

POLYMORPHISMS IN *PFCRT*, *PFMDR1*, *DHFR* GENES AND *IN VITRO* RESPONSES TO ANTIMALARIALS IN *PLASMODIUM FALCIPARUM* ISOLATES FROM BANGUI, CENTRAL AFRICAN REPUBLIC

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Abstract. Drug resistance is probably the greatest challenge to most malaria-control programs. Given the limited resources for other malarial-control measures, rational drug use is crucial. Molecular markers for parasite resistance such as *pfprt*, *pfmdr-1*, and *dhfr* have the potential to be used in an integrated fashion to provide timely information that is useful to policy makers. Therefore, we evaluated polymorphisms in these genes from *Plasmodium falciparum* and their association with *in vitro* antimalarial drug resistance to 135 parasites samples collected in Bangui in 2004. For the *dhfr* gene, we found a strong association between the *dhfr* genotype and chemosensitivity to pyrimethamine. For the *pfprt* gene, we found that haplotypes with mutant-type alleles led to significant changes in the IC50 values for chloroquine, monodesethylamodiaquine, and quinine. We found no correlations for the *pfmdr1* gene. These findings suggest that a regular monitoring and screening for resistance markers for antifolates and for chloroquine could act as an adjunct to *in vivo* trials.

INTRODUCTION

The resistance to antimalarial drugs, especially chloroquine (CQ), of *Plasmodium falciparum* is one of the principal factors contributing to the worldwide increase in morbidity and mortality due to malaria.^{1,2} Different approaches have been developed to monitor the extent of antimalarial drug resistance and to determine the biologic mechanisms by which the parasite has evaded the action of the drug.

The susceptibility of *P. falciparum* to antimalarial drugs is usually assessed by therapeutic responses (*in vivo* test). This method has allowed the thresholds of treatment failure that are crucial for adjusting antimalarial drug policies to be determined.³ However, the risk of loss of patients during long follow-up periods and the logistical demands of clinical response studies in endemic areas have led to the development of laboratory strategies for identifying anti-malaria drug resistance. Laboratory strategies include *in vitro* drug sensitivity tests and evaluation of molecular markers associated with drug resistance.^{4,5} Antimalarial drug sensitivity tested *in vitro* provides information on the frequency of the resistant phenotype among the populations of parasites being transmitted and the possible cross-resistance patterns of anti-malaria drugs.⁶ *In vitro* testing is also useful for evaluating the efficacy of new drugs and for investigating the biologic mechanisms of drug action and resistance.^{7,8} Although this method is useful, its application is limited because samples must be immediately cultured or cryopreserved for transport, parasites must be adapted to culture (which is laborious), and the tests use expensive and radioactive materials.⁹ Although 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 subpopulations of parasites, so that the parasites ultimately assayed may be genetically and phenotypically unrepresentative of the original parasite population.

These limitations of *in vivo* and *in vitro* methods have led to the search for genetic markers of resistance. Potential mo-

lecular markers for parasite resistance to quinoline antimalarials are being identified. The association of CQ resistance and single nucleotide polymorphisms (SNPs) in the *pfprt* (CQR transporter) and *pfmdr1* (multidrug resistance 1) genes are presently well established.^{10–13} For example, the K76T mutation in *pfprt* gene 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 diverse parasite isolates.¹⁴ Polymorphisms in *pfmdr1*, which encodes the *P. falciparum* P glycoprotein homologue 1, modulate chloroquine resistance in mutant *pfprt*-harboring parasites *in vitro*,¹⁵ although their role *in vivo* has yet to be substantiated.¹⁰ The mutations most often cited as potential contributors to chloroquine resistance are *pfmdr1* N86Y and D1246Y. In the same way, several point mutations in *P. falciparum dhfr* and *dhps* genes have been associated with sulfadoxine-pyrimethamine (SP) treatment failure in Africa.^{16–22} Univariate analysis have shown that the *dhfr* triple mutant S108N/N51I/C59R or the *dhps* double mutant A437G/K540E or the *dhfr* and *dhps* quintuple mutant carrying all these 5 mutations are all associated with SP treatment failure in patients.^{19,21,22}

In the Central African Republic (CAR), *Plasmodium falciparum* resistance to CQ has been documented since 1983²³ and to SP since 1987.²⁴ Since 2003, when the latest *in vivo* study was carried out,²⁵ amodiaquine + SP combination has been used as interim first-line treatment, until the best alternative treatments, such as Artemisinin-based Combination Therapies (ACT), become available at low prices in the CAR. We have previously determined, in samples collected in 2004, the *in vitro* susceptibility of *P. falciparum* strains to antimalarials currently being used in the CAR (chloroquine, amodiaquine, quinine, and pyrimethamine), to antimalarials that will be available for future use in this region (artemisinin, halofantrine), and to prophylactic antimalarials (mefloquine, atovaquone, doxycycline).²⁶ We have also previously determined the baseline frequency distribution of the mutant alleles of genes associated with antimalarial drug resistance (*pfprt*, *pfmdr1*, *dhfr*, and *dhps*).²⁷

Here, we aim to complete these studies by determining the utility of the molecular markers such as *pfprt*, *pfmdr1*, and

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dhfr in monitoring antimalarial drug resistance. We have estimated the association of these molecular markers with *in vitro* responses to antimalarial drugs (chloroquine, monodesethylamodiaquine, quinine, dihydroartemisinin, mefloquine, halofantrine, and pyrimethamine) in *Plasmodium falciparum* isolates from Bangui, Central African Republic.

METHODS

***P. falciparum* isolates.** This study was conducted in Bangui, the capital of the CAR, between March and July 2004. Bangui is located beside the Oubangui river in the middle of central Africa, north of the Democratic Republic of the Congo (Geographic coordinates: 7°00' N, 21°00' E). The climate is tropical and rainfall is at its highest from April to November. The average temperature varies from 19–32°C. Malaria transmission occurs throughout the year, with peak transmission at the beginning and the end of the rainy season. Malaria is hyperendemic in this region and *Plasmodium falciparum* is the principal parasite. The prevalence of this parasite in children less than 5 years of age is 31.8%.²⁸

We obtained 381 *P. falciparum* clinical isolates from symptomatic patients from the CAR attending several health centers in Bangui before the patients were treated. Venous blood samples from patients giving informed consent were collected in a tube coated with EDTA (Vaicutainer® tubes, Becton Dickinson, Rutherford, NJ, USA). Giemsa-stained thin and thick blood smears were examined to check for mono-infection with *P. falciparum* and to determine parasite density. Blood samples with a parasite density > 0.1% were assayed *in vitro* within 8 hours of blood extraction. The patients were treated with amodiaquine-sulfadoxine-pyrimethamine combination or quinine, as recommended by the National Malaria Control Program in the CAR.

Ethical approval. As there is no National Ethics Committee in the CAR, study protocols were approved by the expert committee for antimalarial drug policy and the CAR Ministry of Health. Blood samples were obtained from patients after obtaining informed consent during routine malaria diagnosis.

***In vitro* drug sensitivity assay.** Stock solutions of chloroquine, monodesethylamodiaquine, dihydroartemisinin, quinine, mefloquine, and halofantrine were prepared in methanol. The stock solution of pyrimethamine was prepared in ethanol. The final concentration in methanol and ethanol did not exceed 0.05%. Twofold (4-fold for pyrimethamine) serial dilutions of the stock solutions were prepared in distilled water. The solutions tested had concentrations ranging from 12.5–3200 nM for chloroquine, 25–3200 nM for quinine, 7.5–1920 nM for monodesethylamodiaquine, 0.25–64 nM for dihydroartemisinin, 0.25–32 nM for halofantrine, 1.5–400 nM for mefloquine, and 50–40,000 nM for pyrimethamine. Aliquots (20 µL) of each solution (at all concentrations tested) were transferred to 96-well tissue culture plates in triplicate.

The venous blood samples were washed 3 times in RPMI 1640 medium. The white blood cell interface was removed after each wash. The erythrocytes were then resuspended in a volume of complete RPMI 1640 medium (RPMI 1640 medium supplemented with 10% human serum (Serum AbCys®, France, lot no S02909S4190), 25 mM HEPES buffer, and 25 mM sodium bicarbonate) giving a hematocrit of 1.5% and an initial parasitemia of 0.1–0.5%. Para-aminobenzoic acid-free and folic acid-free RPMI 1640 me-

dium was used to assess the *in vitro* sensitivity to pyrimethamine. If the blood sample had a parasitemia greater than 0.5%, fresh uninfected erythrocytes were added to adjust the parasitemia to 0.3%. The technical procedure for the *in vitro* isotopic microtest and calculation of 50% inhibitory concentrations (IC₅₀) has been previously described.²⁶

Extraction of DNA. The DNA template for PCR and the detection of mutant alleles was prepared from the whole blood sample. The blood was centrifuged and the erythrocytes were frozen at –20°C until extraction. Parasite DNA was extracted from 100 µL of thawed red blood cell pellets by treatment with 0.1M NaOH for 3 minutes at 100°C. The supernatant was collected and treated with 250 µL of lysing solution (0.1 M Tris HCl, Triton 100X, 1 M NaCl, SDS 10X, 0.5 M EDTA) and 20 mg/ml proteinase K for 1 hour at 37°C, and then extracted twice with phenol/chloroform (1:1). The DNA was then precipitated with ethanol, resuspended in 100 µL of distilled water, and stored at –20°C.

Amplification by polymerase chain reaction and detection of mutant alleles. Polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis were carried out for 3 genes (dihydrofolate reductase, *dhfr* gene at codons 16, 50, 51, 59, 108, 164; chloroquine resistance transporter, *pfcr* gene at codons 72, 74, 75, 76 and in multidrug resistance 1, *pfmdr1* gene at codons 86 and 1246) to identify the presence of mutant alleles.

A detailed description of these methods is available on the Web at <http://medschool.umaryland.edu/CVD/plowe.html>.

All PCR amplifications were performed with Gene Amp PCR system 9700® thermocycler (Applied Biosystems®, Courtaboeuf, France) and Taq DNA Polymerase Promega® (Promega France, Charbonnières, France). Five microliters of PCR products were incubated with restriction enzymes (New England Biolabs®, Beverly, MA, USA) according to manufacturer's protocols in a 15-µL final reaction. Laboratory strains of *P. falciparum* served as controls (positive and negative) and were included in all PCR and enzyme digests procedures (purified genomic DNA from W2, HB3, and 3D7 reference strains from the Malaria Research Reference Reagent Resource Center, MR4/ATCC, Manassas, Virginia). A subset of 45 samples also was sequenced in Genopole at Institut Pasteur (Paris, France). Briefly, 50 µL of PCR products were purified using polyacrylamide P-100 Gel (Bio-Gel P-100, BioRad®, Marnes-la-Coquette, France) on 96-well plate filtration (Millipore®, St. Quentin en Yvelines, France). Evaluation of template quantity and quality was performed using agarose 1.2% gel and buffer TBE 0.5% (Invitrogen®, Cergy Pontoise, France) on Electrofast micro gel system 96 wells (Fischer Scientific Labosis®, Ottawa, Canada) with DNA quantification kit (Molecular weight, Abgene®, Courtaboeuf, France). Sequencing reactions were performed on a 96-well format, with individual caps (MicroAmp Optical 96-well reaction plate) using BigDye Terminator chemistry (BigDye v 3.1 terminator, Applied BioSystem®, Courtaboeuf, France) on GeneAmp-9700 (Applied BioSystem®, Courtaboeuf, France). Sequencing was realized with 10 µL of EDTA 0.3 mM per well on automatic DNA analyser ABI 3700 (Applied BioSystems®, Courtaboeuf, France). For data analysis, we used SeqScape® software (Applied BioSystem®, Courtaboeuf, France).

msp-1 and msp-2 genotyping. Polymerase chain reaction genotyping was performed as described by Snounou,²⁹ using

repetitive regions found in 2 polymorphic genetic makers, namely *msp-1* (block 2) and *msp-2* (block 3). Allelic variants of *msp-1* (MAD20, K1 and RO33) and *msp-2* (3D7 and FC27) were detected by allelic family-specific nested PCR. All PCR amplifications (Gene Amp PCR system 9700®, Applied Biosystems®, Courtaboeuf, France) contained a positive control (purified genomic DNA from W2, HB3, and 3D7 reference strains from the Malaria Research Reference Reagent Resource Center, MR4/ATCC, Manassas, Virginia for *msp-1* and *msp-2* allelic families) and a negative control (containing no target DNA).

Statistical analysis. The prevalence of each mutant allele was determined without prior knowledge of the *in vitro* susceptibility status of the isolates. We used ANOVA or the non-parametric Mann-Whitney *U* test to compare the geometric means of the IC50 values with respect to the *pfert*, *pfmdr1*, and *dhfr* genotypes. We used Fisher's exact test to assess the statistical associations between point mutations and the *in vitro* resistance phenotypes. We also determined the positive predictive value (PPV) for molecular markers as the probability that a resistant phenotype isolate was restricted to isolates carrying a mutant-type allele. All tests were 2-sided, and $P < 0.05$ were considered statistically significant.

RESULTS

Among 381 blood samples collected from *P. falciparum*-infected individuals, only monoclonal samples (1 genotype found with *msp-1* and *msp-2* genotyping) with most prevalent genotypes and data from the *in vitro* isotopic drug sensitivity assay were analyzed: chloroquine ($N = 134$), monodesethylamodiaquine ($N = 134$), quinine ($N = 135$), dihydroartemisinin ($N = 51$), halofantrine ($N = 24$), mefloquine ($N = 49$), and pyrimethamine ($N = 39$). The mean age of the *falciparum*-infected individuals was 25 years (range, 1–80 years) and 36% were male. The geometric mean of parasitemia was 15,442 parasites/ μ l of blood (range 78–443,071 parasites/ μ l of blood).

Data from the *in vitro* isotopic drug sensitivity assay, the baseline frequency distribution of the mutant alleles of *pfert*, *pfmdr1*, *dhfr*, and *dhps* genes and clonality of infections by *msp-1* and *msp-2* genotyping in *P. falciparum* isolates have been previously described.^{26,27,30} Briefly, mutant alleles of the *pfert* gene were found in two thirds of samples and the frequencies of the deduced haplotype were (amino-acid residues at positions 72, 74, 75, and 76): CIET 45%, CMNK 33.3%, CMNT 12.3%, CIEK 4.7%, SIET 2.3%, SIEK 1.2%, SMET 0.6%, and CINT 0.6%. (CIET, CIEK, SIET, SIEK, SMET, CINT haplotypes were confirmed by sequencing.) The most common allele of the *pfmdr1* gene among the field isolates of *P. falciparum* was 86Y (21.9%). The 1246Y allele was also common (18.0%). Frequencies of deduced haplotype profiles for the *dhfr* gene of *Plasmodium falciparum* isolates were (amino-acid residues at positions 16, 50, 51, 59, 108, and 164): ACIRNI 57.5%, ACIRSI 9.6%, ACNCNI 8.4%, ACNRNI 7.8%, ACNCNI 7.2%, ACNRSI 3.0%, ACICNI 3.0%, ACICSI 2.4%, ACNCTI 0.6%, ACIRSL 0.6%, and ACIRNL 0.6%. (ACIRNI, ACIRSI, ACNRNI, ACICNI, ACNCTI, ACIRSL, and ACIRNL haplotypes were confirmed by sequencing.)

Correlation between polymorphisms in the *pfert* and *pfmdr1* genes and *in vitro* chloroquine susceptibility. For

polymorphisms in the *pfmdr1* gene, we found no significant differences for the geometric means of the IC50 in positions 86 and 1246 ($P > 0.05$, data not shown).

For the *pfert* gene, we compared geometric means of IC50 values for deduced haplotypes in the *pfert* gene and observed significant differences ($P = 0.001$) between haplotype with wild-type alleles (CMNK, $N = 55$, mean IC50 = 50 nM, CI 95% 32–68) and haplotypes with mutant-type alleles: CIET, 3 times higher ($N = 63$, mean IC50 = 151 nM, CI 95% 120–181); CMNT, 2.9 times higher ($N = 10$, mean IC50 = 143 nM, CI 95% 43–242) and CIEK, 2.7 times higher ($N = 6$, mean IC50 = 135 nM, CI 95% 18–280). Figure 1 shows scatter plots of the data.

Haplotypes with mutant-type alleles CIET, CMNT, CIEK were present respectively in 76%, in 75%, and in 55% chloroquine-resistant isolates ($P = 0.005$) and haplotype with wild-type allele CMNK in 85% chloroquine-sensitive isolates ($P = 0.002$). We estimated that deduced haplotypes CIET, CMNT, CIEK were 3.2 times, 2.9 times, and 1.2 times more frequent, respectively, in the chloroquine-resistant phenotype.

The positive predictive value (PPV) of mutant-type I74, E75, and T76 alleles for chloroquine-resistance were estimated to 54%, 20%, and 74%, respectively.

Correlation between polymorphisms in the *pfert* and *pfmdr1* genes and *in vitro* monodesethylamodiaquine susceptibility. For polymorphisms in the *pfmdr1* gene, we found no significant differences for the geometric means of the IC50 values in positions 86 and 1246 ($P > 0.05$, data not shown).

For the *pfert* gene, we found significant increases for geometric mean of IC50 values for *in vitro* monodesethylamodiaquine susceptibility ($P = 0.002$): 2 times higher for the CIET haplotype ($N = 62$, mean IC50 = 52 nM, CI 95% 39–64) than the CMNK haplotypes ($N = 56$, mean IC50 = 26 nM, CI 95% 21–32). Figure 1 shows scatter plots of the data.

The deduced haplotypes CIET was present in 85% monodesethylamodiaquine-resistant isolates ($P = 0.02$) and CMNK haplotype in 95% monodesethylamodiaquine-sensitive isolates ($P = 0.006$). We estimated that deduced haplotypes CIET was 1.6 times more frequent in the monodesethylamodiaquine-resistant phenotype. We estimated the PPV of mutant-type I74, E75, and T76 alleles for monodesethylamodiaquine resistance as 22%, 22%, and 24%, respectively.

Correlation between polymorphisms in the *pfert* and *pfmdr1* genes and *in vitro* quinine susceptibility. For polymorphisms in the *pfmdr1* gene, we found no significant differences for the geometric means of the IC50 values in positions 86 and 1246 ($P > 0.05$, data not shown).

For the *pfert* gene, we also found a significant difference ($P = 0.03$) between the CIET ($N = 64$, mean IC50 = 213 nM, CI 95% 183–244 nM) and CMNK ($N = 55$, mean IC50 = 163 nM, CI 95% 128–159 nM) haplotypes (1.3 times higher for CIET). Figure 1 shows scatter plots of the data.

Correlation between polymorphisms in the *pfert* and *pfmdr1* genes and *in vitro* susceptibilities of others antimalarial drugs tested. For polymorphisms in the *pfmdr1* and *pfert* genes, we found no significant differences ($P > 0.05$, data not shown) for the geometric means of the IC50 values for *in vitro* dihydroartemisinin, halofantrine, and mefloquine susceptibility.

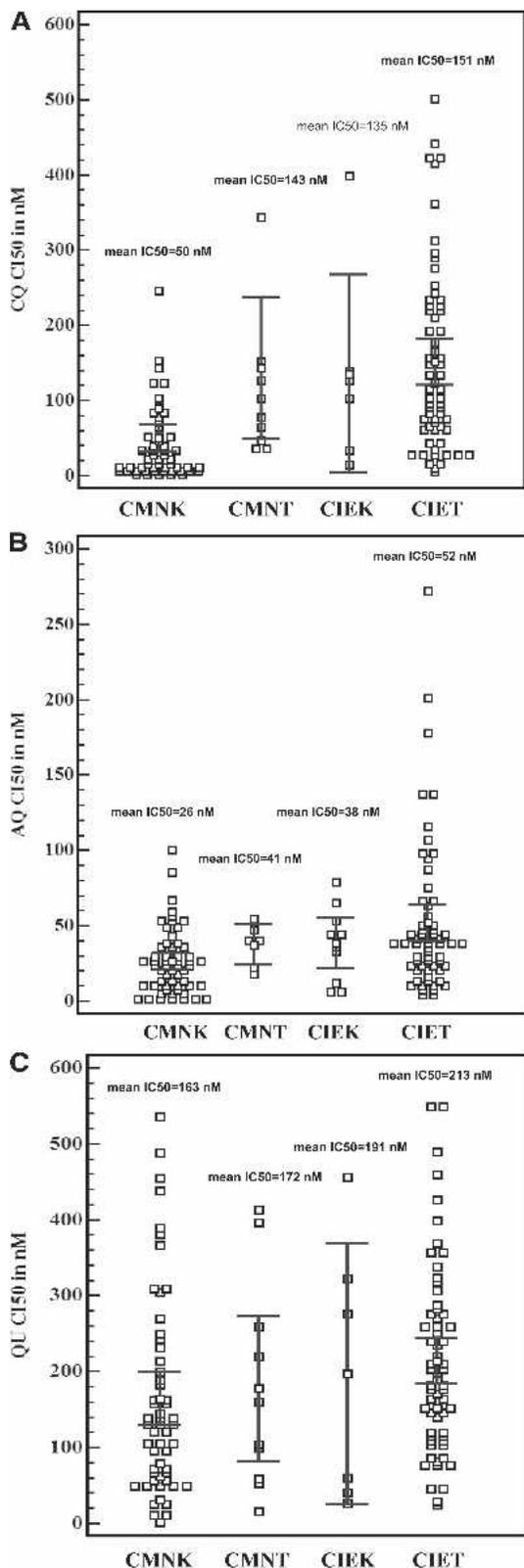


FIGURE 1. *In vitro* susceptibility to chloroquine (A), mono-desethylamodiaquine (B), and quinine (C) in isolates of *Plasmodium falciparum* from the Central African Republic with deduced haplotypes in the *pfert* gene.

Correlation between polymorphisms in *dhfr* and *in vitro* pyrimethamine susceptibility. We found significant increases in the geometric means of the IC50 values for *in vitro* pyrimethamine susceptibility ($P = 0.0002$): 10 times higher for the mutant-type I51 allele ($N = 27$, mean IC50 = 5288 nM, CI 95% 3614–6961 nM) than the wild-type N51 allele ($N = 12$, mean IC50 = 502 nM, CI 95% 118–886 nM); 21 times higher for the mutant-type R59 allele ($N = 31$, mean IC50 = 4742 nM, CI 95% 3201–6283 nM) than the wild-type C59 allele ($N = 8$, mean IC50 = 225 nM, CI 95% 85–489 nM); 21 times higher for the mutant-type N108 allele ($N = 30$, mean IC50 = 4888 nM, CI 95% 3324–6453 nM) than the wild-type S108 allele ($N = 9$, mean IC50 = 238 nM, CI 95% 16–484 nM). We found 100% of wild-type alleles for codons 16 and 50, one sample with mutant-type 164L and one sample with mixed type 164I/L (not analyzed in this study).

For the deduced haplotypes in *dhfr* gene, the geometric means of the IC50 values for *in vitro* pyrimethamine susceptibility increased regularly with the number of mutation ($P = 0.002$): 22 times higher between ACNCNI ($N = 4$, mean IC50 = 299 nM, CI 95% 123–1210) and ACNCSI haplotypes ($N = 5$, mean IC50 = 13.6 nM, CI 95% 2–88), 53 times higher between ACIRSI ($N = 4$, mean IC50 = 720 nM, CI 95% 253–1420) and ACNCSI haplotypes, 80 times higher between ACNRNI ($N = 3$, mean IC50 = 1081 nM, CI 95% 524–2229) and ACNCSI haplotypes, and 332 times higher for ACIRNI haplotype ($N = 23$, mean IC50 = 4323 nM, CI 95% 3140–5951) and ACNCSI haplotypes. Figure 2 shows scatter plots of the data.

The deduced haplotypes ACIRNI, ACIRSI, ACNRNI, and ACNCNI were only present in pyrimethamine-resistant isolates ($P = 10^{-5}$) and the deduced haplotypes ACNCSI in the pyrimethamine-sensitive isolates ($P = 10^{-4}$).

We estimated that the mutant-type I51, R59, and N108 alleles were 27 times, 30 times, 30 times more frequent, respectively, in the pyrimethamine-resistant phenotype. We estimated the PPV of mutant-type I51 and N108 alleles for

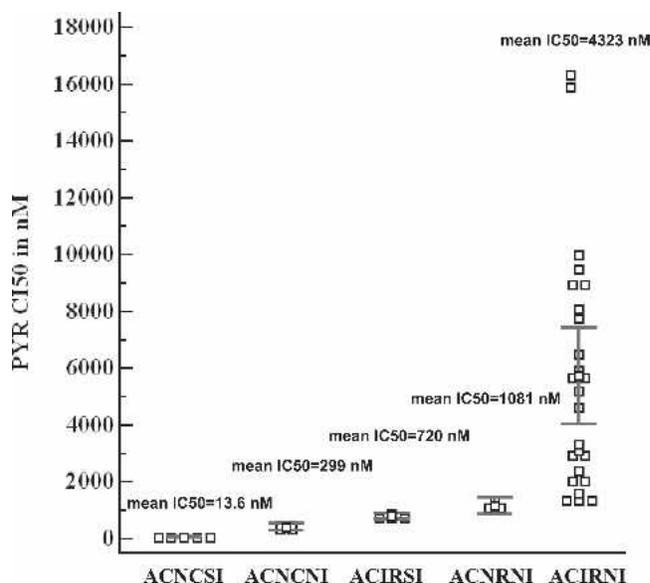


FIGURE 2. *In vitro* susceptibility to pyrimethamine in isolates of *Plasmodium falciparum* from the Central African Republic with the number of mutant allele in the *dhfr* gene.

pyrimethamine-resistant phenotype as 100% for each allele and as 96.8% for the mutant-type R59 allele.

DISCUSSION

Drug resistance is probably the greatest challenge to most malaria-control programs. Given the limited resources for other malarial-control measures, rational drug used is crucial, although economic constraints and the scarcity of drug choice hampers this. Close monitoring of the epidemiology and dynamics of drug resistance are necessary to implement measures to circumvent the problem and to allow the National Malaria Control Program to recommend the best available management of malaria.

We have previously shown a close relationship between *in vivo* and *in vitro* studies in Bangui. We used the standard 14-day WHO 2001 protocol to compare therapeutic responses in children less than 5 years of age with acute uncomplicated *Plasmodium falciparum* malaria in Bangui between February 2002 and March 2004. We found that the overall treatment failure rates with chloroquine, amodiaquine sulfadoxine-pyrimethamine, chloroquine + sulfadoxine-pyrimethamine combination, and amodiaquine + sulfadoxine-pyrimethamine combination were 40.9%, 20.0%, 22.8%, 7.2%, and 0%, respectively.²⁵ Consistent with this were *in vitro* isotopic drug sensitivity assays carried out in Bangui between March and July 2004. They showed that the proportion of resistant isolates was 37% for chloroquine, 15.9% for amodiaquine, 0% for quinine, 0% for dihydroartemisinin, 1.6% for mefloquine, 3.8% for halofantrine, 4.0% for atovaquone, and 83% for pyrimethamine.²⁶ We also determined with the same samples, the baseline frequency distribution of the mutant alleles of genes associated with resistance to chloroquine and sulfadoxine-pyrimethamine. We found mutant alleles of the *pfprt* gene in two thirds of the samples and a high (45%) frequency of the deduced CIET *pfprt* haplotype. The most common alleles of the *pfmdr1* gene among the field isolates of *P. falciparum* were the 86Y (21.9%) and 1246Y (18.0%) alleles. Analysis of *dhfr* gene showed that only 12 isolates carried the wild-type allele (7.2%), whereas many (57.5%) were triple mutants (51I, 59R, 108N).²⁷ More interesting, was to find, for the first time in Central African Republic, the quadruple mutation (51:59/108/164) in 2 samples, using the PCR-RFLP approach, this finding being confirmed by sequencing, subsequently. Unfortunately, IC₅₀ values for *in vitro* pyrimethamine susceptibility were not available for these samples. According to Nzila and colleagues,³¹ this result indicated that the quadruple mutant DHFR exists in Central African Republic where SP is widely used, implying that the useful therapeutic life of the new combination such as chloroquine plus dapsone (LapdapTM) could be very short.

In this present study, we have shown a strong association between the *dhfr* genotype and chemosensitivity to pyrimethamine. Isolates with single or combined *dhfr* I51, R59, or N108 mutations were between 10 and 21 times less susceptible to pyrimethamine. For pyrimethamine, we found a regular increase in IC₅₀ values with the number of *dhfr* mutations, from a 22-fold increase for a single mutant-type allele to a 332-fold increase for a triple mutant. This is consistent with previous studies correlating *dhfr* mutations and chemosensitivity in field samples from Brazil³² and Africa.^{33–35} We also observed a strong relationship between phenotype and geno-

type for pyrimethamine (all except one isolate containing a mutant allele was found to be resistant), the PPVs of mutant-type I51 and N108 alleles for pyrimethamine-resistant phenotype being 100% and the PPV of mutant-type R59 being 96.8%. Therefore, we believe that PCR-RFLP analysis in the *dhfr* gene for codons 51, 59, and 108 may be a quick and reliable method being able to replace the *in vitro* techniques providing particularly poor results for pyrimethamine. However, associations between these molecular markers and clinical SP treatment outcomes seem to be less straightforward. Even if several studies such as Kublin and associates²⁰ showed that the presence together of all 5 *dhfr* and *dhps* mutations that are found in Africa were strongly associated with SP failure, recent study conducted by Francis and colleagues³⁶ strongly suggest that geographic differences in response to antimalarial therapy in Uganda are primarily mediated by acquired immunity associated with malaria transmission intensity, rather than by parasite factors such as molecular markers of antimalarial drug resistance.

We have evaluated the correlation between *pfprt* and *pfmdr1* mutations and *in vitro* responses to chloroquine, monodesethylamodiaquine, quinine, dihydroartemisinin, mefloquine, and halofantrine. We found that for haplotypes with mutant-type alleles in the *pfprt* gene led to significant changes in the IC₅₀ values of chloroquine, monodesethylamodiaquine, and quinine. This is consistent with our previous *in vitro* study²⁶ in which we found significant differences in the IC₅₀ values for monodesethylamodiaquine and quinine in chloroquine-sensitive and chloroquine-resistant isolates, and in which there was a significant correlation between the *in vitro* responses to chloroquine and monodesethylamodiaquine ($r = 0.61$) and to chloroquine and quinine ($r = 0.33$). The cross-resistance between these drugs can be partly explained by similarities in their chemical structures.³⁷ Among the 4 haplotypes (CMNK, CIEK, CMNT, and CIET) found in *pfprt* gene, CMNK haplotype was clearly associated with chloroquine-sensitive and monodesethylamodiaquine-sensitive isolates, whereas the CIEK, CMNT, and CIET haplotypes were associated with higher IC₅₀ values. We observed significant differences in the geometric means of the IC₅₀ values between the CMNK and CIET haplotypes for chloroquine (3-fold), monodesethylamodiaquine (2-fold), and quinine (1.3-fold) and between CMNK and CMNT (2.9-fold), and CMNK and CIEK (2.7-fold) for only chloroquine. According to Djimde and colleagues¹⁰ or Holmgren and associates,³⁸ examination of 76T mutation in the *pfprt* gene sequence can be used with reasonable reliability to predict resistance to chloroquine. Most likely this can be applied in the CAR since PPV for chloroquine resistance of 74%. In the same way, associations of mutations in the *pfprt* gene with clinical treatment outcomes of chloroquine are less straightforward. This is due in part to the complexity of analyzing relationships between multiple mutations on different genes and the multinomial treatment outcomes and in part because treatment outcomes are affected by factors other than parasite resistance, including the host immune response,³⁹ micronutrient levels,⁴⁰ and bioavailability and pharmacokinetics.⁴¹

Much confusion has surrounded the association of different *pfmdr1* alleles to chloroquine resistance, although transfection studies as well as carefully controlled, molecular epidemiologic studies have shown that there are strong associations between polymorphisms and antimalarial resistance.^{42–45}

However, like many other studies,^{46–48} our present findings have failed to find such associations because the presence of both wild-type and mutant-type alleles in our samples were largely independent of their *in vitro* responses. This can be for a variety of reasons, including the inability to control for the inoculum effect,⁴⁹ which can alter the *in vitro* sensitivity erroneously. In your opinion and according to Duraisingh and associates,¹³ there may be true epidemiologic differences in resistance resulting from *pfmdr1* polymorphisms in different populations and differences in the strength of associations between resistance and *pfmdr1* alleles could reflect the different selection histories of these populations. However, correlations between arylaminoalcohols or endoperoxide artemisinin derivatives resistance and amplification of *pfmdr1* gene^{50,51} or new candidate transporter gene⁵² merit further investigation.

In conclusion, these findings suggest that analysis of the *dhfr* and *pfcr* gene may be a reliable tool for the epidemiologic surveillance of antimalarial drug resistance in the CAR. A regular monitoring and screening for resistance markers for antifolates (such as the *dhfr* mutations) and for chloroquine (such as the *pfcr* mutations) could act as an adjunct to *in vivo* trials and should be carried out both in Bangui and in the CAR, to allow the National Malaria Control Program to recommend the best available management of malaria (antimalarial drugs used as first-line treatment or intermittent preventive treatment during pregnancy).

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REFERENCES

- Barat LM, Bloland PB, 1997. Drug resistance among malaria and other parasites. *Infect Dis Clin North Am* 11: 969–987.
- Marsh K, 1998. Malaria disaster in Africa. *Lancet* 352: 924.
- WHO, 2003. Assessment and monitoring of antimalarial drug efficacy for the treatment of uncomplicated falciparum malaria. Geneva, Switzerland. (WHO/HTM/RBM/2003.50). Available online: http://mosquito.who.int/cmc_upload/0/000/017/017/ProtocolWHO.pdf.
- Bickii J, Basco LK, Ringwald P, 1998. Assessment of three *in vitro* tests and an *in vivo* test for chloroquine resistance in *Plasmodium falciparum* clinical isolates. *J Clin Microbiol* 36: 243–247.
- Warhurst DC, 2001. A molecular marker for chloroquine-resistant falciparum malaria. *N Engl J Med* 344: 299–302.
- Warsame M, Wernsdorfer WH, Payne D, Bjorkman A, 2001. Susceptibility of *Plasmodium falciparum* in vitro to chloroquine, mefloquine, quinine and sulfadoxine/pyrimethamine in Somalia: relationships between the responses to the different drugs. *Trans R Soc Trop Med Hyg* 85: 565–569.
- Desjardins RE, Canfield CJ, Haynes JD, Chulay JD, 1979. Quantitative assessment of antimalarial activity in vitro by a semi-automated microdilution technique. *Antimicrob Agents Chemother* 16: 710–718.
- Foot SJ, Cowman AF, 1994. The mode of action and the mechanism of resistance to antimalarial drugs. *Acta Trop* 56: 157–171.
- Bruce-Chwart LJ, Black RH, Canfield CJ, Clyde DF, Peters W, Wernsdorfer WH, 1981. Chemotherapy of malaria. *WHO Monograph Series* 27: 211–224.
- Djimde A, Doumbo OK, Cortese JF, Kayentao K, Doumbo S, Diourte Y, Dicko A, Su XZ, Nomura T, Fidock DA, Welles TE, Plowe CV, Coulibaly D, 2001. A molecular marker for chloroquine-resistant falciparum malaria. *N Engl J Med* 344: 257–263.
- Anderson TJ, Nair S, Sudimack D, Williams JT, Mayxay M, Newton PN, Guthmann JP, Smithuis FM, Tran TH, van den Broek IV, White NJ, Nosten F, 2005. Geographical distribution of selected and putatively neutral SNPs in Southeast Asian malaria parasites. *Mol Biol Evol* 22: 2362–2374.
- Duraisingh MT, Drakeley CJ, Muller O, Bailey R, Snounou G, Targett GA, Greenwood BM, Warhurst DC, 1997. Evidence for selection for the tyrosine-86 allele of the *pfmdr1* gene of *Plasmodium falciparum* by chloroquine and amodiaquine. *Parasitology* 114: 205–211.
- Duraisingh MT, Cowman AF, 2005. Contribution of the *pfmdr1* gene to antimalarial drug-resistance. *Acta Trop* 94: 181–190.
- Fidock DA, Nomura T, Talley AK, Cooper RA, Dzekunov SM, Ferdig MT, Ursos LM, Sidhu AB, Naude B, Deitsch KW, Su XZ, Wootton JC, Roepe PD, Welles TE, 2000. Mutations in the *P. falciparum* digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance. *Mol Cell* 6: 861–871.
- Reed MB, Saliba KJ, Caruana SR, Kirk K, Cowman AF, 2000. *Pgh1* modulates sensitivity and resistance to multiple antimalarials in *Plasmodium falciparum*. *Nature* 403: 906–909.
- Plowe CV, Cortese JF, Djimde A, Nwanyanwu OC, Watkins WM, Winstanley PA, Estrada-Franco JG, Mollinedo RE, Avila JC, Cespedes JL, 1997. Mutations in *Plasmodium falciparum* dihydrofolate reductase and dihydropteroate synthase and epidemiologic patterns of pyrimethamine-sulfadoxine use and resistance. *J Infect Dis* 176: 1590–1596.
- Jelinek T, Ronn AM, Lemnge MM, Curtis J, Mhina J, Duraisingh MT, Bygbjerg IC, Warhurst DC, 1998. Polymorphisms in the dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) genes of *Plasmodium falciparum* and in vivo resistance to sulphadoxine/pyrimethamine in isolates from Tanzania. *Trop Med Int Health* 3: 605–609.
- Nzila AM, Mberu EK, Sulo J, Dayo H, Winstanley PA, Sibley CH, Watkins WM, 2000. 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* 44: 991–996.
- Nzila AM, Mberu EK, Nduati E, Ross A, Watkins WM, Sibley CH, 2002. Genetic diversity of *Plasmodium falciparum* parasites from Kenya is not affected by antifolate drug selection. *Int J Parasitol* 32: 1469–1476.
- Kublin JG, Dzinjalama FK, Kamwendo DD, Malkin EM, Cortese JF, Martino LM, 2002. Molecular markers for failure of sulfa-doxine-pyrimethamine and chlorproguanil-dapsone treatment of *Plasmodium falciparum* malaria. *J Infect Dis* 185: 380–388.
- Eriksen S, Mwankusye S, Mduma A, Kitua G, Swedberg G, Tomson LL, Gustafsson G, Warsame M, 2005. Patterns of resistance and DHFR/DHPS genotypes of *Plasmodium falciparum* in rural Tanzania prior to the adoption of sulfadoxine-pyrimethamine as first-line treatment. *Trans R Soc Trop Med Hyg* 98: 347–353.
- Staedke SG, Sendagire H, Lamola S, Kanya MR, Dorsey G, Rosenthal PJ, 2004. Relationship between age, molecular markers, and response to sulphadoxine-pyrimethamine treatment in Kampala, Uganda. *Trop Med Int Health* 9: 624–629.
- Pierce PF, Milhous WK, Campbell CC, 1987. Clinical and laboratory characterization of a chloroquine-resistant *Plasmodium falciparum* strain acquired in the Central African Republic. *Am J Trop Med Hyg* 36: 1–2.
- Belec L, Delmont J, Vesters I, Testa J, Christian KS, Georges AJ, 1988. Emergence of multiresistant *Plasmodium falciparum* malaria in Central African Republic. *Press Med* 17: 2090–2091.

25. Menard D, Madji N, Manirakiza A, Djalle D, Koula MR, Talarmin A, 2005. Efficacy of chloroquine, amodiaquine, sulfadoxine-pyrimethamine, chloroquine-sulfadoxine-pyrimethamine combination, and amodiaquine-sulfadoxine-pyrimethamine combination in Central African children with noncomplicated malaria. *Am J Trop Med Hyg* 72: 581–585.
26. Menard D, Yapou F, Siadou V, Sana S, Matsika-Claquin MD, Madji N, Talarmin A, 2005. Drug-resistant malaria in Bangui (Central African Republic): an *in vitro* assessment. *Am J Trop Med Hyg* 73: 239–243.
27. Menard D, Djalle D, Yapou F, Manirakiza A, Talarmin A, 2005. Frequency distribution of antimalarial drug-resistant alleles among isolates of *Plasmodium falciparum* in Bangui, Central African Republic. *Am J Trop Med Hyg* 74: 205–210.
28. UNICEF, 2001. Enquête à indicateurs multiples, MICS 2000. Editions B, ed. *Rapport Final Bangui* Bangui: Ministère du Plan, RCA, 130–131.
29. Snounou G, Zhu X, Siripoon N, 1999. Biased distribution of *msp1* and *msp2* allelic variants in *Plasmodium falciparum* populations in Thailand. *Trans R Soc Trop Med Hyg* 93: 369–374.
30. Menard D, Dolmazon V, Matsika-Claquin MD, Manirakiza A, Yapou F, Nambot M, Talarmin A, 2006. Genetic diversity and genotype multiplicity of *Plasmodium falciparum* infections in symptomatic individuals living in Bangui (CAR). *Trans R Soc Trop Med Hyg*.
31. Nzila A, Ochong E, Nduati E, Gilbert K, Winstanley P, Ward S, Marsh K, 2005. Why has the dihydrofolate reductase 164 mutation not consistently been found in Africa yet? *Trans R Soc Trop Med Hyg* 99: 341–346.
32. Peterson DS, Di Santi SM, Povoia M, Calvosa VS, Do Rosario VE, Wellems TE, 1991. Prevalence of the dihydrofolate reductase Asn-108 mutation as the basis for pyrimethamine-resistant falciparum malaria in the Brazilian Amazon. *Am J Trop Med Hyg* 45: 492–497.
33. Basco LK, Eldin de Pecoulas P, Wilson CM, Le Bras J, Mazabraud A, 1995. Point mutations in the dihydrofolate reductase-thymidylate synthase gene and pyrimethamine and cycloguanil resistance in *Plasmodium falciparum*. *Mol Biochem Parasitol* 69: 135–138.
34. Nzila-Mounda A, Mberu EK, Sibley CH, Plowe CV, Winstanley PA, Watkins WM, 1998. Kenyan *Plasmodium falciparum* field isolates: correlation between pyrimethamine and chlorocycloguanil activity *in vitro* and point mutations in the dihydrofolate reductase domain. *Antimicrob Agents Chemother* 42: 164–169.
35. Khalil I, Ronn AM, Alifrangis M, Gabar HA, Satti GM, Bygbjerg IC, 2003. Dihydrofolate reductase and dihydropteroate synthase genotypes associated with *in vitro* resistance of *Plasmodium falciparum* to pyrimethamine, trimethoprim, sulfadoxine, and sulfamethoxazole. *Am J Trop Med Hyg* 68: 586–589.
36. Francis D, Nsobya SL, Talisuna A, Yeka A, Kamya MR, Machelkano R, Dokomajilar C, Rosenthal PJ, Dorsey G, 2006. Geographic differences in antimalarial drug efficacy in Uganda are explained by differences in endemicity and not by known molecular markers of drug resistance. *J Infect Dis* 193: 978–986.
37. Pradines B, Mabika Mamfoumbi M, Parzy D, Owono Medang M, Lebeau C, Mourou Mbina JR, Doury JC, Kombila M, 1998. *In vitro* susceptibility of Gabonese wild isolates of *Plasmodium falciparum* to artemether, and comparison with chloroquine, quinine, halofantrine and amodiaquine. *Parasitology* 117: 541–545.
38. Holmgren G, Gil JP, Ferreira PM, Veiga MI, Obonyo CO, Bjorkman A, 2006. Amodiaquine resistant *Plasmodium falciparum* malaria *in vivo* is associated with selection of *pfprt* 76T and *pfmdr1*86Y. *Infect Genet Evol.* 6: 309–314.
39. Wellems TE, Plowe CV, 2001. Chloroquine-resistant malaria. *J Infect Dis* 184: 770–776.
40. Van Hensbroek MB, Morris-Jones S, Meisner S, Jaffar S, Bayo L, Dackour R, Phillips C, Greenwood BM, 1995. Iron, but not folic acid, combined with effective antimalarial therapy promotes haematological recovery in African children after acute falciparum malaria. *Trans R Soc Trop Med Hyg* 89: 672–676.
41. Watkins WM, Mberu EK, Nevill CG, Ward SA, Breckenridge AM, Koech DK, 1990. Variability in the metabolism of proguanil to the active metabolite cycloguanil in healthy Kenyan adults. *Trans R Soc Trop Med Hyg* 84: 492–495.
42. Foote SJ, Kyle DE, Martin RK, Oduola AM, Forsyth K, Kemp DJ, Cowman AF, 1990. Several alleles of the multidrug-resistance gene are closely linked to chloroquine resistance in *Plasmodium falciparum*. *Nature* 345: 255–258.
43. Babiker HA, Pringle SJ, Abdel-Muhsin A, Mackinnon M, Hunt P, Walliker D, 2001. High-level chloroquine resistance in Sudanese isolates of *Plasmodium falciparum* is associated with mutations in the chloroquine resistance transporter gene *pfprt* and the multidrug resistance gene *Pfmdr1*. *J Infect Dis* 183: 1535–1538.
44. Nagesha HS, Din-Syafuriddin, Casey GJ, Susanti AI, Fryauff DJ, Reeder JC, Cowman AF, 2001. Mutations in the *pfmdr1*, *dhfr* and *dhps* genes of *Plasmodium falciparum* are associated with *in-vivo* drug resistance in West Papua, Indonesia. *Trans R Soc Trop Med Hyg* 95: 43–49.
45. Von Seidlein L, Duraisingh MT, Drakeley CJ, Bailey R, Greenwood BM, Pinder M, 1997. Polymorphisms in the *pfmdr1* gene and chloroquine resistance in *Plasmodium falciparum* in the Gambia. *Trans R Soc Trop Med Hyg* 91: 450–453.
46. Basco LK, Ringwald P, 1997. *pfmdr1* gene mutation and clinical response to chloroquine in Yaounde, Cameroon. *Trans R Soc Trop Med Hyg* 91: 210–211.
47. Haruki K, Bray PG, Ward SA, Hommel M, Ritchie GY, Chloroquine resistance of *Plasmodium falciparum*: further evidence for a lack of association with mutations of the *pfmdr1* gene. *Trans R Soc Trop Med Hyg* 88: 694.
48. Pillai DR, Labbe AC, Vanisaveth V, Hongvangthong B, Pomphida S, Inkathone S, Zhong K, Kain KC, 2001. *Plasmodium falciparum* malaria in Laos: chloroquine treatment outcome and predictive value of molecular markers. *J Infect Dis* 183: 789–795.
49. Duraisingh MT, Jones P, Sambou I, von Seidlein L, Pinder M, Warhurst DC, 1999. Inoculum effect leads to overestimation of *in vitro* resistance for artemisinin derivatives and standard antimalarials: a Gambian field study. *Parasitology* 119: 435–440.
50. Price RN, Uhlemann AC, Brockman A, McGready R, Ashley E, Phaipun L, Patel R, Laing K, Looareesuwan S, White NJ, Nosten F, Krishna S, 2004. Mefloquine resistance in *Plasmodium falciparum* and increased *pfmdr1* gene copy number. *Lancet* 364: 438–447.
51. Wilson CM, Volkman SK, Thaithong S, Martin RK, Kyle DE, Milhous WK, Wirth DF, 1993. Amplification of *pfmdr1* associated with mefloquine and halofantrine resistance in *Plasmodium falciparum* from Thailand. *Mol Biochem Parasitol* 57: 151–160.
52. Mu J, Ferdig MT, Feng X, Joy DA, Duan J, Furuya T, Subramanian G, Aravind L, Cooper RA, Wootton JC, Xiong M, Su XZ, 2003. Multiple transporters associated with malaria parasite responses to chloroquine and quinine. *Mol Microbiol* 49: 977–989.