IDENTIFICATION AND ANALYSIS OF DIHYDROFOLATE REDUCTASE ALLELES FROM PLASMODIUM FALCIPARUM PRESENT AT LOW FREQUENCY IN POLYCLONAL PATIENT SAMPLES

SOMNATH MOOKHERJEE, VINCENT HOWARD, ALEXIS NZILA-MOUANDA, WILLIAM WATKINS, AND CAROL HOPKINS SIBLEY

Department of Genetics, University of Washington, Seattle, Washington; Kenya Medical Research Institute/Wellcome Trust Collaborative Program, Nairobi, Kenya; Department of Pharmacology and Therapeutics, University of Liverpool, Liverpool, United Kingdom

Abstract. As resistance to chloroquine spreads in sub-Saharan Africa, pyrimethamine plus sulfadoxine (PSD) is increasingly used as a first-line treatment for falciparum malaria. Populations of Plasmodium falciparum (Pf) resistant to PSD have been selected quickly in other regions. The resistance is strongly correlated with point mutations in dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS), the two targets of the drug. It is critical to identify drug-resistant Pf-DHFR alleles that are present at a low frequency in these populations since alleles that confer drug resistance will be quickly selected by PSD use. It is difficult to identify these rare alleles by standard molecular techniques. We have designed a yeast expression system that facilitates the identification and rapid analysis of Pf-DHFR alleles that confer PSD resistance, even when they are present at very low frequency in polyclonal patient samples. We analyzed samples from patients in Kilifi, Kenya collected between 1992 and 1995. We determined the prevalence of the drug-sensitive and drug-resistant alleles in patient samples analyzed in parallel by an allele-specific enzyme digestion (ASED) assay. We identified a pyrimethamine-resistant allele (S108N) present at a frequency of <1% in a sample that was scored as only S108 by ASED. In addition, a novel pyrimethamine-resistant allele (I164M) was isolated twice, once each from two different patient samples. This approach will allow determination of the prevalence of Pf-DHFR alleles that confer pyrimethamine resistance in particular regions, and the rapid identification of novel alleles that confer drug resistance.

Dihydrofolate reductase (DHFR) catalyzes the reduction of 7,8-dihydrofolate to 5,6,7,8-tetrahydrofolate, an essential step in the production of methyl groups for biosynthesis.\(^1,2\) Blocking the folate pathway at DHFR prevents the synthesis of deoxothymidine, methionine, and histidine, all essential metabolites for parasite growth. Pyrimethamine, clociguanil, chlorcycloguanil, cycloguanil, and the experimental WR-99210 are antimalarial drugs that competitively inhibit DHFR in Plasmodium falciparum (Pf), the parasite that causes the most virulent form of human malaria.\(^2-6\)

Effective antimalarial chemotherapy is becoming increasingly difficult due to the spread of multiple drug-resistant strains of P. falciparum.\(^2,9\) In contrast to most antimalarial drugs, the mechanism of resistance to antifolates has been well characterized. Point mutations in the DHFR of drug-resistant P. falciparum correlate with varying levels of resistance to these inhibitors.\(^3,10-24\) Studies in vitro of purified P. falciparum DHFR have shown that a single mutation from serine to asparagine at position 108 (S108N) reduces binding of pyrimethamine to the active site of the enzyme. Additional mutations from asparagine to isoleucine at residue 51 (N51I), cysteine to arginine at position 59 (C59R), and isoleucine to leucine at 164 (I164L) all augment the resistance.\(^25-29\)

The DHFR inhibitors are always used clinically in combination with sulfonamides or sulfones.\(^2,6,30\) Currently, pyrimethamine plus sulfadoxine (PSD) is replacing chloroquine as the first-line antimalaria drug in many countries in Africa.\(^30\) Unfortunately, alleles with mutations at S108N, N51I, and C59R have already been observed in this region; this combination is strongly correlated with decreased susceptibility to PSD.\(^30,14,16,18,31-34\) Trials of a more potent combination, chlorproguanil and dapsone (Lap/Dap) are in progress.\(^35\) In anticipation of the future deployment of new antifolates, and the continuing use of current antifolates, it is necessary to assess the distribution of Pf-DHFR genotypes currently prevalent in the field. Additionally, it is desirable to identify novel Pf-DHFR alleles that may confer a selective advantage against new antifolates or combinations of antifolates. In both cases, it is advantageous to screen efficiently large numbers of samples isolated from patients.

DNA sequencing is the most thorough approach to identify new and currently known alleles, and will remain the ultimate goal for characterization of mutations important for antifolate resistance. However, it is a relatively inefficient method by which to screen large numbers of samples. Furthermore, sequencing from field samples is not a reliable means of screening populations where mixed infections may be present, a common situation in African samples.\(^10\) Characterization of P. falciparum mutants by culturing in the laboratory is also a time-consuming strategy, and it has the disadvantage that it selects only those parasites that can adapt to tissue culture. The use of allele-specific oligonucleotide polymerase chain reaction (ASO-PCR) and allele-specific enzyme digestion (ASED) assays has been extremely useful in identifying already known point mutations,\(^10,14,15,17,24,31,36-43\) but these methods are not sensitive enough to detect an allele present at a low level in a mixed population.

We have developed a recombinant yeast expression system that allows the analysis of Pf-DHFRs in an in vivo system. We transformed a Saccharomyces cerevisiae strain without endogenous DHFR activity with a single copy plasmid bearing the wild type (S108) Pf-DHFR gene and showed that it complemented the DHFR deficiency in the yeast.\(^44\) These yeast strains were sensitive to the DHFR inhibitors at levels similar to those used to inhibit wild type Plasmodium in vivo and in vitro.\(^45\) In a similar manner, when the DHFR-
deficient yeast strain was transformed with the S108N or S108N + N51I Pf-DHFR alleles, each strain showed antifolate sensitivity that paralleled the sensitivity of the original *P. falciparum* strain. Thus, the ability of transgenic yeast bearing Pf-DHFR alleles to grow in the presence of DHFR inhibitors can function as a surrogate test of drug sensitivity in the *P. falciparum* strain from which the DHFR allele was isolated.

The analysis of Pf-DHFRs amplified from field samples through expression in this system permits the efficient extraction and analysis of DHFR alleles from mixed population samples. This is ensured by cloning the Pf-DHFR coding region onto a yeast plasmid that carries a centromere, so that each transgenic yeast strain can carry only one Pf-DHFR allele. We describe in the present study a step-wise process through expression in this system permits the efficient extraction and analysis in liquid culture.

**Materials and Methods**

**Yeast and malaria parasite strains.** The dfrl mutant TH5 *S. cerevisiae* strain (MATa leu2–3,112 trpl ura3–52 dfrl:: URA3 tup1) was generously provided by Dr. Tun Huang (University of Alberta, Edmonton, Alberta, Canada). Yeast were cultured for all experiments at 30°C, on minimal, drop-out, and rich media using standard yeast genetics techniques. The *tup1* mutation is critical to the maintenance of the strain because it increases the permeability of the yeast to dTMP and growth of the *dfrl* mutant requires supplementation of the media with 100 μg/ml of dTMP (Sigma, St. Louis, MO).

The Pf-DHFR genes in the reference stains were cloned from strain SL/D6 (S108 allele), Honduras (S108N allele), or Mikenga (S108N + N51I), as previously described. The DNA samples were isolated from field isolates collected during a clinical trial of the antifolate combination Lari-Dap in Kilifi, Kenya between 1993 and 1995. The patients were part of the Lap/Dap study. Blood samples were spotted onto filter paper, dried, and stored at room temperature. Parasite DNA was extracted from the samples using saponin lysis and Chelex-100 (Bio-Rad Laboratories, Hercules, CA) as described previously. The Pf-DHFR genes from individual patient parasite DNA samples were amplified by a PCR using a nested procedure. For the first amplification, the primers were 5′-CAC GAT TGA TAC ATA AAG-3′ (139A) and 5′-TAA AAT AAA CAA CAT C-3′ (140A), respectively. Reactions in a total volume of 50 μl contained 1 μM of each primer, 200 μM dNTP, 1.5 units of Vent polymerase (New England Biolabs, Beverly MA), 1× Thermopol buffer (New England Biolabs), and 5 μl of parasite DNA extract. Reactions were amplified in a Thermocycler PTC 200 (MJ Research, Watertown MA) under the following conditions: initial denaturation at 94°C for 3 min, denaturation at 94°C for 30 sec, annealing at 50°C for 45 sec, elongation at 72°C for 45 sec, repeated 34 times, followed by a final elongation at 72°C for 10 min. Upstream and downstream primers used in the second amplification were 5′-GAA CTG CGG GAT CCT ATG ATG GAA CAA GTC TGC G-3′ (71) and 5′-GAT TGA GCG CGG CCG TCA TAT GAC ATG TAT-3′ (86), respectively. The Vent polymerase was used to minimize errors during the PCR. Reactions were done in a total volume of 100 μl, using the same conditions as the first amplification, along with 5 μl of the first amplification product. Amplification conditions were the same as in the first amplification except that the annealing temperature was 55°C and there was no final elongation.

**Allele-specific enzyme digestion.** Allele-specific enzyme digestion assays have been designed, in some cases using mutagenic primers, to allow the detection of all known point mutations in the *P. falciparum* DHFR. Since previous studies have shown that the S108N mutation is the first step toward antifolate resistance in field samples from this region, mutations at codon 108 were used as a metric for antifolate resistance in this study, in the manner described previously. Briefly, the amplification product described was digested with restriction enzymes Alu I and Bsr I (New England Biolabs) in accordance with the manufacturer’s instructions. The DHFR genes amplified from the SL/D6, Mikenga, and Honduras lines were digested in parallel as controls. Digestion of the PCR product by *Alu I* indicates presence of the Ser 108 allele, digestion by *Bsr I* indicates presence of the Asn 108 allele, and partial digestion by both enzymes indicates a polyclonal sample.

**Expression in yeast.** The plasmid used in this experiment is a derivative of the Sikorski and Hieter shuttle vector pRS314 bearing the 600 basepair (bp) region 5′ of the *S. cerevisiae* DHFR coding region, 20 bp from the immediate 5′ region of the Pf-DHFR, the first 34 bp of the Pf-DHFR coding region, bases 710–785 from the 3′ end of the DHFR domain, and the 400 bp that comprise the transcription and translation terminator from the *S. cerevisiae* 3′ DHFR sequence as shown in Figure 1. This vector, designed by Joseph Cortese (University of Maryland School of Medicine, Baltimore, MD), has a unique introduced *Nco I* site at po-

![Figure 1](image-url)

**Figure 1.** Gap vector used in producing the *Plasmodium falciparum* dihydrofolate reductase (PF-DHFR)–dependent yeast strains. The pRS314 vector formed the basis for the DHFR gap vector. The yeast promoter derives from the 600 basepair (bp) region 5′ of the *Saccharomyces cerevisiae* DHFR coding sequence; region 1 is sequence 5′ of the first ATG in the *P. falciparum* coding sequence, 20-bp long in the vector and 12-bp long in the insert, region 2 is the first 34 bases of the Pf-DHFR coding sequence, region 3 comprises bases 710–785 of the Pf-DHFR coding sequence; the yeast terminator is the 400 bp 3′ of the *S. cerevisiae* DHFR coding sequence. A unique *Nco I* site was engineered at base 36 in the *P. falciparum* DHFR sequence to allow easy generation of the linearized vector. Cen = *S. cerevisiae* centromere sequence; Trp = *S. cerevisiae* (TRP1) gene.
sition 35 within the Pf-DHFR. The \textit{dfrr::URA3} yeast (TH5) were transformed with approximately 1–2 \(\mu\)g of the linearized vector and 0.25–1 \(\mu\)g of the population of DHFR molecules produced by PCR amplification of the genomic DNA isolated from each patient sample. Insert-vector mixtures were transformed into the yeast by standard lithium acetate–mediated methods.\textsuperscript{54} Transformation mixes were plated on tryptophan deficient synthetic media supplemented with 100 \(\mu\)g/ml of dTMP (Sigma). Homologous recombination of the vector and the insert repairs the gap in the vector, as shown in Figure 1. The exact site of recombination within each homologous regions is unknown, but the resulting DHFR must contain at least amino acids 12–236 derived from the introduced DHFR sequence. This region encompasses all of the amino acids known to confer pyrimethamine resistance in the DHFR enzyme.

**Replica plate assay.** The pyrimethamine, chlorocyclocuguanil, cycloguanil, clociguanil, and WR 99210 were provided by Drs. David Jacobus and Norman Jensen (Jacobus Pharmaceutical Company, Princeton, NJ). Drug stocks were dissolved in dimethysulfoxide (Sigma) at concentrations of 5 or 10 mg/ml and dilutions were made in DMSO. The growth of yeast dependent upon S108 (D6), S108N (Honduras), and S108N + N51I (Mikenga) alleles was used a standard to compare the drug sensitivity of the yeast transformed with unknown DHFR alleles. Verified transformants were patched onto complete media supplemented with dTMP and allowed to grow for 3–4 days. These patches were then double replica plated onto plates with 10\(^{-6}\) M drug. The double replica plating is essential because the drugs work by inhibiting growth, so only actively growing yeast are sensitive. The appropriate amount of drug to establish these concentrations was spread on the surface of complete media prior to the replica plating, and allowed time to be absorbed. Plates were scored after 3–5 days of growth at 30\(^{\circ}\)C.

**Liquid culture 50\% inhibitory concentration (IC\(_{50}\)) assay.** Quantitative drug sensitivity assays were performed in liquid culture on clones of interest selected from the plate screening. The same drugs and stock drug solutions were used for both assays. For each clone, an equal concentration of actively growing cells (approximately 2 \times 10^6 cells/ml) was treated with the appropriate concentration of drug or solvent. The assay was performed in 96-well plates in the manner described previously.\textsuperscript{46} The degree of growth of each culture was ascertained by the absorbance at 650 nm after 24 hr. Inhibition curves were calculated in each case by plotting the percentage of growth of treated cultures (relative to the solvent control), versus the drug concentration of the treated cultures. Each point was assayed in duplicate.

**Spoke assay.** Spoke assays were performed in the manner described previously.\textsuperscript{46} Briefly, clones of interest and control strains were patched radially onto complete plates, and allowed to grow for 3–4 days. These patches were then double replica plated onto two complete plates. Ten microliters of 5 or 10 mg/ml of drug was added to the center of the first plate, and 10 \(\mu\)l of DMSO was added to the center of the control plate. Plates were scored after 3–5 days of growth.

**RESULTS**

Previous studies of samples isolated from the Kilifi area of coastal Kenya had demonstrated that at least half of the samples were polyclonal (contained at least two different DHFR alleles). In the current study, a set of four samples was chosen in which two were positive by ASED for Ser 108, one was positive for Asn 108, and one was obviously polyclonal, being positive for both Ser 108 and Asn 108. Table 1 summarizes these results. We had two goals in this study; first, to estimate the relative frequency of antifolate resistant alleles in each sample, and second, to identify quickly any DHFR alleles with unusual drug-sensitivity phenotypes and isolate them for further molecular analysis.

The DHFR domain was amplified in a nested manner with genomic DNA from each sample as the template. If a sample contained several different DHFR alleles, they should all be amplified roughly in proportion to their prevalence in the initial template population. The population of these linear molecules encoding DHFR(s) was cotransformed with the linearized plasmid pEH2-GR7 into the DHFR deficient yeast strain TH5\textsuperscript{44} and selected on plates lacking tryptophan, all as previously described.\textsuperscript{45} As shown in Figure 1, homologous recombination allowed the reconstitution of a circular plasmid that expressed the Pf-DHFR under the control of the yeast DHFR promoter. The yeast centromere sequence on the plasmid ensured that each yeast transformed expressed only a single copy of the plasmid.\textsuperscript{50} We assume that each DHFR allele in the original sample was amplified with the same efficiency and that there was no bias in the recombination that reconstitutes the plasmids. If these assumptions are met, the distribution of DHFR alleles in the transformant colonies should reflect the original distribution in the patient sample.

To determine the phenotype of each yeast colony, transformants were patched onto complete plates lacking dTMP, and double replica plated onto complete plates containing 10\(^{-6}\) M pyrimethamine. Plates had reference strains dependent upon the yeast DHFR coding region or the S108, S108N, or S108N + N51I allele. We had previously established that this concentration distinguishes the pyrimethamine-sensitive S108 allele from all of the pyrimethamine-resistant alleles that have been identified.\textsuperscript{45} This simple plating then allows one to estimate the fraction of transformants with drug sensitivity comparable to the pyrimethamine-sensitive SL/D6 line. Figure 2 shows a sample of these data. Field sample 9 was scored as S108 alone by ASED. However, analysis of 311 individual colonies revealed two that grew on the plate containing 10\(^{-6}\) M pyrimethamine. The

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* The dihydrofolate reductase (DHFR) alleles amplified from the indicated patients samples were assayed by ASED to identify the presence or absence of the S108N mutation. The same DHFR amplification product was transformed into TH5 yeast with the gapped vector as described in the Materials and Methods. Transformants were arrayed on a plate without dTMP and three days later double replica-plated onto a plate with 10\(^{-6}\) M pyrimethamine, as shown in Figure 2. Plates were scored after four days growth at 30\(^{\circ}\)C.
two resistant clones isolated were shown to have the S108N mutation by ASED and by sequence of the whole DHFR coding region. A screen of the same magnitude did not identify any pyrimethamine-resistant colonies in field sample 14.

Although it is clearly of most interest to identify minor fractions of pyrimethamine-resistant alleles, rare sensitive alleles in predominantly resistant populations can also be readily detected. Field sample 50 was scored as N108 by ASED; however, the yeast screen showed seven colonies with a pyrimethamine-sensitive phenotype. Field sample 39 was scored as N108 by ASED, and 73% of the colonies showed a pyrimethamine-resistant phenotype, as expected. A minority component was not detected in field sample 14, although it is possible that rare alleles would have been detected had far larger numbers of clones been screened. Table 1 summarizes these experiments.

A number of DHFR inhibitors are currently in use or in clinical trials in east Africa. Because these drugs exert such a potent selective force, it is critical to establish efficient methods for identifying alleles that confer resistance to each drug. The ASO-PCR and ASED are two approaches, but they failed to identify resistant alleles that are present at low levels in the population. To examine the drug sensitivity of the DHFR alleles isolated from these patient populations, transformants derived from field sample 9, as well as 8, 30, and 45 from the same patient pool were arrayed on master plates, and then replica plated to a series of plates with a set concentration of a number of different drugs. We chose these strains because they showed different drug sensitivity phenotypes in the initial screening on pyrimethamine. The three reference strains dependent upon S108, S108N, or the S108N + N51I DHFR alleles were included in all analyses. The yeast were replica plated to plates containing pyrimethamine, cycloguanil, chlorcycloguanil, and clociguianil, or WR99210 at concentrations ranging from 10⁻⁸ to 10⁻³ M. Figure 3 shows two examples of such an experiment in which the strains of interest were tested against pyrimethamine and clociguianil. Two of the unknown strains did not form patches on 10⁻⁶ M pyrimethamine, similar to the S108 allele. One unknown strain grew on 10⁻⁶ M but not on 10⁻⁵ M pyrimethamine, like the S108N reference strain. Five of the eight test strains showed phenotypes comparable to the S108 + N51I strain. Comparison of the growth of the field-derived and reference stains showed that strongly drug-resistant strains were easily identified by this quick screen. Comparisons of the reference and experimental strains on the plates with clociguianil showed only one field-derived allele was highly resistant to clociguianil.

Examination of the relative growth of the same strain on plates containing the five drugs also demonstrated the relative activity of the drugs. Figure 4 shows the same array of control and test strains replica plated onto plates with 10⁻⁵ M of each drug. As one would expect from previous studies on these drugs, the relative potency of the drugs was pyrimethamine < cycloguanil < chlorcycloguanil < clociguianil < WR99210. It is particularly striking that the only drug of this group that was effective against all the field-derived strains was WR99210.

We also tested some of these strains with an even simpler technique. In this assay, the strains of interest are radially arranged, replica plated and a small amount of concentrated drug is added to the center of a plate. The radius of inhibition of each strain is a quick measure of the relative sensitivity of the strains on the plate to that drug. Figure 5 shows the three reference strains and four strains derived from field samples arrayed opposite these standard controls. The plates shown were spotted with 10 μl of 10 mg/ml pyrimethamine or WR99210. The growth of each strain toward the concentrated drug compared with the reference strains allowed a quick estimate of the relative potency of that drug against the field isolates. For example, strain 4, like the S108 allele, is highly sensitive to pyrimethamine but less sensitive to WR99210, whereas strains 1 and 2 more closely resemble the phenotype of the S108N + N51I allele.

Most yeast strains derived from field-isolated DHFR genes showed phenotypes similar to one of the three reference strains. This was easily observed in plate assays, and was further quantified in liquid culture assays. A sensitive and a resistant strain isolated from a single patient sample were tested for pyrimethamine sensitivity in comparison with the S108, S108N, and S108N + N51I controls (Figure 6). The cells were dispensed in a series of wells in a 96-well plate with increasing concentrations of pyrimethamine. After 24 hr, growth was monitored by measuring the optical density at 650 nm. The growth relative to the control with
solvent alone is plotted. The IC$_{50}$ values calculated for the field-derived strains show a high degree of similarity to the standards; 30-B2 had an IC$_{50}$ of approximately $1 \times 10^{-9}$ M, like that of the S108 dependent strain, and 30-A2 had an IC$_{50}$ of approximately $6 \times 10^{-6}$ M, like the S108N + N51I reference strain. Analysis of the DHFR alleles carried in these two strains demonstrated that these mutations were indeed present.

Since a large number of samples can easily be screened by the replica plating, it is also possible to identify phenotypes that result from rare alleles at the DHFR locus. For example, in addition to the low frequency S108N + N51I mutants described, one strain was isolated from an otherwise homogenous field sample that demonstrated slightly elevated resistance to all the antifolates tested, relative to the Mikenga control. When sequenced, the DHFR mutations present in this strain were found to be S108N + C59R, consistent with the DHFR genotype of the K1 strain. Even more interesting, three yeast strains were detected because they showed resistance to pyrimethamine, but grew more slowly than the reference strains. One had a change from tyrosine to histidine at amino acid 57 and two had the same mutation, a novel change from isoleucine to methionine at position 164. It is possible that these novel alleles were generated during the

FIGURE 3. Assay of drug sensitivity of unknown dihydrofolate reductase (DHFR) alleles by replica plating. Four control strains (boxed) were grown on complete plates along with eight different yeast strains dependent upon DHFR alleles isolated from patients with polyclonal infections. After 3–4 days, each master plate was double replica plated to complete plates with either pyrimethamine or clociguanil at the indicated concentrations. Growth was photographed after four days at 30°C.
amplification with Vent® polymerase, and were not present in the original sample. However, the two I164M mutations were observed in different patient samples, making it likely that this allele existed in the wild populations of *P. falciparum*.

**DISCUSSION**

As molecular methods have been developed, they have been applied increasingly to analysis of pathogens in populations of affected individuals. Mutations in the DHFR gene of *P. falciparum* are a major component that determines sensitivity of the parasites to Fansidar and the experimental combination antifolate Lap/Dap. If one wishes to evaluate the sensitivity of malaria parasites to a DHFR inhibitor in a geographic region, three questions about the Pf-DHFR alleles in a given population need to be addressed. 1) What already known mutant alleles are present in the population? 2) What is the frequency of each of the mutant alleles? and 3) Are there any novel mutants that confer drug resistance?

There are a number of methods currently used for analysis of mutations in a population. The ASO-PCR and ASED have been extremely useful for surveillance of common mutations that are known to confer drug resistance.10,14,15,17,31,36–42,57 There are two problems with this approach. First, only alleles that have been previously identified are surveyed. Second, polyclonal infections are common at least in African samples,10,15,16,58 and this approach does not allow very precise estimation of the frequency of the mutant alleles identified. In addition, it is clear that a patient sample that contains a minor proportion of an allele that confers strong drug resistance is frequently not identified as polyclonal. In our experiments, the ASO detected minor alleles when they were greater than about 10%. If that minor allele confers drug resistance, it will be easily selected by treatment of that patient with a drug to which that allele confers resistance. It has been shown by several groups that treatment of a patient with such a mixed population easily selects the resistant parasites and produces a rapid recrudescence of the infection.10,16 Greater sensitivity of the ASO can be attained by blotting the PCR product and probing, but on the routine basis required for larger surveys, this would be extremely labor intensive. Direct sequencing of the gene that encodes the drug target avoids the necessity for concentrating only on previously identified alleles, but is also not well suited to large-scale surveys. If one aimed to estimate proportions of alleles in a population and to identify rare alleles, the amount of sequencing would also be prohibitively expensive. Some techniques intermediate between these two approaches have been used in other systems. For example, single-strand conformation polymorphisms have been used to survey populations for alleles different from a set of reference standards59 and surveillance of complete DNA sequences with DNA arrays may ultimately prove feasible. Analysis of polyclonal samples would also be problematic with these more sophisticated approaches.

The analysis in yeast of DHFR alleles from patient samples can complement the approaches above by efficiently identifying rare novel alleles, and roughly estimating the frequency of each mutant allele in a population. In the experiments described earlier, we analyzed relatively small numbers of yeast colonies from each patient sample. Assay of 300–400 separate colonies is easily accomplished on about 10 plates, and the patching takes about 90 min. This number yields a fairly accurate estimate the relative frequency of drug-sensitive and drug-resistant alleles in the sample. The replica plating is an excellent first screen, but is not meant to be extremely quantitative. For example, patches at the periphery of the plate are sometimes not transferred as efficiently as those in the center. One can see this in Figure 2 where the control patches at the bottom of the no drug plates...
FIGURE 5. Rapid radial assay of dihydrofolate reductase (DHFR) inhibitors. The four reference strains and four strains with unknown DHFR alleles were streaked on complete plates and grown for 3–4 days. These plates were then double replica plated to a complete plate, followed by the application of 10 μl of 10 mg/ml of pyrimethamine (PYR) or WR-99210 dissolved in dimethylsulfoxide to the center. Inhibition was measured by the distance from the center of the plate to which the yeast grew in four days at 30°C.

FIGURE 6. Quantitative assay of the 50% inhibitory concentration (IC_{50}) for pyrimethamine. Three reference strains and two yeast strains dependent upon unknown dihydrofolate reductase alleles were grown in pyrimethamine concentrations ranging from 0 to 10^{-5} M for 24 hr. Growth of the yeast was measured optically and is plotted as the growth in wells with drug relative to growth in no drug. Clone 30-B2 has an IC_{50} of approximately 1 \times 10^{-4} M, like the S108 dependent strain, and clone 30-A2 has an IC_{50} of approximately 6 \times 10^{-6} M, like the S108N + N51I reference strain.
were not all uniformly transferred. With this in mind, any interesting colonies can be more thoroughly examined in liquid culture.

We assumed that the PCR amplification and the transformation into yeast are equally efficient for all alleles. This may not be the case. In early amplification cycles with limiting template, the annealing of the primer to any particular template is a stochastic process, and if a rare allele were initially amplified it could be over-represented in the final population of DNA molecules produced. In these samples, the template is abundant, and the fraction of colonies that fail to grow on the pyrimethamine plate appears to estimate fairly accurately the original proportions of the sensitive and resistant parasites among the amplified products. This fraction should then yield a reasonable estimate of the original prevalence in the patient sample. With this approach, even an allele present at only 1% can be detected easily, and one that is even less frequent could be identified with some extra effort.

A novel allele will be identified in this system only if it has an unusual drug-response phenotype in yeast. If a new mutation confers the same sensitivity to each inhibitor as one of the reference strains, its novelty will not be recognized in the screen. While there is a limitation, the major focus in screening patient samples is the identification of alleles that pose a major threat of clinically important drug resistance, and that is unlikely to be missed here. The screen for novel alleles was conducted using plates with $10^{-4}$ M pyrimethamine, a concentration that discriminates the fully pyrimethamine-sensitive phenotype from all others. A screen for alleles with clinically important levels of pyrimethamine resistance would simply require replica plating to plates with higher levels of drug. For example, a concentration of $5 \times 10^{-5}$ M pyrimethamine distinguishes the triple mutant (N51I + C59R + S108N) from the highly resistant quadruple mutant (N51I + C59R + S108N + I164L) (Lau H, Munning J, unpublished data). A screen for this highly resistant phenotype among African samples would be an extremely useful adjunct to other surveillance methods.

Both novel alleles identified in this screen occurred in regions of the DHFR protein already implicated in drug resistance. Mutations at position 50, 51, and 59 are all known to enhance pyrimethamine resistance in combination with S108N. The proximity of the novel mutation Y57H to these known mutations suggests that amino acid 57 may also be an important position for interaction of pyrimethamine with DHFR. Even more interesting, a commonly observed mutation at position 164, I164L, confers extremely high levels of pyrimethamine resistance when combined with the triple mutant, N51I + C59R + S108N. When this quadruple mutation has been observed in patient samples, it has been strongly correlated with clinical failure of PSD. The I164L mutation has not yet been observed in any African samples. If the I164M mutation also confers high level pyrimethamine resistance, the quadruple could easily be generated by recombination, since the triple mutant is extremely common in this region of Kenya. This raises the alarming possibility that clinical failure of PSD could be very rapidly follow selection of this allele in east Africa. We amplified all DHFR sequences with Vent® polymerase to reduce the introduction of errors in the PCR. The published error frequency of this polymerase is still about $5 \times 10^{-5}$, so this step could still have been the source of the novel mutations. However, the isolation of the I164M allele from reactions initiated with two independent patient samples strongly suggests that this allele is in the population of *P. falciparum* sampled in this study. Whatever their origin, the apparent pyrimethamine-resistant phenotype of these alleles will provide interesting information on the importance of these amino acids in the interaction of DHFR inhibitors with the active site of the *P. falciparum* DHFR enzyme.

It is important to emphasize that the IC$_{50}$ of a particular drug that we measure in yeast is a complex function. The effectiveness of the drug depends both upon the permeability of the yeast to the drug and the level of enzyme expression in the cell. Since the yeast strains compared here are isogenic, the entry of the drug should be the same in all of the strains. The plasmid that controls expression of the DHFR is also identical in all of the strains, so we assume that it is valid to compare the relative effectiveness of a particular drug against yeast strains dependent upon different DHFR alleles. We included the reference DHFR alleles in all of these assays to allow a rapid classification of the relative drug sensitivity phenotypes of the DHFR alleles under study.

In this study, we have assessed only the phenotype of the DHFR alleles from the patient samples. Clinically, all of these DHFR inhibitors are used in combination with sulfonamides or sulfones. A number of studies have shown that resistance of *P. falciparum* to PSD is correlated with point mutations in both DHFR and dihydropteroate synthase (DHPS). Analysis of both genes will likely be required to correlate completely the molecular analysis with the response of parasites to the combination drug. We are currently developing a similar system that will allow us to analyze the drug responses of *P. falciparum* DHPS genes as well.

The current study is a small one, performed to establish the feasibility of rapid screening of DHFR alleles. The method requires only a small amount to genomic DNA, and thus is well-adapted to analysis of either current samples, or those collected in the past. A number of groups have shown that blood samples fixed and stained for routine diagnosis provide DNA of sufficient quality to provide a template for PCR amplification. That opens the possibility of studying the drug sensitivity in yeast of DHFR alleles collected before antifolate drugs were introduced in a particular locale, and following the changes in allele frequencies as the drugs moved into common use. The combination of ASO-PCR and ASED with the analysis of DHFR and DHPS alleles in yeast will provide powerful tools for these analyses. Systematic study will allow the tracking of DHFR and DHPS phenotypes in defined regions, and perhaps allow us to design strategies that will slow the selection of the drug-resistant alleles responsible for the clinical failure of antifolate drugs.

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