker/prevalence of therapeutic failure

Application

Measure prevalence of molecular marker
↓
Measure prevalence of parasitological resistance and therapeutic failure
↓
Calculate GRI and GFI
↓
Use molecular surveys to predict prevalence of resistance and failure
↓
Measured prevalence of marker/established GRI = predicted prevalence of resistance

Measured prevalence of marker/established GFI = predicted prevalence of failure

Fig. 1. Definitions of genotype-resistance and genotype-failure indices and schema for their subsequent application for surveillance of drug-resistant malaria and treatment failure.

diverse parasite isolates (Fidock et al., 2000a). The 76T mutation is always accompanied by a set of other *pfcrt* mutations, but the specific set of accompanying mutations is different in different geographic regions (Wellems and Plowe, 2001). This observation, and the finding that a naturally occurring parasite isolate that harbors the full complement of mutations except 76T is chloroquine sensitive but can be rendered resistant by the addition of 76T (Fidock et al., 2000b; Sidhu et al., 2002), firmly established the key role of the 76T mutation in chloroquine resistance *in vitro* and suggested that the other mutations are necessary to maintain protein function (which remains unknown) in the presence of 76T. Several field studies described in a recent review have since confirmed the absolute specificity of the *pfcrt* K76T to clinical chloroquine resistance (Wellems and Plowe, 2001).

Polymorphisms in *pfmdr1*, which encodes the *P. falciparum* P glycoprotein homologue 1, modulate chloroquine resistance in mutant *pfcrt*-harboring parasites *in vitro* (Reed et al., 2000), although their role *in vivo* has yet to be substantiated (Djimde et al., 2001a). The mutations most often cited as potential contributors to chloroquine resistance are *pfmdr1* N86Y and D1246Y.

Molecular basis of antifolate resistance

Pyrimethamine and chlorcycloguanil (the active metabolite of chlorproguanil) bind and inhibit dihydrofolate reductase (DHFR), and the sulfa drugs (including sulfadoxine and dapsone) inhibit dihydropteroate synthase (DHPS) by acting as analogues of *p*-aminobenzoic acid (PABA), a folate precursor (Foote and Cowman, 1994). Single nucleotide polymorphisms in the genes encoding the target enzymes of the antifolate drugs have been known for several years to be associated with *in vitro* resistance to these drugs. *In vitro* resistance of *P. falciparum* to pyrimethamine and to chlorcycloguanil is due to specific point mutations in *P. falciparum* DHFR, which is encoded by a bi-functional gene (*dhfr-ts*) also encoding thymidylate synthase (Peterson et al., 1988, 1990; Cowman et al., 1988; Zolg et al., 1989; Foote et al., 1990).

The *dhfr* mutations alter the shape of the active site cavity where the DHFR inhibitors bind the enzyme, resulting in differential binding affinities for the different drugs. A single point mutation causing a Ser → Asn change at codon 108 causes pyrimethamine resistance with a only a moderate loss of susceptibility to chlorcycloguanil. The addition of Asn → Ile51 and/or Cys → Arg59 mutations confers higher levels of pyrimethamine resistance. Ile → Leu164, when combined with Asn108 and Ile51 and/or Arg59, confers high-level resistance to both drugs.

Point mutations in the gene encoding DHPS have similarly been associated with *in vitro* resistance to the sulfa drugs and sulfones. This gene (*dhps-pppk*) is bi-functional, also encoding hydroxymethylpterin pyrophosphokinase. Mutations associated with decreased susceptibility to sulfas include Ser → Ala436, Ala → Gly437, Ala → Gly581, and Ser → Phe436 coupled with Ala → Thr/Ser613 (Brooks et al., 1994; Triglia and Cowman, 1994; Wang et al., 1997b; Robson et al., 1992). A Lys → Glu540 DHPS mutation was discovered in Bolivia (Plowe et al., 1997) and also appears to be linked with *in vitro* resistance to sulfa drugs (Triglia et al., 1997). Both the DHFR and DHPS mutations occur in a progressive, step-wise fashion, with higher levels of *in vitro* resistance occurring in the presence of multiple mutations (Plowe et al., 1997; Wang et al., 1997a).

Molecular markers for resistance

These resistance-conferring mutations in parasite genes have been unequivocally linked to *in vitro* resistance, but demonstrating clear associations between molecular markers and clinical treatment outcomes has been less straightforward. This is due in part to the complexity of analyzing relationships between multiple mutations on different genes and the multinomial treatment outcomes listed in the Appendix and in part because treatment outcomes are affected by factors other than parasite resistance, including the host immune response (Wellems and Plowe, 2001), micronutrient levels (van Hensbroek et al., 1995) and bioavailability and pharmacokinetics (Watkins et al., 1990). Nevertheless, molecular markers are now available that can predict resistance to SP (Kublin et al., 2002) and chloroquine (Djimde et al., 2001a) with reasonable reliability.

In the case of chloroquine, *pfcrt* 76T provides a single marker for chloroquine resistance, but establishing markers for SP resistance has been more difficult. In one study, pre-treatment DHFR and DHPS genotypes and treatment outcomes from a longitudinal trial of SP and LapDap for uncomplicated falciparum malaria were analyzed using a standardized system for interpreting PCR results. The presence together of all five DHFR and DHPS mutations that are found in Africa were strongly associated with SP failure and there was a statistical interaction between DHFR and DHPS mutations (Kublin et al., 2002). This 'quintuple mutant' associated with SP treatment failure was not associated with LapDap failure. Just two mutations, DHFR Arg59 and DHPS Glu540, were over 90% sensitive and specific for the presence of all five DHFR and DHPS mutations. It is likely, but not yet proven, that DHFR and DHPS mutations that are widespread in Southeast Asia and South America would cause resistance to LapDap, but the critical mutations are either rare (DHPS A581G) or absent (DHFR I164L) in surveys of clinical samples in Africa.

Mutations in *pfmdr1* (Price et al., 1999) and *pfcrt* (Cooper et al., 2002) have been found in laboratory studies to be associated with changes in susceptibility to artemisinin derivatives, but these *in vitro* differences are not associated with clinical failure, and clinical resistance has yet to be documented for this class of drug. Markers for resistance to most other antimalarial drugs are lacking because mechanisms of resistance are not yet understood at the molecular level. Molecular markers are beginning to be applied as tools for surveillance for SP and chloroquine resistance but are, at present, still primarily used as research tools. Molecular markers can provide direct and convincing evidence of selection for resistant parasites by antimalarial drug treatment (Curtis et al., 1998; Diourte et al., 1999; Nzila et al., 2000a; Djimde et al., 2001a) or prophylaxis (Doumbo et al., 2000). Using the prevalence of molecular markers to measure selective pressure provides a means of assessing the ability of different combinations of antimalarial drugs to deter resistance to the drugs for which markers are available.

Genotype-resistance and genotype-failure indices

Although the molecular markers for chloroquine and SP resistance have been associated with *in vivo* resistance in many field studies, their application as tools for surveillance has been challenging, in part because the prevalence rates of molecular markers for resistance have been found to be higher than the prevalence of *in vivo* drug resistance in nearly all studies. This problem was addressed by studies in Mali, a country that uses chloroquine as its first-line antimalarial therapy. The investigators calculated ratios between rates of the chloroquine-resistant *pfcrt* genotype and therapeutic and parasitological outcomes of *in vivo* studies conducted at multiple sites over three years (Djimde et al., 2001b). A genotype-resistance index (GRI) for each site was calculated by dividing the prevalence of the resistant genotype (*pfcrt* 76T) by the prevalence of *in vivo* chloroquine resistance (RI, RII and RIII) at that site. A genotype-failure index (GFI) was similarly calculated as the ratio of resistant genotype to chloroquine therapeutic failure (early and late treatment failures) at each site. The Mali studies showed that GRIs and GFIs increased dramatically with age, reflecting acquired immunity and a higher proportion of older persons who cleared parasites with the chloroquine-resistant genotype when treated with chloroquine. This observation has led to the suggestion that the ability to clear drug-resistant parasites may serve as a good model for protective immunity (Djimde et al., in press).

After controlling for age, both GRIs and GFIs ranged from 1.6 to 2.8 at all study sites over the three-year period. This study introduced the intriguing possibility that the ratios between the prevalence rates of resistant genotypes and *in vivo* outcomes may remain stable over time even as rates of both change, and that once these ratios, or indices, are established, molecular surveys could be used to predict *in vivo* treatment outcomes on a much broader scale than is possible using standard *in vivo* efficacy studies. Where GRIs and GFIs have been established and validated, it should be possible to collect finger-stick filter paper blood samples routinely at outlying health facilities and to transport these samples at ambient temperature to a central laboratory where the prevalence of the molecular marker can be quickly assessed for each site. Predictions of rates of resistance and failure will be somewhat imprecise but still useful. For example, in Mali it is now possible to measure the prevalence of *pfcrt* 76T and predict *in vivo* chloroquine failure and resistance rates of approximately half of the prevalence of the molecular marker. At the very least, molecular surveys can be used to target the scarce resources required to conduct *in vivo* studies: sites with very low rates of resistant genotypes can be safely monitored by periodic molecular surveys, while sites where the prevalence of resistance markers is high or rising might warrant confirmatory *in vivo* efficacy studies.

Further studies are needed to validate this model in other and more varied epidemiological settings and to refine the

model to determine whether molecular surveys can reliably predict high-level (RII and RIII) resistance and early treatment failures. The stability over time of the indices in Mali could be attributable to relatively small differences among sites and years in the levels of malaria transmission and acquired immunity. Similar studies at sites with much higher *vs* lower malaria transmission intensities or highly seasonal *vs* year-round transmission might yield very different results. One can speculate that just as the indices increased with age in Mali, they would be higher in settings with higher levels of transmission intensity and immunity. Where transmission is very low, few persons might be expected to clear chloroquine-resistant parasites when treated with chloroquine, so the prevalence rate of *in vivo* resistance would approach that of *pfcrt* 76T, yielding very low GRIs and GFIs; in a setting of much higher transmission and immunity, most people may have enough immunity to clear these resistant infections, resulting in higher GRIs and GFIs even after adjusting for age. Alternatively, if GRIs and GFIs are found to be similar and stable in such different settings, the model would be far more useful in that indices would not have to be established in each setting before they could be applied there.

It will also be important to validate this model with other antimalarial drugs, most importantly SP. If ratios between the prevalence rate of the two mutations that predict the presence of quintuple DHFR and DHPS mutants and the prevalence of SP parasitological resistance and treatment failure are found to be stable after adjusting for age and at different sites with different epidemiological characteristics, the GRI and GFI model may provide a good tool for mapping SP resistance. This would be useful not only where SP is already being employed, to monitor for declining efficacy, but where SP is being considered as a replacement for chloroquine.

Monitoring resistance after cessation of drug use

If removing a drug from use were to result in the disappearance of parasites resistant to that drug, the possibility of rotating the limited number of safe, effective and affordable antimalarial drugs could be considered. Removing antimicrobial drug pressure may or may not compromise the fitness of resistant microorganisms and result in selection of drug-sensitive phenotypes (Levin et al., 2000; Enne et al., 2001). Incomplete withdrawal of the drug, cross-resistance to other drugs and compensatory mutations that reduce the cost of resistance to the fitness of the resistant microbe without jeopardizing the resistance itself (Sherman et al., 1996) may allow resistance to persist even after an antimicrobial drug has been withdrawn from use.

In 1993, Malawi became the first sub-Saharan African country to switch from chloroquine to the antifolate combination SP as the first-line antimalarial nationwide (Bloland et al., 1993). Since 1993, SP has been the only treatment for uncomplicated malaria available in government health facilities, where it is dispensed without prescription. Chloroquine is legally available only by prescription, and a national information campaign was largely successful in convincing health practitioners and the public to accept SP as the standard treatment for malaria. Some other eastern and southern African countries, including Kenya in 1999 (Shretta et al., 2000), have attempted to institute similar changes in national drug policy in the face of rising chloroquine resistance, with more mixed results.

Recent studies have found that the cessation of chloroquine use in Malawi was followed by reemergence and predominance of chloroquine-sensitive *P. falciparum*. The prevalence of the chloroquine-resistant *pfcrt* 76T genotype decreased steadily from 85% in 1992 to 13% in 2000 in Blantyre, a large city in central Malawi. In 2001, chloroquine cleared 100% of 63 asymptomatic *P. falciparum* infections, no isolates were resistant to chloroquine *in vitro*, and no infections with the chloroquine-resistant *pfcrt* genotype were detected (Kublin et al., 2003). This same study found that in 1999, 92% of *P. falciparum* infections carried this mutation in neighboring Zambia, where chloroquine remains the first-line drug. Another study found a 17% prevalence of *pfcrt* 76T in 1998 and only 2% in 2000 in a district further north in Malawi (Mita et al., 2003), confirming that the withdrawal of chloroquine from Malawi has resulted in the return of chloroquine-sensitive falciparum malaria there. This is most likely explained by a cost to the fitness of the parasite of the chloroquine resistance-conferring *pfcrt* mutations and selection for parasites with wild-type *pfcrt* in the absence of chloroquine drug pressure, although this remains to be demonstrated.

These studies provide a rationale for conducting controlled trials of chloroquine efficacy in areas where chloroquine use has been substantially reduced for a period of years. If such trials confirm a return of chloroquine's clinical efficacy, governments can consider withdrawing chloroquine and switching to other drugs on an interim basis, knowing that they may be able to later reintroduce chloroquine, which is unparalleled in its safety and low cost. As such antimalarial drug policy changes are considered and implemented, careful and integrated use of *in vivo*, *in vitro* and molecular assays for resistance have the potential to provide timely and practical information to help guide these policies. Already, molecular surveys have been integrated into the National Malaria Control Program in Mali and used to provide timely advice in a malaria epidemic in northern Mali (A. A. Djimde, A. Dolo, S. Diakite, A. Ouattara, C. V. Plowe and O. K. Doumbo, unpublished). Laboratories capable of performing basic molecular assays are well established in many African countries. The challenge now for scientists and policy makers is to work together to exploit advances in our understanding of the molecular mechanisms of drug-resistant malaria in order to make rational and informed decisions about malaria chemotherapy and prophylaxis policies.

Appendix. Definitions of *in vivo* therapeutic efficacy and parasitological resistance

Therapeutic efficacy outcomes

Early treatment failure:

danger signs or severe malaria on post-treatment days 1, 2 or 3, with parasitemia;

axillary temperature of >37.5°C on day 2 with parasitemia greater than the day 0 level;

axillary temperature of >37.5°C on day 3 in the presence of parasitemia; or

parasitemia on day 3 of >25% of day 0 level.

Late treatment failure:

failure to meet any of the criteria for early treatment failure;

development of danger signs or severe malaria in the presence of parasitemia during days 4–14; or

axillary temperature of >37.5°C in the presence of parasitemia during days 4–14.

Adequate clinical response:

failure to meet any of the criteria of early or late treatment failure;

absence of parasitemia on day 14 irrespective of temperature; or

axillary temperature of <37.5°C irrespective of the presence of parasitemia.

Parasitological resistance outcomes

RIII: no reduction in parasitemia, or reduction to >25% of day 0 level, by day 3.

RII: reduction in parasitemia to <25% of day 0 level without clearance leading to re-treatment or followed by persistent parasitemia.

RI: initial clearance of parasites indicated by negative thick smear after day 0, with subsequent positive thick smear by day 14.

Sensitive: clearance of parasites by day 14 with no recurrence of parasitemia.

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