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MAJOR ARTICLE

Rare, Highly Pyrimethamine-Resistant Alleles of the *Plasmodium falciparum* Dihydrofolate Reductase Gene from 5 African Sites

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In eastern and southern Africa, there has been a rapid increase in the prevalence of alleles with mutations in the *Plasmodium falciparum* dihydrofolate reductase gene *(dhfr)* associated with increased risk of clinical failure of sulfadoxine-pyrimethamine (S/P). Molecular methods for surveillance of these mutations are now widespread, but the usual analysis detects only the most prevalent allele in a polyclonal sample. We used a yeast-expression system to identify rare, highly pyrimethamine-resistant alleles of *dhfr* in isolates from 5 African countries—Kenya, Tanzania, Malawi, Gabon, and Nigeria. Only the isolates from Nigeria yielded significant numbers of novel resistant alleles, and only 1 of the alleles from any location showed a >3-fold increase in resistance to S/P or to chlorproguanil-dapsone. Overall, these results suggest that *dhfr* alleles that confer high levels of resistance to antifolates are rare, even in eastern and southern Africa, where pyrimethamine has been intensively used.

As the effectiveness of chloroquine for treatment of *Plasmodium falciparum* infections has decreased, countries in Africa have shifted to sulfadoxine-pyrimethamine (S/P) as the recommended treatment. Malawi made that change in 1993, and other countries in eastern and southern Africa have recently followed suit [1, 2]. The pyrimethamine component of S/P is a competitive inhibitor of the essential enzyme dihydrofolate reductase (DHFR; Enzyme Commission number 1.5.1.3), and sulfadoxine inhibits dihydropteroate synthase (DHPS; Enzyme Commission number 2.5.1.15), a key enzyme in folate biosynthesis [3]. Point mutations in the *dhfr* gene are the principal mode by which resistance to pyrimethamine evolves. The ease of molecular analysis by polymerase chain reaction (PCR) has produced substantial literature on the prevalence of the polymorphisms in *P. falciparum dhfr* that are responsible for resistance to pyrimethamine [3–18]. The most common mutation correlated with resistance to pyrimethamine is a change from serine to asparagine at codon 108 (S108N) [19, 20], and additional mutations at codons 51 and 59 progressively increase the resistance to pyrimethamine [3, 21–23].

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In many sites in eastern and southern Africa, there has been a rapid increase in the prevalence of the 51I/59R/108N triple alleles, and this change is associated with an increased risk of clinical failure of treatment with S/P [11, 14, 15, 24–30]. The increasing failure of S/P stimulated the development of chlorproguanil-dapsone (LapDap; GlaxoSmithKline). Like S/P, this drug is a combination: chlorcycloguanil, the metabolite of chlorproguanil, inhibits DHFR, and dapsone inhibits DHPS. Chlorproguanil-dapsone is clinically effective, even in areas where resistance to S/P is high [24, 25, 31–33], and it has recently been introduced in a number of countries [34]. Parasites that carry *dhfr* alleles with 1 additional mutation, an isoleucine-to-leucine change at codon 164 (51I/59R/108N/164L), are no longer sensitive to S/P or chlorproguanil-dapsone [35]. These quadruple mutants became common in Southeast Asia after only ~6 years of S/P use, and new drugs were required for treatment [36].

In many sites, polyclonal infections are common, and the sensitivity of the standard PCR [37] is not sufficient to detect alleles that include less than ~10% of the parasites in an isolate. If molecular analysis is to be useful as an early warning of rare but potentially highly drug-resistant alleles, then methods for their detection at very low levels are needed. We have used a yeast-expression system [38, 39] to rapidly screen samples isolated from patients in 5 African countries—Kenya, Tanzania, Malawi, Gabon, and Nigeria.

METHODS

The *P. falciparum* DNA examined was collected as part of the phase 3 safety and efficacy trial comparing the new, fixed-combination antifolate drug chlorproguanil-dapsone with S/P [34]. This was a double-blind, placebo-controlled study, with 1480 patients in the chlorproguanil-dapsone arm and 370 patients in the S/P arm. The samples were collected between March and December 2000 at 5 sites: Kisarawe (Tanzania), Kilifi (Kenya), Blantyre (Malawi), Lambaréné (Gabon), and Ibadan (Nigeria). The samples examined in the present study were blood spots from 25 patients who were judged by use of microscopy to have parasites on day 14 after their initial treatment. Samples obtained before treatment (day 0) and at day 14 were analyzed for each patient. Informed consent was obtained from all patients or their parents for the study from which these samples derived. The study met both US Department of Health and Human Services guidelines and the guidelines established by the home institutions in each study site. Permission was granted for analysis of the parasite DNA in our laboratory.

Genomic *P. falciparum* DNA was initially extracted from dried blood spots on filter paper by use of methanol precipitation or by use of the blood sample DNA extraction protocol from a commercial kit (QIAamp; QIAGEN). The *dhfr* gene was amplified by use of PCR with PfuTurbo polymerase (Stratagene) with proofreading activity, to minimize replication errors during PCR. Samples that yielded interesting alleles were reanalyzed and resequenced to ensure that the alleles were not PCR artifacts. The 50-μL reaction contained 1 μL of PfuTurbo polymerase, 1× PCR buffer, 0.5 μmol/L each primer, 0.4 mmol/L dNTPs, and 5–10 μL of template DNA. The 5′ primer used was CTCCTTTTTATGATGGAACAAGTCTGCGACGTTTTCG, and the 3′ primer used was TCATATGACATGTATCTTTGTCATCATTCTTTAAAGGC. The cycling parameters were as follows: initial denaturation for 3 min at 94°C, 30 cycles of denaturation for 30 s at 94°C, annealing for 45 s at 50°C, elongation for 1 min at 72°C, and final extension for 10 min at 72°C. For each amplification, a water control was performed and transformed into yeast; these yielded no colonies, confirming that no contamination had occurred during the PCRs.

The *Saccharomyces cerevisiae* yeast strain TH5 lacks endogenous DHFR activity and has been described elsewhere [39]. The yeasts were transformed with a gapped plasmid GR7 [8, 39] and the linear *dhfr* products of the PCR by use of a high-efficiency lithium acetate protocol [40]. Transformants were plated onto medium lacking tryptophan and deoxythymidine monophosphate. Colonies were arrayed on master plates and sequentially replica-plated onto plates containing rich medium and 5×10^{-4} mol/L pyrimethamine dissolved in DMSO (Pierce) and onto control plates with rich medium and DMSO only. The drug concentration was determined to allow growth of only the colonies that were more resistant to pyrimethamine than those that expressed the 51I/59R/108N *dhfr* genotype. In addition, 5 unselected isolates from each sample were chosen at random from plates containing no drug and were processed for automated sequencing (MegaBACE; Amersham Biosciences).

The IC_{50} assays were performed to obtain quantitative measures of drug sensitivity, as described elsewhere [38]. In brief, the growth of the yeast in this assay depends on the resistance to antifolate of the *dhfr* allele expressed. Yeasts were grown in 96-well plates in pyrimethamine or chlorcycloguanil ($0–5 \times 10^{-4}$ mol/L) or WR99210 ($0–10^{-5}$ mol/L) (all drugs were a gift from Jacobus Pharmaceutical). The growth of the yeast in each well was assessed by reading the optical density at 650 nm after ~24 h of incubation at 30°C. The numerical IC_{50} value was calculated from the slope and intercept of the line defined by the 2 data points that bracket 50% relative growth. IC_{50} assays were performed at least twice for each allele, to ensure reproducibility.

RESULTS

The aim of the present study was to identify rare alleles of *dhfr* that encode enzymes with a very high level of resistance to pyrimethamine. The samples examined were from patients in the phase 3 trial of the new antifolate combination, chlorproguanil-dapsone [34]. The 25 patients studied were treated with either S/P or chlorproguanil-dapsone and remained parasite-

mic 14 days after treatment. Parasite DNA from both the day-0 and the day-14 samples was isolated, and the *dhfr* domain of the *dhfr-ts* gene was amplified and integrated into a yeast shuttle plasmid by homologous recombination. The centromere on the plasmid ensures that each resulting yeast colony contains only 1 *dhfr* allele, allowing for the unambiguous identification of individual alleles from samples that are polyclonal. The basic strategy is outlined in figure 1.

The standard allele-specific oligonucleotide or restriction-digestion methods [37] identify alleles that are present at a frequency of ~10%, but our goal was to detect rare resistant alleles. Both approaches use PCR, but our emphasis on rare alleles made it crucial to ensure that we did not generate these rare alleles in the amplification reaction. To test this, we amplified the triple-mutant allele (51I/59R/108N) with the high-fidelity polymerase, PfuTurbo, cloned the products of the reaction into yeast, and screened the resulting colonies with the same protocol used for analysis of the field samples. We screened 1100 individual colonies on plates with 5×10^{-4} mol/L pyrimethamine. This level of drug was chosen to allow growth of colonies that are only slightly more resistant than those with the 51I/59R/108N allele, to ensure that we did not miss any alleles that fell into this group. As a result, about half of the colonies we observed on these screening plates carried the 51I/59R/108N allele with no modification. In this control experiment, only 19 of 1100 colonies grew on the selective plates. The sequence of the *dhfr* gene in these 19 colonies and in 20 colonies from a plate with no drug was determined. No synonymous or nonsynonymous mutations were detected in any of these sequences; all carried a *dhfr* allele with only the "parental" 51I/59R/108N genotype and no additional mutations. In addition, only 2 synonymous mutations were detected among 12,616 *dhfr* alleles sequenced from the Kenyan, Tanzanian, Malawian, and Gabonese samples. The very low number of silent changes indicated that the few mutations we did observe were most likely to have originated from the samples and did not result from high levels of polymerase errors during the amplification.

The analysis of the isolates was done in 2 parts. First, the yeast colonies were plated with no drug selection, and the *dhfr* was sequenced from at least 5 independent colonies. These sequences allow the identification of the predominant alleles in the sample, including any alleles that carry simple polymorphisms unrelated to drug resistance. The initial amplification of all 25 samples collected before treatment was successful; 2 samples from the Tanzanian set and 2 from the Nigerian set did not yield any product after amplification of the sample collected on day 14. The list of unselected genotypes in table 1 summarizes these data.

In 4 of the 5 locations, the triple-mutant allele (51I/59R/108N) was clearly prevalent, and this genotype comprised 80% of the alleles observed overall. Among the 3 patients from Kisarawe, Tanzania, 1 sample showed only the triple-mutant allele,

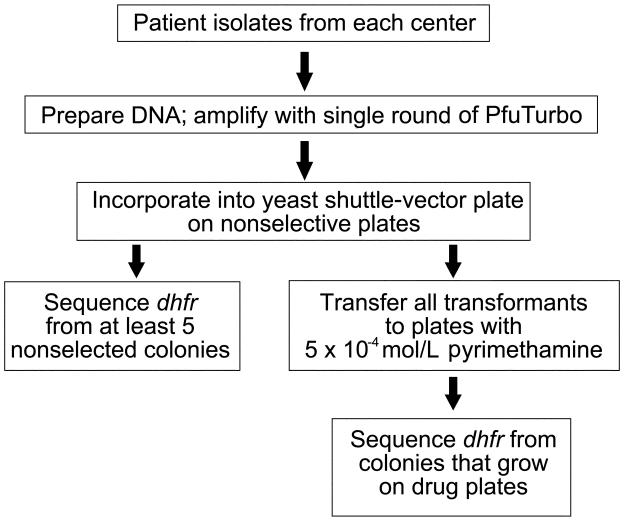

Figure 1. The overall plan for the study. Day-0 and day-14 isolates from the 25 patients who still retained parasites 14 days after treatment with either chlorproguanil-dapsone or sulfadoxine-pyrimethamine were studied as indicated.

but the other 2 were predominantly wild type *(wt)*. Four novel alleles were observed; 1 patient from Tanzania and 1 from Gabon each had a single allele that carried a mutation in addition to 51I/59R/108N. Two patients from Nigeria had a single novel allele, and 1 patient carried 3 different alleles. We have previously intensively mutagenized *P. falciparum dhfr,* to identify mutations that could encode a functional, resistant enzyme [41, 42].

The transformed colonies were then arrayed on plates and sequentially replica plated onto plates that contained 5×10^{-4} mol/L pyrimethamine. This concentration permits robust growth of yeast that depends on the highly pyrimethamine-resistant Southeast Asian allele, 51I/59R/108N/164L, but inhibits the growth of yeast colonies that carry only the 51I/59R/108N genotype. Figure 2 shows an example of such an experiment. All colonies that grew vigorously on the drug plates were isolated, and the DNA sequence of *dhfr* was determined. Table 1 summarizes these data, as well. As noted above, about half of the colonies tested carried only the 51I/59R/108N genotype. The most common new allele isolated, 51I/59R/108N/213A, was observed in all sites except Tanzania. We observed 2 other alleles that had been isolated previously from the field or in the laboratory [42, 43]—a Kenyan allele with a fourth mutation at codon 188 (51I/59R/108N/188K) and a Gabonese allele with a change at 189 (51I/59R/108N/189R).

In contrast, the samples from Nigeria had 6 novel alleles with numerous mutations. Two of these included the same 213A mutation (51I/59R/108N/213A), and a third carried a N51I/C59R/D91N/S108N/V213A genotype. Two other patients in the Nigerian set each carried a novel quadruple-mutant allele, 51I/

Table 1. Summary of the novel pyrimethamine-resistant alleles identified in the 5 African sites.

Site	Total no. of patients	Colonies with unselected genotypes: No. *wt*	51/108	59/108	51/59/108	Mutations found without selection	Total	Novel pyrimethamine-resistant genotypes: N51I/C59R/S108N plus single aa change	Other genotypes
Kilifi, Kenya	6	6	10	7	42	…	65	**N188K** (day 0) and **V213A** (day 14)	C59R/S108N/**V213A** (day 0)
Kasarawe, Tanzania	3	10	4	0	9	K19M	24	…	…
Blantyre, Malawi	3	0	2	0	28	…	30	**V213A** (day 0) and **V213A** (×2) (day 14)	…
Lambaréné, Gabon	5	0	0	0	59	K56R	60	**S189R V213A** (day 14)	…
Ibadan, Nigeria	8	0	10	0	80	E25G (x 2) T215A M92I	95	**I200M V213A** (day 0) and **P113L V213A I164V** (×2) (day 14)	N51I/S108N/**V213A** N51I/C59R/**D91N**/ S108N/**V213A** (day 0) and **C50Y**/N51I/C59R/ **E84K/V86M**/S108N/ **I164V** (×2) (day 14)
Total (%)	**25**	**16 (5.8)**	**26 (9.4)**	**7 (2.5)**	**218 (79.5)**	…	**274**	…	…

NOTE. Unselected genotypes are those identified by sequencing of the *Plasmodium falciparum dhfr* allele from at least 5 randomly chosen colonies. Novel pyrimethamine-resistant genotypes are those observed in colonies that grew on the selective plates with 5×10^{-4} mol/L pyrimethamine. Bold type indicates mutations in addition to the triple mutant. aa, amino acid; *wt,* wild type; ×2, mutation was found in 2 different samples.

59R/108N/164V, but the mutation at codon 164 differed from the quadruple allele observed in Southeast Asia, 51I/59R/108N/164L. In both patients, this 164V mutation was also observed in combination with 3 additional changes, to produce an allele with 7 differences from the *wt* (C50Y/51I/59R/E84K/V86M/108N/I164V). To confirm that this highly mutant allele was not generated by the PCR amplification, we performed a second independent amplification of 1 of these samples. The same highly mutant allele was isolated in both analyses, making it extremely unlikely that it is a PCR artifact.

The Nigerian samples also carried several silent substitutions not seen elsewhere; 3 of 8 patients carried parasites with a GAA→GAG substitution at codon 202. Among these, 1 isolate also carried a parasite with a silent GAT→AAT change at codon 19, and another isolate carried a parasite with a TTG→TTA change at codon 73. These numerous synonymous and nonsynonymous changes distinguished the Nigerian samples from those at the other 4 sites. Since the polymerase and protocol used for all of the samples were identical, this disparity also suggests that the changes observed were not simply generated by the PCR amplification.

On the basis of both the number of times we had isolated an allele and our previous in vitro mutagenesis [41, 42], we chose some of these alleles and performed IC_{50} assays to estimate in yeast the level of drug resistance of the enzymes that they encode. We determined the sensitivity of yeast dependent on the canonical mutant *dhfr* alleles (51I/59R/108N and 51I/59R/108N/164L) to pyrimethamine, chlorcycloguanil (the active metabolite of chlorproguanil), and the experimental DHFR inhibitor (WR99210) and compared them with novel mutant alleles. Each yeast line was grown in 0–10^{-4} mol/L pyrimethamine or chlorcycloguanil and 0–10^{-5} mol/L WR99210, and the IC_{50} value (the concentration of drug that inhibited growth of the yeast 50%, compared with the growth in the absence of drug) was determined. Figure 3 shows average IC_{50} curves for each allele analyzed. Figure 4*A* tabulates the actual IC_{50} values for each drug, and figure 4*B* summarizes the fold change in sensitivity between the triple mutant and each novel allele.

The plating screen was designed to identify yeast colonies that were more resistant to pyrimethamine than the triple-mutant allele, and the IC_{50} values show the expected trend: the novel mutants confer a higher level of resistance to pyrimethamine than the 51I/59R/108N allele. However, the IC_{50} values of lines that carry alleles with 1 additional mutation (indicated with bold type) at 213 (51I/59R/108N/**213A**) or 51I/59R/108N/**164V** are roughly 3-fold higher, still substantially lower than the 51I/59R/108N/**164L**, for which an IC_{50} value for pyrimethamine cannot even be measured. Additional mutations in these alleles (51I/59R/**91N**/108N/**213A**) or (**C50Y**/51I/59R/**E84K/V86M**/108N/**I164V**) showed somewhat lower IC_{50} values, demonstrating that the additional mutations do not contribute to the resistance to pyrimethamine. Only the alleles with a mutation at codon 188 (51I/59R/108N/**188K**) or codon 189 (51I/59R/108N/**189R**) showed resistance to pyrimethamine comparable to that of the canonical quadruple-mutant N108/I51/R59/L164 allele; all 3 strains are so insensitive to pyrimethamine that an IC_{50} value cannot be measured in this system.

As expected, the IC_{50} values for chlorcycloguanil were uniformly lower than those measured for pyrimethamine but followed a similar pattern: the alleles with an additional mutation at 213A or 164V were >3-fold more resistant to chlorcycloguanil than 51I/59R/108N alone, and the additional mutations slightly decreased resistance. The strains that carried the 188K, 189R, and 51I/59R/108N/164L alleles were substantially more resistant

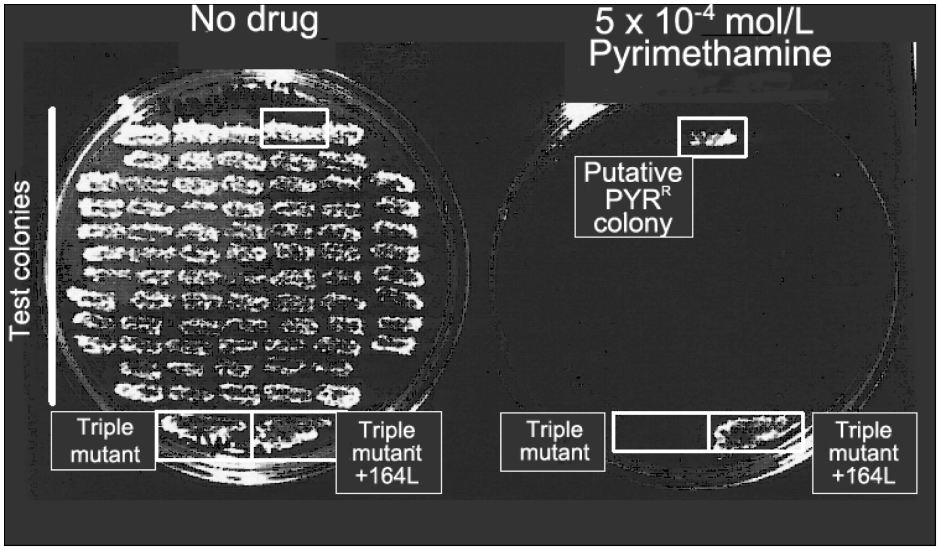

Figure 2. A representative selective plate used to screen for pyrimethamine-resistant colonies. The *dhfr* coding region was amplified with PfuTurbo polymerase (Stratagene) and inserted into the single-copy yeast plasmid by homologous recombination. All resulting colonies were arrayed as shown without drug, and 5 were randomly chosen for determination of the *dhfr* sequence. The colonies were then sequentially replica plated onto plates with 5×10^{-4} mol/L pyrimethamine, and growth of each colony was assessed after 3 and 5 days at 30°C. The "triple-mutant yeast" is dependent on the 51I/59R/108N genotype, and the triple +164L (51I/59R/108N/164L) mutant is the highly resistant mutant commonly found in Southeast Asia. The plasmid from the single colony that grew on the selective plate was then isolated, and the sequence of the *Plasmodium falciparum dhfr* was determined.

than the strains carrying the triple mutants (51I/59R/108N) to chlorcycloguanil, and, as observed for pyrimethamine, no IC_{50} value could be measured.

The experimental inhibitor WR99210 is extremely effective against yeasts that carry *P. falciparum dhfr* alleles with ≤3 mutations [39, 41–43]. Even the canonical quadruple-mutant allele is quite sensitive to WR99210; in yeast, it has an IC_{50} value of ~3×10^{-7} mol/L, compared with values in the 2–4×10^{-8} mol/L range measured for the other alleles. All of the novel alleles except 51I/59R/108N/**188K** and 51I/59R/108N/**189R** were also extremely sensitive to WR99210, with IC_{50} values in the range of 10^{-8} mol/L. Like the 51I/59R/108N/**164L** allele, strains that depended on the 51I/59R/108N/**188K** allele were 10–20-fold less sensitive to that inhibitor, with an IC_{50} value of ~4×10^{-7} mol/L. The 51I/59R/108N/**189R** allele conferred only a 6-fold increase, with an IC_{50} value of 1.3×10^{-7} mol/L.

In the present study, we isolated 10 novel alleles from 25 patients from a wide geographic area, and only the samples from Nigeria yielded more than a few resistant alleles. In contrast, we previously identified 38 novel alleles with the same method from only 6 isolates from a single location, Muheza, Tanzania [43]. Table 2 compares these results directly.

The very small numbers of patients and the method we used preclude a direct estimation of the prevalence of any particular allele, but the paucity of novel alleles in the current data set is clear. For example, 3 of the 6 patients from Muheza carried the N51I/59R/108N/**164L** mutation, which is associated with clinical failure of S/P in Southeast Asia. In this wider screen, quadruple-mutant alleles with a 164V mutation were identified in the Nigerian patients, but the 164L change was not observed in any of the isolates identified in this screen.

DISCUSSION

In many sites in eastern and southern Africa, there has been a rapid increase in the prevalence of the triple-mutant 51I/59R/108N alleles associated with an increased risk of clinical failure of treatment with S/P [3–16]. This trend is clearly reflected here, as well; the triple-mutant allele was the most common in 4 of the 5 sites in which the trial was conducted, and this genotype comprised ~80% of the alleles sequenced in our study. We designed the present study to identify rare *dhfr* alleles that are likely to confer high levels of resistance to pyrimethamine. The 17 isolates from 4 of the 5 sites in this study yielded only 4 novel resistant alleles, and only 2 of these (51I/59R/108N/188K and 51I/59R/108N/189R) are in regions of the molecule known to confer extremely high levels of resistance to pyrimethamine [41, 42, 44]. The isolates from the fifth site, Nigeria, did not follow this pattern. From the 8 Nigerian patients, we isolated 6 highly mutant alleles, including a novel quadruple-mutant allele with a mutation from isoleucine to valine at codon 164, carried by 2 different patients. Although the Southeast Asian quadruple mutant carries a mutation at the same position, that mutation encodes an isoleucine-to-leucine change. The enzymes encoded by the genotypes that carried the 164V change did not confer levels of resistance comparable to the Southeast

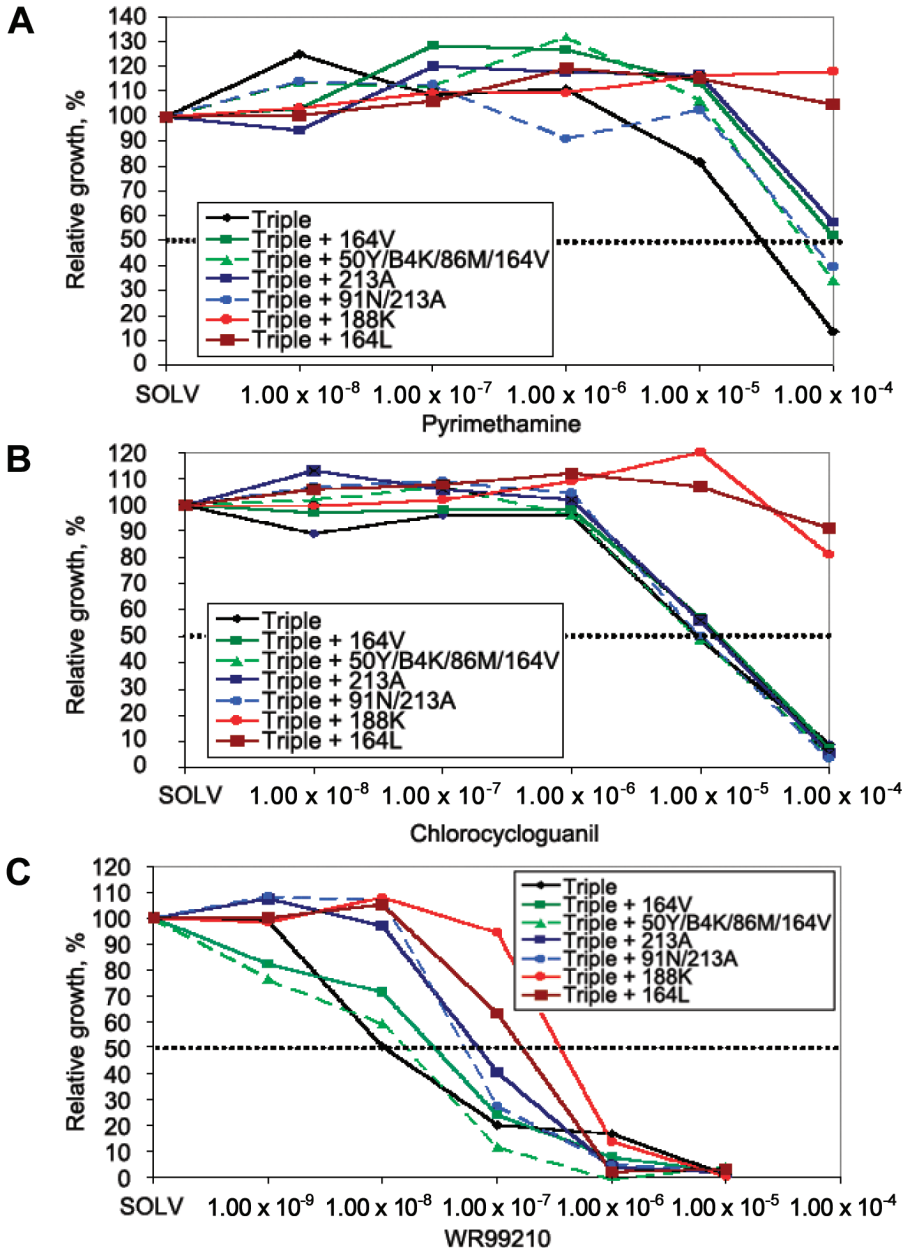

Figure 3. The sensitivity of yeast dependent on the novel *dhfr* alleles to pyrimethamine, chlorcycloguanil, and WR99210. Each strain was grown in liquid in a 96-well plate with the indicated concentrations of each drug. After 18–24 h, the growth of yeast in each well was monitored by reading the optical density at 650 nm. These curves show the average of 2–3 separate determinations, and the IC_{50} values in figure 4*A* were determined from the graphs as shown. Because of the much higher potency of WR99210, the scales differ slightly in panel *C;* the highest concentration of pyrimethamine and chlorcycloguanil used was 10^{-4} mol/L, but 10^{-5} mol/L was used for WR99210. SOLV, solvent alone (no drug).

Asian allele for any of the drugs tested. Overall, these results suggest that alleles that confer high levels of resistance to antifolates are still rare in sub-Saharan Africa, even in eastern and southern Africa, where pyrimethamine has been intensively used.

The very small numbers of patients and the method we have used preclude a direct estimation of the prevalence of any particular allele, but the paucity of novel alleles in the current data set, compared with the data from Muheza, is clear. In addition, 3 of the 6 patients from Muheza carried the N51I/59R/108N/164L mutation, which is associated with clinical failure of S/P in Southeast Asia. In this wider screen, quadruple-mutant alleles with a 164V mutation were identified in the Nigerian patients, but the 164L change was not observed in any of the isolates identified in this screen.

Antifolate drugs have been used intensively in both areas for longer periods than in most others in Africa, and there are high

A

Genotype of *dhfr* allele	Pyrimethamine, µmol/L (SD)	Chlorocycloguanil, µmol/L (SD)	WR99210, µmol/L (SD)
51I/59R/108N **(triple)**	34 (4.9)	6.1 (1.1)	0.021 (0.01)
51I/59R/108N/**213A**	105 (6.3)	22 (4.3)	0.050 (0.02)
51I/59R/**D91N**/108N/**213A**	60 (0.8)	10 (1.4)	0.045 (0.003)
C50Y/51I/59R/**E84K/V86M**/108N/**I164V**	57 (5.0)	16 (3.1)	0.025 (0.01)
51I/59R/108N/**164V**	106 (5.0)	23 (3.2)	0.037 (0.01)
51I/59R/108N/**188K**	†	†	**0.39 (0.05)**
51I/59R/108N/**189R**	†	†	**0.13 (0.06)**
51I/59R/108N/**164L (quadruple)**	†	†	**0.34 (0.04)**

B

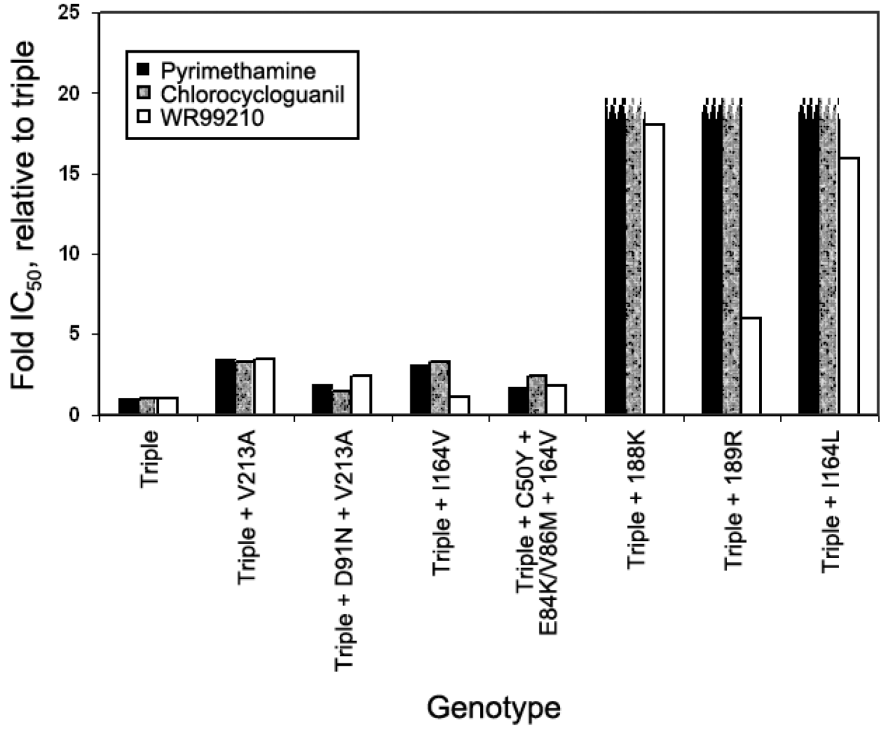

Figure 4. The comparison of drug sensitivity of selected alleles of *Plasmodium falciparum dhfr* to pyrimethamine, chlorcycloguanil, and WR99210. The IC_{50} values for 2–4 experiments were calculated, and the average is reported in panel *A*. A graphical presentation of the fold change in sensitivity of each novel allele, compared with the triple-mutant allele, (51I/59R/108N) is shown. †, The level of resistance to that drug was so high that no IC_{50} value could be measured. *B, Jagged bars,* an IC_{50} value could not be measured, so the fold increase over the triple mutant allele could not be calculated.

levels of resistance to S/P in both areas [26, 45, 46]. The dominance of triple-mutant alleles in all of the study locations makes it clear that S/P-driven selection for mutant alleles of *dhfr* is certainly in progress at all 5 locations, as well [14, 24, 47–50]. Whatever the selection pressure, the sites in Muheza and Ibadan appear to have a deeper "reservoir" of alleles that are more resistant to pyrimethamine than the triple mutant 51I/59R/108N, compared with the sites in Kenya, Malawi, and Gabon.

Any method that requires PCR amplification runs the risk of generating mutants simply from polymerase errors. Several observations suggest that this has not been a problem in the present study. First, the amplification of the triple-mutant allele with the high-fidelity PfuTurbo polymerase produced no resistant mutants in the >1000 colonies that we assessed, and there was a striking paucity of silent substitutions. Second, 2 samples from patients in Tanzania had only *wt* parasites in the nonselected colonies, and drug selection also yielded no resistant alleles. Third, by use of in vitro mutagenesis, we have previously generated a diverse set of alleles that can confer a high level of resistance to pyrimethamine in this assay. This result demonstrates that genotypes other than those observed here could be generated by polymerase errors and isolated by

Table 2. Comparison of screens of samples from 6 locations.

Variable	Muheza, Tanzania[a]	Kenya, Tanzania, Malawi, and Gabon	Nigeria
Isolates screened, no.	6	17	8
Yeast colonies screened, no.	3994	12,616	7374
Novel alleles identified, no.	38	4	6

[a] Data are summarized from reference [34].

use of this method [42]. However, in these field samples, 1 allele, 51I/59R/108N/213A, was isolated in 4 of 5 sites and in samples from 7 different patients, an unlikely occurrence if these alleles were simply being generated at random during the reaction. Finally, most of the alleles observed have a single new mutation on a background of the common triple-mutant genotype. These changes could not be generated by the polymerase "jumping" from 1 template to another during the amplification, another error that has been observed at low levels in PCRs [51]. The highly mutant alleles from Nigeria could have resulted from such an aberration, but the fact that the same genotype was isolated from 2 individual patients and twice during independent analyses of isolates from the same patient greatly reduces the probability that these also are PCR artifacts. Thus, we are confident that the novel *dhfr* alleles identified in this screen were present at low levels in the samples.

One goal of the present study was to provide a baseline for molecular surveillance of mutations in *dhfr* that are likely to evolve in response to antifolate drug use. We have previously used intensive mutagenesis in vitro to identify mutations of the *P. falciparum dhfr* gene that encode functional, drug-resistant forms of the enzyme [41, 42]. In both previous studies of *dhfr* alleles isolated from patients' samples, the same 2 regions of the molecule with a disproportionate number of resistant mutations have been identified: aa 188–191 and 213–215. Mutations in aa 50–59, 108, and 164 are all observed frequently in field isolates [43, 52]. Alleles with additional mutations in aa 188–191 or aa 213–215 have also been observed in vitro and at low levels in the Muheza study [43]. The isolation of mutations at codons 188 and 189 and at 213 in the current broader sample follows this same pattern. Taken together, these data suggest that it would be prudent to include assay of aa 188–191 and aa 213–215 of the gene in a surveillance program designed to provide early warning of selection of antifolate-resistant populations of *P. falciparum* [18].

The sample set analyzed here derives from a single time point, and a temporal study of changes in prevalence of rare resistant alleles will be required to determine whether these alleles "in the background" comprise raw material for selection by S/P. High-throughput methods with greater sensitivity than the current allele-specific PCR methods will be needed to identify resistant alleles even when they are at low prevalence, and some steps have already been made in that direction [15, 53–55].

Recent molecular studies show that, in highland areas of low transmission close to Muheza, the triple-mutant alleles of *dhfr* share the same extended haplotype, suggesting that a single triple-mutant allele of *dhfr* has invaded the *P. falciparum* population and spread under drug selection [56]. More-extensive studies in Southeast Asia and South America support this "selective sweep" model of the selection process [57, 58]. This outcome is surprising, since the increasing resistance to pyrimethamine requires simply the acquisition of 1 additional point mutation after another, and one might have predicted that mutant alleles could arise repeatedly in situ on many different haplotypes. The sample set analyzed here derives from a single time point, and a temporal study of changes in prevalence of rare resistant alleles will be required to determine whether these alleles "in the background" comprise raw material for selection by chlorproguanil-dapsone.

Chlorproguanil-dapsone has recently been introduced in a number of African countries, including those in the present study [34]. As yet, there are no clinical data that would allow us to assess whether the modest increases in resistance to chlorcycloguanil that we observed in the yeast system would compromise the clinical efficacy of chlorproguanil-dapsone. However, the set of mutations that confer high levels of resistance to chlorcycloguanil overlaps substantially with the set of mutations that confer resistance to pyrimethamine [42, 59], so selection of any of these novel alleles would certainly head in the wrong direction. On the basis of the results of the present study, only the N108/I51/R59/188K and the N108/I51/R59//S189R alleles are likely to substantially increase resistance to a level comparable to that of the 51I/59R/108N/164L allele [35]. Each of these alleles was found only once, suggesting that there may not yet be a reservoir of these alleles, at least in the sites tested in the phase 3 trial.

The biguanide prodrugs of the WR99210 class are currently in preclinical development [60, 61], and the excellent effectiveness of WR99210 against all of the alleles examined here is encouraging. On the basis of the data reported here, we would expect that none of the alleles we isolated would show serious levels of resistance to drugs of the WR99210 class; definitive information on this conclusion will depend, of course, on direct clinical trials.

What can we learn from a limited "snapshot" of this kind? Such a small sample cannot be extrapolated to general predictions about resistance to antifolates in the sites at which the original study was done and certainly cannot be expanded to conclusions about nearby locations. Variation even within geographically or temporally related sites is far too high for those kinds of predictions to be reliable [15, 62–64]. However, our approach does allow qualitative comparisons, and it identifies locations such as Muheza and Ibadan, where there has been apparently strong selection for antifolate resistance in the past.

In the sites sampled in Kenya, Tanzania, Malawi and Gabon, S/P has been used for almost 10 years, and the triple-mutant allele is prevalent. Under these circumstances, the low "harvest" of resistant alleles is encouraging. This suggests that the combination of chlorproguanil-dapsone may have a reasonably useful therapeutic life in those locations. The presence of these alleles underlines the importance of developing and implementing sensitive, high-throughput methods and of creating and maintaining regional databases to serve as better early warning systems for the emergence of alleles associated with drug resistance.

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References

1. Bloland PB, Kachur SP, Williams HA. Trends in antimalarial drug deployment in sub-Saharan Africa. J Exp Biol **2003**; 206:3761–9.
2. East African Network for Monitoring Antimalarial Treatment (EANMAT). The efficacy of antimalarial monotherapies, sulphadoxine-pyrimethamine and amodiaquine in East Africa: implications for sub-regional policy. Trop Med Int Health **2003**; 8:860–7.
3. Cowman AF. The mechanisms of drug action and resistance in malaria. Molec Genet Drug Resist **1997**; 3:221–46.
4. Plowe CV, Kublin JG, Doumbo OK. *P. falciparum* dihydrofolate reductase and dihydropteroate synthase mutations: epidemiology and role in clinical resistance to antifolates. Drug Resist Updates **1998**; 1:389–96.
5. Basco LK, Eldin de Pecoulas P, Wilson CM, Le Bras J, Mazabraud A. Point mutations in the dihydrofolate reductase-thymidylate synthase gene and pyrimethamine and cycloguanil resistance in *Plasmodium falciparum*. Mol Biochem Parasitol **1995**; 69:135–8.
6. Wang P, Lee CS, Bayoumi R, et al. Resistance to antifolates in *Plasmodium falciparum* monitored by sequence analysis of dihydropteroate synthetase and dihydrofolate reductase alleles in a large number of field samples of diverse origins. Mol Biochem Parasitol **1997**; 89:161–77.
7. Jelinek T, Ronn AM, Curtis J, et al. High prevalence of mutations in the dihydrofolate reductase gene of *Plasmodium falciparum* in isolates from Tanzania without evidence of an association to clinical sulfadoxine/pyrimethamine resistance. Trop Med Int Health **1997**; 2:1075–9.
8. Cortese JF, Plowe CV. Antifolate resistance due to new and known *Plasmodium falciparum* dihydrofolate reductase mutations expressed in yeast. Mol Biochem Parasitol **1998**; 94:205–14.
9. Jelinek T, Kilian AH, Kabagambe G, von Sonnenburg F. *Plasmodium falciparum* resistance to sulfadoxine/pyrimethamine in Uganda: correlation with polymorphisms in the dihydrofolate reductase and dihydropteroate synthetase genes. Am J Trop Med Hyg **1999**; 61:463–6.
10. Diourte Y, Djimde A, Doumbo OK, et al. Pyrimethamine-sulfadoxine efficacy and selection for mutations in *Plasmodium falciparum* dihydrofolate reductase and dihydropteroate synthase in Mali. Am J Trop Med Hyg **1999**; 60:475–8.
11. Nzila-Mounda A, Mberu EK, Sibley CH, Plowe CV, Winstanley PA, Watkins WM. Kenyan *Plasmodium falciparum* field isolates: correlation between pyrimethamine and chlorcycloguanil activity in vitro and point mutations in the dihydrofolate reductase domain. Antimicrob Agents Chemother **1998**; 42:164–9.
12. Doumbo OK, Kayentao K, Djimde A, et al. Rapid selection of *Plasmodium falciparum* dihydrofolate reductase mutants by pyrimethamine prophylaxis. J Infect Dis **2000**; 182:993–6.
13. Biswas S, Escalante A, Chaiyaroj S, Angkasekwinai P, Lal AA. Prevalence of point mutations in the dihydrofolate reductase and dihydropteroate synthetase genes of *Plasmodium falciparum* isolates from India and Thailand: a molecular epidemiologic study. Trop Med Int Health **2000**; 5:737–43.
14. Kublin JG, Dzinjalamala FK, Kamwendo DD, et al. Molecular markers for failure of sulfadoxine-pyrimethamine and chlorproguanil-dapsone treatment of *Plasmodium falciparum* malaria. J Infect Dis **2002**; 185:380–8.
15. Pearce RJ, Drakeley C, Chandramohan D, Mosha F, Roper C. Molecular determination of point mutation haplotypes in the dihydrofolate reductase and dihydropteroate synthase of *Plasmodium falciparum* in three districts of northern Tanzania. Antimicrob Agents Chemother **2003**; 47:1347–54.
16. Le Bras J, Durand R. The mechanisms of resistance to antimalarial drugs in *Plasmodium falciparum*. Fundam Clin Pharmacol **2003**; 17:147–53.
17. Hyde JE. Mechanisms of resistance of *Plasmodium falciparum* to antimalarial drugs. Microbes Infect **2002**; 4:165–74.
18. Plowe CV. Monitoring antimalarial drug resistance: making the most of the tools at hand. J Exp Biol **2003**; 206:3745–52.
19. Cowman AF, Morry MJ, Biggs BA, Cross GA, Foote SJ. Amino acid changes linked to pyrimethamine resistance in the dihydrofolate reductase-thymidylate synthase gene of *Plasmodium falciparum*. Proc Natl Acad Sci USA **1988**; 85:9109–13.
20. Peterson DS, Walliker D, Wellems TE. Evidence that a point mutation in dihydrofolate reductase-thymidylate synthase confers resistance to pyrimethamine in falciparum malaria. Proc Natl Acad Sci USA **1988**; 85:9114–8.
21. Hyde JE. The dihydrofolate reductase-thymidylate synthase gene in the drug resistance of malaria parasites. Pharmacol Ther **1990**; 48:45–59.
22. Sirawaraporn W, Sathitkul T, Sirawaraporn R, Yuthavong Y, Santi DV. Antifolate-resistant mutants of *Plasmodium falciparum* dihydrofolate reductase. Proc Natl Acad Sci USA **1997**; 94:1124–9.
23. Sibley CH, Hyde JE, Sims PFG, et al. Pyrimethamine/sulfadoxine resistance in *Plasmodium falciparum:* what next? Trends Parasitol **2001**; 17:582–8.
24. Nzila AM, Nduati E, Mberu EK, et al. Molecular evidence of greater selective pressure for drug resistance exerted by the long-acting antifolate pyrimethamine/sulfadoxine compared with the shorter-acting chlorproguanil/dapsone on Kenyan *Plasmodium falciparum*. J Infect Dis **2000**; 181:2023–8.
25. Mutabingwa T, Nzila A, Mberu E, et al. Chlorproguanil-dapsone for treatment of drug-resistant falciparum malaria in Tanzania. Lancet **2001**; 358:1218–23.
26. Mutabingwa TK, Maxwell CA, Sia IG, et al. A trial of proguanil-dapsone in comparison with sulfadoxine-pyrimethamine for the clearance of *Plasmodium falciparum* infections in Tanzania. Trans R Soc Trop Med Hyg **2001**; 95:433–8.
27. Checchi F, Durand R, Balkan S, et al. High *Plasmodium falciparum* resistance to chloroquine and sulfadoxine-pyrimethamine in Harper, Liberia: results in vivo and analysis of point mutations. Trans R Soc Trop Med Hyg **2002**; 96:664–9.
28. Plowe CV, Cortese JF, Djimde A, et al. Mutations in *Plasmodium falciparum* dihydrofolate reductase and dihydropteroate synthase and epidemiologic patterns of pyrimethamine-sulfadoxine use and resistance. J Infect Dis **1997**; 176:1590–6.
29. Kamya MR, Dorsey G, Gasasira A, et al. The comparative efficacy of chloroquine and sulfadoxine-pyrimethamine for the treatment of uncomplicated falciparum malaria in Kampala, Uganda. Trans R Soc Trop Med Hyg **2001**; 95:50–5.
30. Talisuna AO, Nalunkuma-Kazibwe A, Bakyaita N, et al. Efficacy of sulphadoxine-pyrimethamine alone or combined with amodiaquine or chloroquine for the treatment of uncomplicated falciparum malaria in Ugandan children. Trop Med Int Health **2004**; 9:222–9.
31. Amukoye E, Winstanley PA, Watkins WM, et al. Chlorproguanil-dapsone: effective treatment for uncomplicated falciparum malaria. Antimicrob Agents Chemother **1997**; 41:2261–4.
32. Sulo J, Chimpeni P, Hatcher J, et al. Chlorproguanil-dapsone versus sulfadoxine-pyrimethamine for sequential episodes of uncomplicated

falciparum malaria in Kenya and Malawi: a randomised clinical trial. Lancet **2002**; 360:1136–43.
33. Winstanley P, Watkins W, Muhia D, Szwandt S, Amukoye E, Marsh K. Chlorproguanil/dapsone for uncomplicated *Plasmodium falciparum* malaria in young children: pharmacokinetics and therapeutic range. Trans R Soc Trop Med Hyg **1997**; 91:322–7.
34. Alloueche A, Bailey W, Barton S, et al. Comparison of chlorproguanil-dapsone with sulfadoxine-pyrimethamine for the treatment of uncomplicated falciparum malaria in young African children: a double-blind randomised controlled trial. Lancet **2004**; 363:1843–8.
35. Wilairatana P, Kyle DE, Looareesuwan S, et al. Poor efficacy of antimalarial biguanide-dapsone combinations in the treatment of acute, uncomplicated falciparum malaria in Thailand. Ann Trop Med Parasitol **1997**; 91:125–32.
36. White NJ. Antimalarial drug resistance: the pace quickens. J Antimicrob Chemother **1992**; 30:571–85.
37. Duraisingh MT, Curtis J, Warhurst DC. *Plasmodium falciparum:* detection of polymorphisms in the dihydrofolate reductase and dihydropteroate synthetase genes by PCR and restriction digestion. Exp Parasitol **1998**; 89:1–8.
38. Sibley CH, Brophy VH, Cheesman S, et al. Yeast as a model system to study drugs effective against apicomplexan proteins. Methods **1997**; 13:190–207.
39. Wooden JM, Hartwell LH, Vasquez B, Sibley CH. Analysis in yeast of antimalaria drugs that target the dihydrofolate reductase of *Plasmodium falciparum.* Mol Biochem Parasitol **1997**; 85:25–40.
40. Ito H, Fukuda Y, Murata K, Kimura A. Transformation of intact yeast cells treated with alkali cations. J Bacteriol **1983**; 153:163–8.
41. Ferlan JT, Mookherjee S, Okezie IN, Fulgence L, Sibley CH. Mutagenesis of dihydrofolate reductase from *Plasmodium falciparum:* analysis in *Saccharomyces cerevisiae* of triple mutant alleles resistant to pyrimethamine or WR99210. Mol Biochem Parasitol **2001**; 113:139–50.
42. Hankins EG, Warhurst DC, Sibley CH. Novel alleles of the *P. falciparum dhfr* highly resistant to pyrimethamine and chlorcycloguanil, but not WR99210. Mol Biochem Parasitol **2001**; 117:91–102.
43. Hastings MD, Bates SJ, Blackstone EA, Monks SM, Mutabingwa TK, Sibley CH. Highly pyrimethamine-resistant alleles of dihydrofolate reductase in isolates of *Plasmodium falciparum* from Tanzania. Trans R Soc Trop Med Hyg **2002**; 96:674–6.
44. Hastings IM, D'Alessandro U. Modelling a predictable disaster: the rise and spread of drug-resistant malaria. Parasitol Today **2000**; 16:340–7.
45. Ronn AM, Msangeni HA, Mhina J, Wernsdorfer WH, Bygbjerg IC. High level of resistance of *Plasmodium falciparum* to sulfadoxine-pyrimethamine in children in Tanzania. Trans R Soc Trop Med Hyg **1996**; 90:179–81.
46. Falade CO, Salako LA, Sowunmi A, Oduola AM, Larcier P. Comparative efficacy of halofantrine, chloroquine and sulfadoxine-pyrimethamine for treatment of acute uncomplicated falciparum malaria in Nigerian children. Trans R Soc Trop Med Hyg **1997**; 91:58–62.
47. Aubouy A, Jafari S, Huart V, et al. DHFR and DHPS genotypes of *Plasmodium falciparum* isolates from Gabon correlate with in vitro activity of pyrimethamine and cycloguanil, but not with sulfadoxine-pyrimethamine treatment efficacy. J Antimicrob Chemother **2003**; 52: 43–9.
48. Nzila AM, Mberu EK, Sulo J, et al. Towards an understanding of the mechanism of pyrimethamine-sulfadoxine resistance in *Plasmodium falciparum:* genotyping of dihydrofolate reductase and dihydropteroate synthase of Kenyan parasites. Antimicrob Agents Chemother **2000**; 44: 991–6.
49. Kyabayinze D, Cattamanchi A, Kamya MR, Rosenthal PJ, Dorsey G. Validation of a simplified method for using molecular markers to predict sulfadoxine-pyrimethamine treatment failure in African children with falciparum malaria. Am J Trop Med Hyg **2003**; 69:247–52.
50. Plowe CV, Kublin JG, Dzinjalamala FK, et al. Sustained clinical efficacy of sulfadoxine-pyrimethamine for uncomplicated falciparum malaria in Malawi after 10 years as first line treatment: five year prospective study. BMJ **2004**; 328:545.
51. Tanabe K, Sakihama N, Farnert A, et al. In vitro recombination during PCR of *Plasmodium falciparum* DNA: a potential pitfall in molecular population genetic analysis. Mol Biochem Parasitol **2002**; 122:211–6.
52. Mookherjee S, Howard V, Nzila-Mouanda A, Watkins W, Sibley CH. Identification and analysis of dihydrofolate reductase alleles from *Plasmodium falciparum* present at low frequency in polyclonal patient samples. Am J Trop Med Hyg **1999**; 61:131–40.
53. Abdel-Muhsin AM, Ranford-Cartwright LC, Medani AR, et al. Detection of mutations in the *Plasmodium falciparum* dihydrofolate reductase *(dhfr)* gene by dot-blot hybridization. Am J Trop Med Hyg **2002**;67: 24–7.
54. Cheesman SJ, de Roode JC, Read AF, Carter R. Real-time quantitative PCR for analysis of genetically mixed infections of malaria parasites: technique validation and applications. Mol Biochem Parasitol **2003**; 131:83–91.
55. Ranford-Cartwright LC, Johnston KL, Abdel-Muhsin AM, Khan BK, Babiker HA. Critical comparison of molecular genotyping methods for detection of drug-resistant *Plasmodium falciparum.* Trans R Soc Trop Med Hyg **2002**; 96:568–72.
56. Roper C, Pearce R, Bredenkamp B, et al. Antifolate antimalarial resistance in southeast Africa: a population-based analysis. Lancet **2003**; 361: 1174–81.
57. Nair S, Williams JT, Brockman A, et al. A selective sweep driven by pyrimethamine treatment in southeast Asian malaria parasites. Mol Biol Evol **2003**;20: 1526–36.
58. Cortese JF, Caraballo A, Contreras CE, Plowe CV. Origin and dissemination of *Plasmodium falciparum* drug-resistance mutations in South America. J Infect Dis **2002**; 186:999–1006.
59. Curtis J, Maxwell CA, Msuya FH, Mkongewa S, Alloueche A, Warhurst DC. Mutations in *dhfr* in *Plasmodium falciparum* infections selected by chlorproguanil-dapsone treatment. J Infect Dis **2002**; 186:1861–4.
60. Jensen NP, Ager AL, Bliss RA, et al. Phenoxypropoxybiguanides, prodrugs of DHFR-inhibiting diaminotriazine antimalarials. J Med Chem **2001**; 44:3925–31.
61. Canfield CJ, Milhous WK, Ager AL, et al. PS-15: a potent, orally active antimalarial from a new class of folic acid antagonists. Am J Trop Med Hyg **1993**; 49:121–6.
62. Carter R, Mendis KN, Roberts D. Spatial targeting of interventions against malaria. Bull World Health Organ **2000**; 78:1401–11.
63. Kyes S, Harding R, Black G, et al. Limited spatial clustering of individual *Plasmodium falciparum* alleles in field isolates from coastal Kenya. Am J Trop Med Hyg **1997**; 57:205–215.
64. Abdel-Muhsin AA, Mackinnon MJ, Awadalla P, et al. Local differentiation in *Plasmodium falciparum* drug resistance genes in Sudan. Parasitology **2003**; 126:391–400.