FREQUENCY DISTRIBUTION OF ANTIMALARIAL DRUG-RESISTANT ALLELES AMONG ISOLATES OF PLASMODIUM FALCIPARUM IN BANGUI, CENTRAL AFRICAN REPUBLIC

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Abstract. We determined the baseline frequency distribution of mutant alleles of genes associated with resistance to chloroquine and sulfadoxine-pyrimethamine in Plasmodium falciparum isolates in Bangui, Central African Republic. Mutant alleles of the P. falciparum chloroquine resistance transporter (pfcrt) gene were found in all samples and the frequency of the deduced CIET pfcrt haplotype was high (45%). The most common allele of the P. falciparum multidrug resistance 1 (pfmdr1) gene among the field isolates of P. falciparum was 86Y (21.9%). The 1246Y allele was also common (18.0%). Of the 167 P. falciparum isolates in which the dihydrofolate reductase gene was studied, only 11 carried the wild-type allele (6.6%) whereas many (50.3%) were quadruple mutants (50R, 51I, 59R, 108N). The frequency of the 436A mutant allele of the dihydropteroate synthase gene was high (74.3%), but the frequencies of the 437G (18.6%) and 540E (5.2%) mutant alleles were low. Molecular analyses of antimalarial drug-resistant alleles of P. falciparum isolates in Bangui strongly suggest the widespread distribution of chloroquine and pyrimethamine resistance and to a lesser extent sulfadoxine resistance.

INTRODUCTION

Malaria is a major infectious disease worldwide and is a leading cause of morbidity and mortality, particularly among children. In Africa, more than 90% of cases and between 1.5 and 3 million deaths occur in children less than five years of age. Chloroquine has been the drug of choice for treating Plasmodium falciparum malaria for more than 50 years. However, the use of chloroquine as a prophylactic drug and as a treatment for malaria is being limited because of the spread of chloroquine-resistant P. falciparum strains throughout most malaria-endemic areas.

In the Central African Republic, P. falciparum resistance to chloroquine and sulfadoxine-pyrimethamine has been documented since 1983 and 1987, respectively. The most recent in vivo study based on the World Health Organization standard protocol (2001) was conducted in Bangui in 2002. It showed that the overall rates of treatment failure were 40.9% with chloroquine, 20.0% with amodiaquine, 22.8% with sulfadoxine-pyrimethamine, 7.2% with the chloroquine plus sulfadoxine-pyrimethamine combination, and 0% with the amodiaquine plus sulfadoxine-pyrimethamine combination. In accordance with in vivo results, in vitro isosensitive drug sensitivity assays in Bangui in 2004 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. No multi-resistant isolates (showing resistance to more than three drugs) were found. These findings resulted in the Ministry of Health of the Central African Republic Ministry of Health replacing chloroquine with the amodiaquine plus sulfadoxine-pyrimethamine combination. This combination was to be used as an interim first-line antimalarial treatment until better, alternative treatments, such as artemisinin-based combination therapies, became available at low prices in the Central African Republic.

To complete the description of resistance in Bangui, Central African Republic, we assessed the drug resistance status of the local malaria parasite population using molecular markers. Analysis of the molecular basis of antimalarial drug resistance over the last few decades has showed various mutant alleles of the P. falciparum chloroquine resistance transporter (pfcrt) and the P. falciparum multidrug resistance 1 (pfmdr1) genes that are associated with resistance to chloroquine. Similarly, mutant alleles of the dihydrofolate reductase (dhfr) and dihydropteroate synthase (dhps) genes had also been implicated in P. falciparum resistance to antifolates and sulfa drugs, respectively. There have been numerous epidemiologic studies on the frequency distribution of the alleles in P. falciparum isolates in different geographic regions. These studies have been invaluable in evaluating the spread of drug resistance in many countries.

The purpose of this study was to determine the baseline frequency distribution of the mutant alleles of genes associated with resistance to chloroquine and sulfadoxine-pyrimethamine in P. falciparum isolates from Bangui, the capital of the Central African Republic before artemisinin-based combination therapies are used as first-line antimalarial drugs.

MATERIALS AND METHODS

Study site. The location of Bangui is shown in Figure 1. The Central African Republic is a subtropical country bordered by Sudan to the east, Cameroon to the west, Chad to the north, and the Republic of Congo to the south. It has an area of 632,000 km² and an estimated population of 3,823,929. Bangui is located near the Oubangui River (7°N, 21°E). The climate is tropical and rainfall is highest from April to November. The average temperature varies from 19°C to 32°C. Malaria transmission occurs throughout the year, with peaks at the beginning and the end of the rainy season. Malaria is hyperendemic in this region and P. falciparum is the predominant malaria species. The prevalence of this parasite in children less than five years of age is 31.8%.

Sample collection. This study was conducted between March and July 2004. Clinical isolates of P. falciparum were

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obtained from symptomatic Central African patients before they were treated. These patients attended several health centers (in the northern districts of Boy Rabe and Gobongo and the southern districts of La Kouanga and Ouango). Blood samples, collected in a tube coated with EDTA (Vacutainer® tubes; Becton Dickinson, Rutherford, NJ), were obtained from patients who provided informed consent during routine malaria diagnosis. Giemsa-stained thin and thick blood smears were examined to check for mono-infection with *P. falciparum* and to determine parasite density. The patients were treated with the amodiaquine-sulfadoxine-pyrimethamine combination or quinine, as recommended by the National Malaria Control Program in the Central African Republic.

Ethical approval. Since there is no National Ethics Committee in the Central African Republic, study protocols were reviewed and approved by the expert committee for antimalarial drug policy and the Central African Republic Ministry of Health.

Extraction of DNA. The DNA template for a polymerase chain reaction (PCR) and 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 0.1 M NaOH for three minutes at 100°C. After centrifugation, the supernatant was collected, treated with 250 μL of lysing solution (150 mM Tris-HCl pH 7.5, 1% [v/v] Triton 100X, 150 mM NaCl, 1% sodium dodecyl sulfate, 1 mM EDTA) and proteinase K (20 mg/mL) for one hour at 37°C, and 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 PCR and detection of mutant alleles. The PCR and restriction fragment length polymorphism (RFLP) analysis were conducted for four genes (*dhfr*, *dhps*, *pfcrt*, and *pfmdr1*) to identify the presence of mutant alleles. A detailed description of these methods is available from http://medschool.umaryland.edu/CVD/plowe.html.

Statistical analysis. Data were analyzed using Epi-Info 2000 software (Centers for Disease Control and Prevention, Atlanta, GA) and MedCalc version 8.0.0.1 software (MedCalc Software, Mariakerke, Belgium). Fisher’s exact test was used to compare two variables with two categories. Spearman’s correlation coefficients were calculated to assess associations between codons in wild-type and mutant alleles.

RESULTS

A total of 386 blood samples from *P. falciparum*-infected individuals were examined for the presence of mutations in the *pfcrt*, *pfmdr1*, *dhfr*, and *dhps* genes: only 267 (69.2%) gave PCR results. There were 135 samples (50.5%) that contained mixed infections of both wild-type and mutant alleles. Allele frequencies of mutations in *pfcrt* and *pfmdr1* are shown in Table 1 and in *dhfr* and *dhps* in Table 2.

Mutant alleles of the *pfmdr1* and *pfcrt* genes. Analysis of *pfmdr1* gene PCR products indicated that mutant alleles of this gene were present in 55 (31.8%) of 173 samples. The most common allele of the *pfmdr1* gene in the field isolates of *P. falciparum* was 86Y (21.9%). The 1246Y allele was also observed at a lower frequency (18.0%).

TABLE 1
Allele frequencies of mutations in *pfcrt* and *pfmdr1* in isolates of symptomatic individuals from Bangui, Central African Republic with uncomplicated *Plasmodium falciparum* malaria in 2004*

<table>
<thead>
<tr>
<th>Genes</th>
<th>Codons</th>
<th>Amino acids</th>
<th>Counts</th>
<th>%</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>pfcrt</em></td>
<td>C</td>
<td>W</td>
<td>168</td>
<td>95.4</td>
<td>91.2–98.0</td>
</tr>
<tr>
<td>72</td>
<td>C/S</td>
<td>Mixed</td>
<td>1</td>
<td>0.6</td>
<td>0–3.1</td>
</tr>
<tr>
<td>S</td>
<td>M</td>
<td>7</td>
<td>4.0</td>
<td>1.6–8.1</td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>W</td>
<td>79</td>
<td>44.6</td>
<td>37.2–52.3</td>
<td></td>
</tr>
<tr>
<td>74</td>
<td>M/I</td>
<td>Mixed</td>
<td>18</td>
<td>10.2</td>
<td>6.1–15.6</td>
</tr>
<tr>
<td>I</td>
<td>M</td>
<td>80</td>
<td>45.2</td>
<td>37.7–52.8</td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>W</td>
<td>3</td>
<td>1.7</td>
<td>0.3–4.8</td>
<td></td>
</tr>
<tr>
<td>75</td>
<td>N/E</td>
<td>Mixed</td>
<td>4</td>
<td>2.2</td>
<td>0.6–5.6</td>
</tr>
<tr>
<td>E</td>
<td>M</td>
<td>172</td>
<td>96.1</td>
<td>92.1–98.4</td>
<td></td>
</tr>
<tr>
<td>L</td>
<td>W</td>
<td>72</td>
<td>40.0</td>
<td>32.8–47.6</td>
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<tr>
<td>76</td>
<td>K/T</td>
<td>Mixed</td>
<td>33</td>
<td>18.3</td>
<td>13.0–24.8</td>
</tr>
<tr>
<td>T</td>
<td>M</td>
<td>75</td>
<td>41.7</td>
<td>34.4–49.2</td>
<td></td>
</tr>
<tr>
<td><em>pfmdr1</em></td>
<td>N</td>
<td>W</td>
<td>175</td>
<td>78.1</td>
<td>72.1–83.4</td>
</tr>
<tr>
<td>86</td>
<td>N/Y</td>
<td>Mixed</td>
<td>25</td>
<td>11.2</td>
<td>7.4–16.0</td>
</tr>
<tr>
<td>Y</td>
<td>M</td>
<td>24</td>
<td>10.7</td>
<td>7.0–15.5</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>W</td>
<td>146</td>
<td>82.0</td>
<td>75.6–87.4</td>
<td></td>
</tr>
<tr>
<td>1246</td>
<td>D/Y</td>
<td>Mixed</td>
<td>18</td>
<td>10.1</td>
<td>6.1–15.5</td>
</tr>
<tr>
<td>Y</td>
<td>M</td>
<td>14</td>
<td>7.9</td>
<td>4.4–12.8</td>
<td></td>
</tr>
</tbody>
</table>

*pfcrt* = *P. falciparum* chloroquine resistance transporter; *pfmdr1* = *P. falciparum* multidrug resistance gene 1; CI = confidence interval; W = wild-type allele; M = mutant allele.
Mutant alleles of the pfcr gene were found in all samples (171 of 171). Deduced haplotype frequencies in the pfcr gene are shown in Table 3. There was a significant correlation in wild-type and mutant alleles between codons 74 and 76 (P < 0.005) and codons 59 (P = 0.002), codons 51 and 108 (P < 10^{-6}), codons 50 and 51 (P = 0.002), codons 51 and 59 (P < 10^{-6}), codons 51 and 108 (P = 0.005) and codons 59 and 108 (P = 0.0005).

**Mutant alleles of the dhfr and dhps genes.** Mutant alleles of the dhfr gene were found in all but 11 samples. Deduced haplotype frequencies for the dhfr gene are shown in Table 4. The most prevalent haplotype carried four mutant alleles: S6N, I59R, 59R, and T108N. The mutant allele with a mutation at codon 16 was not detected in any of our samples examined. In one isolate, the 108T mutation was found as a single mutation.

Significant correlations were found in wild-type and mutant alleles between codons 50 and 51 (P = 0.002), codons 50 and 59 (P < 10^{-6}), codons 50 and 108 (P = 0.002), codons 51 and 59 (P < 10^{-6}), codons 51 and 108 (P = 0.005) and codons 59 and 108 (P = 0.0005).

**DISCUSSION**

Until recently, our knowledge of the epidemiology of drug-resistant malaria was based on the collection of in vivo data...
from symptomatic patients to whom different antimalarial drugs were administered and, to a lesser extent, on in vitro drug sensitivity assays. The limitations of these methods for studying drug-resistant malaria and elucidating molecular mechanisms of resistance to some antimalarial drugs have stimulated the use of a third approach based on molecular markers for resistance.\textsuperscript{20} Thus, to complete recent in vivo\textsuperscript{4} and in vitro\textsuperscript{5} data on drug-resistant malaria in Bangui, Central African Republic, we determined the baseline frequency distribution of the mutant alleles of genes associated with resistance to chloroquine and sulfadoxine-pyrimethamine in the \textit{P. falciparum} population. Samples collected in 2004 for in vitro drug sensitivity assays were used for testing for markers of drug resistance.\textsuperscript{5}

There is still no single, universally accepted PCR protocol to amplify the target genes. Consequently, we used a PCR-RFLP technique. Compared with other techniques used to determine DNA sequences, for example hybridization with DNA probes or direct sequencing of amplified fragments, the PCR-RFLP technique has been found to be robust\textsuperscript{21} and is widely used in other African countries. It was also the easiest technique for us to use because our laboratory was already equipped with the necessary material.

We found mixed infections of both wild-type and mutant alleles in more than half of the samples. This is not surprising and is consistent with our previous study on the genetic diversity and clone multiplicity of \textit{P. falciparum} infections in symptomatic individuals living in Bangui. In this study, we showed a high percentage of multiclonal infections (42.7%) with merozoite surface protein 1 [\textit{msp} 1] gene and 76.7% with the merozoite surface protein 2 [\textit{msp} 2] gene), with a mean of 1.7 genotype with \textit{msp} 1 and 2.8 genotypes with \textit{msp} 2, which probably reflects a high rate of transmission (unpublished data).

The baseline frequency distribution of the mutant alleles of genes associated with resistance to chloroquine was consistent with previous studies in Africa. We found mutant alleles of the \textit{pfct} gene in all samples and high frequencies of the \textit{pfct} alleles 74I, 75E and 76T, as has been described in numerous African countries, such as Cameroon,\textsuperscript{22} Gabon,\textsuperscript{23} Democratic Republic of Congo,\textsuperscript{24} Sudan,\textsuperscript{25} Nigeria,\textsuperscript{26} Senegal,\textsuperscript{27} Uganda,\textsuperscript{28} and Mauritania,\textsuperscript{29} where chloroquine has been widely used. We also found close relationships between the overall in vivo rate of treatment failure (40.9%), the proportion of resistant isolates estimated by in vitro isotopic drug sensitivity assays (37%), and the frequency of the deduced CIET \textit{pfct} haplotype (45%), as already shown in Lambaréné in Gabon.\textsuperscript{23} Mutant alleles 86Y and 1246Y in \textit{pfmdr1} gene, which are often cited as potential contributors to resistance to chloroquine, were also analyzed. Unlike previous reports in Africa,\textsuperscript{30,31} we found only a low prevalence of mutant alleles 86Y and 1246Y in isolates from Bangui and these two alleles were found in only 16 (9.2%) of 173 isolates. The low prevalence of the \textit{pfmdr1} Y1246 allele is consistent with other data from Africa.\textsuperscript{32,33} This polymorphism has been found mostly in isolates originating from South America.\textsuperscript{34,35} However, the presence of the Y1246 mutant allele in a few isolates from Bangui may indicate the introduction of new strains into this region.

Of the 167 \textit{P. falciparum} isolates examined in the \textit{dhfr} gene, only 11 carried the wild-type allele (6.6%). There was a high frequency (50.3%) of quadruple mutants (50R, 51I, 59R, 108N). Thus, as suggested by Basco and others,\textsuperscript{20} there seemed to be a drastic change from a wild-type allele to quadruple mutants, rather than a gradual accumulation of mutations in the \textit{dhfr} gene. These results are consistent with other studies in neighboring countries,\textsuperscript{20,36} and imply a high degree of resistance to pyrimethamine.\textsuperscript{37} It was more surprising to find a high 436A mutant allele frequency, but low frequencies of the 437G mutant allele (18.6%) and 540E mutant allele (5.2%) in the \textit{dhps} gene. However, this is consistent with the overall in vivo treatment failure rate of 22.8% with sulfadoxine-pyrimethamine in 2002. Regular evaluation of these two molecular markers in the \textit{dhps} gene would be valuable in monitoring the therapeutic efficacy of sulfadoxine-pyrimethamine. It is unclear whether these results reflect drug pressure due to the use of sulfadoxine-pyrimethamine as the second-line antimalarial treatment or of antibiotics containing antifolates (e.g., cotrimoxazole). Cross-resistance between sulfadoxine-pyrimethamine and trimethoprim-sulfamethoxazole has been described.\textsuperscript{37} Strains with mutant alleles N108, N108-I51, and N108-R59 in the \textit{dhfr} gene are less susceptible to both pyrimethamine and trimethoprim than wild-type isolates,\textsuperscript{38} and a high rate of bacterial uropathogen resistance to trimethoprim-sulfamethoxazole has been observed in Bangui.\textsuperscript{39} Intermittent use of these drugs could contribute to pyrimethamine resistance. Moreover, the extensive use of trimethoprim-sulfamethoxazole as prophylaxis against human immunodeficiency virus (HIV) infection-associated opportunistic infections probably makes a large contribution to pyrimethamine resistance because HIV seropositivity has now reached 15% in Bangui.\textsuperscript{40}

In conclusion, molecular analyses of antimalarial drug-resistance alleles of \textit{P. falciparum} isolates in Bangui clearly show the widespread distribution of chloroquine- and pyrimethamine-resistant isolates, and to a lesser extent, sulfadoxine-resistant isolates. The molecular markers used in this study are valuable tools for describing the epidemiology of drug-resistant \textit{P. falciparum}. Regular monitoring of molecular markers both in Bangui and the rest of the Central African Republic would help the National Malaria Control Program to identify and recommend the best available treatment of malaria in this country.

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