GENETICS OF DRUG-RESISTANT \textit{PLASMODIUM FALCIPARUM} MALARIA IN THE VENEZUELAN STATE OF BOLIVAR

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Abstract. The state of Bolivar in Venezuela experiences episodic outbreaks of multidrug-resistant \textit{Plasmodium falciparum} malaria. We obtained \textit{P. falciparum}-infected blood samples in Bolivar in 1998–2000, and performed molecular assays for mutations conferring resistance to the antifolate combination of sulfadoxine-pyrimethamine (SP) and to chloroquine. All infections carried the dihydrofolate reductase (\textit{dhfr}) S108A and N51I mutations, and 45% of the infections had the \textit{dhfr} C50R mutation, which has been implicated in mid-level resistance to SP. Two dihydropteroate synthase (\textit{dhps}) mutations also involved in SP resistance, A581G and K540E, were detected in 90% and 67% of the samples, respectively. The \textit{dhfr} I164L mutation, which confers high-level resistance, was not identified. The \textit{P. falciparum} chloroquine resistance transporter (\textit{pfcr}) K76T mutation, which is critical for chloroquine resistance, was found in 167 of 168 infections. Six \textit{dhfr/dhps} allelotypes and four \textit{pfcr}-resistant alleles were observed. Their interrelationships suggest a semi-clonal propagation of \textit{P. falciparum} malaria in Bolivar, and an invasion of multi-resistant pathogens from Brazil. Despite national restrictions on the use of SP and chloroquine, genotypic resistance to these therapies remains widespread in Bolivar.

INTRODUCTION

South America has experienced a resurgence of malaria in recent decades, paralleling both the disinclination toward insecticide spraying\textsuperscript{1} and the spread of parasite drug resistance.\textsuperscript{2} The disease contributes to the impoverishment of rural communities in the Amazon,\textsuperscript{3} and endangers some of the already vulnerable indigenous peoples of the Americas.\textsuperscript{3,5} Malaria is endemic in the Venezuelan Amazon, with \textit{Plasmodium vivax} being the predominant pathogen.\textsuperscript{2} The Bolivar State, a 238,000 km\textsuperscript{2} region in southern Venezuela with a population exceeding 900,000 (Venezuelan Central Information Office Caracas, 1991), contributes more than 60% of the nationally reported cases of malaria, 20% of which are caused by \textit{P. falciparum} (Venezuelan Rural Endemic Division, 1995), the deadly human malaria and the focus of this study. In addition to Amerindian communities, the humid tropical forests of Bolivar are frequented by itinerant, multiethnic gold and diamond miners as well as agricultural workers who are subject to epidemic malaria outbreaks at the beginning and end of the rainy season. It was reported in 2001 that the overall malaria prevalence in Bolivar was 3.4 infections per 1,000 inhabitants (Weekly Epidemiologic Report, Venezuela Ministry of Health and Social Development, April 2001), although this is probably an underestimate, and does not reflect the serious morbidity and mortality that can follow the sudden and unpredictable outbreaks of drug-resistant disease.\textsuperscript{6}

Multi-drug resistance remains a formidable obstacle in the control of falciparum malaria in South America.\textsuperscript{5} The world’s first documented case of clinical chloroquine failure was described in Venezuela in 1960,\textsuperscript{7} and by the 1980s resistance to this safe and affordable antimalarial drug was almost ubiquitous in the malarious Amazon. Like chloroquine, pyrimethamine was used in the 1950s as a single-agent prophylactic in malaria suppression trials conducted in local populations in Venezuela.\textsuperscript{8} The trials were largely unsuccessful, but pyrimethamine was used again in the 1970s in the synergistic antifolate combination sulfadoxine-pyrimethamine (SP), which remained a useful treatment for chloroquine-resistant malaria into the 1980s, although \textit{in vivo} and \textit{in vitro} SP resistance was documented in Venezuela as early as 1977.\textsuperscript{9} By the mid 1980s, high-level parasitologic resistance to SP was evident in much of the Amazon.\textsuperscript{10,11} As a consequence of multidrug resistance in Venezuela, chloroquine use was banned by the National Program of Malaria Therapeutics in 1986, and SP use was banned in 1998. However, chloroquine is still occasionally used for treatment of uncomplicated disease. Currently, mefloquine is the drug of choice for uncomplicated malaria, and mefloquine in combination with parenteral quinine is used for treatment of complicated disease.

The molecular mechanisms of \textit{P. falciparum} antifolate resistance have been reviewed.\textsuperscript{12} Point mutations conferring specific amino acid alterations in the parasite’s dihydrofolate reductase (\textit{DHFR}), the target of pyrimethamine, and dihydropteroate synthase (\textit{DHPS}), where the sulfonamides act, have been identified in all antifolate-resistant strains and isolates of \textit{P. falciparum}. Two mutant \textit{dhfr} alleles are of special relevance in South America, the C50R/N51I/S108N mutation, which confers mid-level pyrimethamine resistance \textit{in vitro},\textsuperscript{13} and the N51I/S108N/I164L mutation, which confers high-level resistance,\textsuperscript{13} and whose appearance in a geographic area signals the end of the usefulness of SP. The prevalence of both alleles generally maps to the regions of the Amazon where antifolate resistance is most substantial,\textsuperscript{14} while the antecedent N51I/S108N allele confers only low-level resistance and is widespread in malarious regions of the continent. An array of mutations in \textit{dhps} usually accompanies parasites harboring mutant \textit{dhfr} alleles, with the A437G generally arising with N51I, and K540E and A581G with the \textit{dhfr} alleles C50R and I164L.\textsuperscript{14} While the extent of the contribution of \textit{dhps} mutations to \textit{in vivo} SP resistance has been a matter of contention,\textsuperscript{15,16} a recent study using multivariate analysis showed that the combined \textit{dhps} A437G/K540E was associated with SP failure both independently and in conjunction with \textit{dhfr} N51I/C59R/S108N.\textsuperscript{17}

Chloroquine resistance in \textit{P. falciparum} is conferred by mutations in the parasite \textit{pfcr}, which encodes a putative transporter of the digestive vacuole.\textsuperscript{18} A K76T mutation is an absolute requirement for the \textit{in vitro} and \textit{in vivo} chloroquine

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resistance phenotypes in naturally occurring infections. The K76T is accompanied by an array of more variable pfcr mutations whose roles in resistance are beginning to be elucidated.\textsuperscript{19} Multiple resistance pfcr alleles have been identified in South America.\textsuperscript{18} Additional parasite and host factors are undoubtedly involved in producing the in vivo chloroquine resistance phenotype.\textsuperscript{18, 20}

In this study, we estimate the regional prevalence of these drug resistance mutations from blood samples obtained from P. falciparum-infected individuals in the Bolivar State in 1998–2000. We define multiple alleles of dhfr, dhps, and pfcr and use their interrelated prevalences as a measure of the degree of clonality of the Venezuelan parasite populations, and to suggest the origin of the most drug-resistant pathogens. Our findings have implications for future antimalarial drug policy in this South America country.

MATERIALS AND METHODS

Study area and sample collection. Giemsa-stained thick blood smears positive for P. falciparum were obtained from 48 malaria diagnosis centers representing six of the 10 municipalities in the Bolivar State of Venezuela (Cedeño, Sucre, Par, Raul Leoni, Sifontes, and Gran Sabana). The slides were made for the routine microscopic diagnosis of malaria in 1998–2000 for symptomatic patients from whom written informed consent was obtained in accordance with the Ethical Committee of Malariology of the Ministry of Health of Venezuela. Malaria transmission is seasonal in Bolivar, peaking at the beginning (April-June) and end (October-December) of the rainy season. Itinerant labor in the mining and agricultural industries is the region’s principal employment. Patients were 95% males and 5% females, with 93% miners and 7% representing indigenous Amerindians. The miners were predominantly Venezuelan, but included citizens of neighboring countries. Ages ranged from 12 to 68 years, with a mean of 32. Parasitemias of the malaria-infected slides ranged from 0.001% to 4%.

Allelotyping of infected blood samples. Detailed polymerase chain reaction (PCR) and mutation analysis protocols are presented on our web site (http://medschool.umd.edu/CVD/plowe.html). The DNA was initially extracted from the blood smears by the methanol-fixation/heat-extraction method detailed elsewhere.\textsuperscript{20} Subsequently, PCR sensitivity was found to improve after performing a protease digestion and spin-column purification of the detached blood smear according to manufacturer’s recommendations (QI Amp DNA Mini Kit; Qiagen, Valencia, CA). Five to eight microliters of the final eluate served as template in each 25-\muL primary PCR performed as previously described.\textsuperscript{20} Allele-specific restriction analyses (ASRA) of polymorphic codons of dhfr (50, 51, 59, 108, and 164), dhps (437, 540, and 581), and pfcr (72, 74, 75, and 76), were performed as described on the web site. The pfcr amplicons from 13 infections with various 70-domain alleles as determined by ASRA were sequenced by standard methods to verify the assay results.

Statistical analysis. The proportion of specific alleles within allelotypes was compared using the chi-square test for two-tailed significance at $P < 0.05$ (Epi Info 6.0; Centers for Disease Control and Prevention, Atlanta GA).

RESULTS

Analysis of dhfr/dhps mutations. Extraction of DNA was attempted for PCR-based mutation analysis on approximately 200 P. falciparum-infected blood smears obtained in 1998–2000 from diverse regions of Bolivar (Figure 1), of which 168 yielded informative results at all or some of the polymorphic codons under study. The overall frequencies of the antifolate resistance mutations in dhfr and dhps were similar to those observed in a more limited study conducted in 1995 in northeastern Bolivar.\textsuperscript{21} Of the known dhfr mutations, all but the C50R were either absent or universal, with C50R being present in 45% of the 130 infections assayed. The dhfr S108A and the N511 mutations were detected in all infections examined (85 slides for codon 108 and 60 for codon 51, with infections chosen to represent each of the dhfr/dhps and pfcr allelotypes described in this report), while the C59R and I164L mutations were not detected (50 infections assayed for codon 59 and 81 for codon 164, with infections including samples representing each of the dhfr/dhps and pfcr allelotypes). It should be noted that of the infections examined at dhfr codon 164, four consistently gave results indicating a mixture of the wild and mutant codons. These slides of these infections were processed at the same time, and were of low parasitemia, this requiring extensive amplification and use of the entire sample. Since only 6% of the remaining slide extracts exhibited polyclonality at any of the other dhfr or dhps codons, and the four aforementioned infections were found not to harbor identical dhps and pfcr alleles, which would be expected for a rare mutation in the Bolivar cohort, we ascribed the mutant products to low-level contamination with mutant dhfr amplicons from other studies, and the ambiguity was not further pursued. Ninety percent of 144 infections were found to harbor dhps A581G, and 67% of 137 had the dhps K540E mutation, a mutation not previously examined in Venezuela. The dhps 437 codon was assayed in two infections from each dhfr/dhps allelotype, and confirmed to be mutant in each case. Similar to dhfr S108N, dhps A437G is nearly ubiquitous in the malarious Amazon, and was found in all the Bolivar isolates studied in 1995.\textsuperscript{21}

Allelotypes of dhfr/dhps. One hundred twenty-seven infections for which the dhfr and dhps mutations were fully characterized at the variable codons (dhfr 50 and dhps 540 and 581) were segregated into dhfr/dhps allelotypes (Table 1). Eight samples exhibited mixed codon identities at dhfr 50 and/or dhps 540; these were presumed to represent polyclonal infections and were excluded from further analysis. As shown, six of a possible eight dhfr/dhps allelotypes were identified, designated RA-RF, with 34% of the infections comprising RF harboring the full array of dhfr and dhps mutations detected in this survey. Two of the allelotypes, RB and RD, which harbored dhfr C50R without the complete array of dhfr and dhps mutations, were rare, together comprising only 3% of the infections.

Mutation analysis of pfcr. The pfcr K76T mutation critical to chloroquine resistance was detected in 167 of 168 infections examined. A single sample had the wild type codon at pfcr 76 as determined by ASRA and confirmed by direct sequencing of the PCR amplicon. This exceptional infection also harbored wild-type codons at pfcr 72, 74, and 75, suggesting that the parasite harbored a bona fide sensitive pfcr allele, and not a resistant allele that had reverted at the critical 76 codon, as
has been seen in one African isolate.\textsuperscript{22} We sought to more fully characterize the \textit{pfcrt} alleles in Venezuela by identifying the polymorphisms at codons 72, 74, and 75 in a subset of 91 infections, representing the three designated regions of Bolivar and the various \textit{dhfr/dhps} allelotypes. As shown in Table 2, four previously known arrays of polymorphisms in the \textit{pfcrt} 70 domain were found among the K76T-harboring infections, which we designate by the parasite strain from which they were first observed.\textsuperscript{22} Sixty-four percent of the infections had the DIV allele previously identified in Brazil\textsuperscript{23} and Bolivia, and among antifolate-resistant infections in Peru.\textsuperscript{24} The Dd2 allele, identified in Africa and Asia but not previously observed in South America,\textsuperscript{22} was present in 4% of the infections, and the Jav type first characterized in Colombia\textsuperscript{22} was found at a similar frequency. The \textit{pfcrt} allele of the Brazilian strain 7G8\textsuperscript{22} that has also been detected in Peru\textsuperscript{24} comprised 21\% of the infections.

**Drug resistance allele associations.** There were striking associations between the \textit{pfcrt} and \textit{dhfr/dhps} allelotypes among the Venezuelan parasites, as shown in Figure 2. The DIV allele was the most prevalent \textit{pfcrt} among most mutant \textit{dhfr/dhps} allelotypes. Ninety-four percent of 33 RF infections and 80\% of 25 RE shared the DIV \textit{pfcrt}, which was found in only 25\% of 24 RC and 17\% of 12 RA infections. The preponderance of DIV alleles among the RF and RE compared with the RC and RA allelotypes was highly significant ($P < 0.0001$). The three rare RB and RD parasites, which similar to RF harbor C50R, also had the DIV \textit{pfcrt} allele. Thus, 94\% of the \textit{dhfr} C50R, 88\% of the \textit{dhps} K540E, and 70\% of \textit{dhps} A581G mutants carried the DIV \textit{pfcrt}, while wild-type infections at these codons carried this allele at lower frequencies: 46\%, 28\%, and 23\%, respectively. Most of the parasites with the RE or RF \textit{dhfr/dhps} allelotypes not carrying the DIV \textit{pfcrt} (73\%) carried its 7G8 Brazilian counterpart.

### Table 1

<table>
<thead>
<tr>
<th>Allelotype</th>
<th>\textit{dhfr} codon</th>
<th>\textit{dhps} codon</th>
<th>% infections (n)</th>
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</thead>
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<tr>
<td>RA</td>
<td>Cys Ile Asn Ile</td>
<td>Gly Lys Ala</td>
<td>13 (12)</td>
</tr>
<tr>
<td>RB</td>
<td>Arg Ile Asn Ile</td>
<td>Gly Lys Ala</td>
<td>1 (1)</td>
</tr>
<tr>
<td>RC</td>
<td>Cys Ile Asn Ile</td>
<td>Gly Lys Gly</td>
<td>25 (24)</td>
</tr>
<tr>
<td>RD</td>
<td>Arg Ile Asn Ile</td>
<td>Gly Lys Gly</td>
<td>1 (1)</td>
</tr>
<tr>
<td>RE</td>
<td>Cys Ile Asn Ile</td>
<td>Gly Glu Gly</td>
<td>26 (25)</td>
</tr>
<tr>
<td>RF</td>
<td>Arg Ile Asn Ile</td>
<td>Gly Glu Gly</td>
<td>34 (33)</td>
</tr>
</tbody>
</table>

*Bold amino acids denote mutant codons.*

### Table 2

<table>
<thead>
<tr>
<th>Allele</th>
<th>\textit{pfcrt} codon</th>
<th>% infections (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HB3</td>
<td>Cys Met Asn Lys</td>
<td>1 (1)</td>
</tr>
<tr>
<td>DIV</td>
<td>Ser (TCT)\textsuperscript{*}</td>
<td>Met Asn Thr</td>
</tr>
<tr>
<td>7G8</td>
<td>Ser (AGT)</td>
<td>Met Asn Thr</td>
</tr>
<tr>
<td>Jav</td>
<td>Cys Met Glu Thr</td>
<td>5 (5)</td>
</tr>
<tr>
<td>Dd2</td>
<td>Cys Ile Glu Thr</td>
<td>4 (4)</td>
</tr>
</tbody>
</table>

*Bold amino acids denote mutant codons.*

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\textsuperscript{*}Figure 1. Map of Bolivar State showing municipalities. The inset shows Bolivar State in Venezuela. Circles indicate malaria field sites from which study samples were obtained, and are identified according to their location in the western (W), central (C), and eastern (E) regions designated in this study.
Regional analysis. We divided the Bolivar State into eastern, western, and central regions (Figure 1) to determine if there were large geographic differences in the distribution of the drug resistance mutations in 1998–2000. As shown in Figure 3, the pfcr1 and dhfr/dhps allelotypes were not exclusive to a particular region of the state. RF, the most mutant dhfr/dhps allelotype, was evident in 30–50% of the infections in each region, and there was a corresponding prevalence of the DIV pfcr1 allelic most often present with RF. The infrequently found pfcr1 Dd2 and Jav alleles were confined to the west and central areas, where their associated RA dhfr/dhps allelotype was more prevalent. We performed a limited microsatellite analysis on RF infections from various regions of Bolivar using the ca1 marker, which is not linked to the drug resistance loci analyzed in this study. Ninety-two percent of 25 isolates yielded a ca1 product of an identical size. A more extensive analysis of five RF isolates from the eastern region found an identical profile using four microsatellite markers.

DISCUSSION

Our findings complement two earlier molecular epidemiologic studies conducted on *P. falciparum* in Venezuela. Urdaneta and others examined 54 malaria isolates obtained in 1995 from a hospital in northeastern Bolivar for the antifolate resistance mutations in dhfr and dhps. Sixty-four percent of the isolates had dhfr C50R, while the dhfr mutations at codons 51 and 108, as well as the dhps mutations at 437 and 581, were ubiquitous. Urdaneta and others subsequently used the random amplified polymorphic DNA fingerprinting technique to demonstrate the restricted genetic diversity of these parasites and to provide evidence for their clonal propagation.

The blood samples in our survey were obtained from across Bolivar in 1998–2000, largely from adult males in the mining industry. However, relative to 1995, they do not demonstrate a rapid increase in antifolate resistance mutations. Most encouragingly, dhfr I164L, the last and most formidable mutation to arise under antifolate drug pressure, whose appearance signals the end of the usefulness of SP and which is already prevalent in Bolivia, Brazil, and Peru, did not appear to be present in our survey. The absence of dhfr I164L and the incomplete prevalence of C50R conferring mid-level *in vitro* resistance are consistent with the 20% incidence of parasitologic failures for SP measured in Bolivar in 1995. The relatively low failure rates for SP in the face of the extensively mutated dhps found in the Bolivar surveys support the view that dhfr mutations are the primary determinants of SP resistance, with the dhps mutations playing a secondary, augmentative role.

In contrast to the antifolate resistance mutations, the pfcr1 K76T mutation critical to chloroquine resistance, and identified in multiple pfcr1 alleles whose significance is unknown, was found to be all but ubiquitous in Bolivar, since it has been found in almost all of South America, including at least two of Venezuela’s neighbors, Brazil and Colombia. However, rates of high-level (RII and RIIP) parasitologic resistance were only 20% in the 1995 chloroquine efficacy study, which reinforces the role of additional parasite and host factors in clinical chloroquine failure.

Our allelic analysis at the drug resistance loci provides further evidence for the clonal propagation of *P. falciparum* malaria in Venezuela, albeit in an imperfect manner that might be best described as semi-clonal. Indeed, our findings suggest that antifolate drug pressure is a principal force in shaping these pathogen populations. Parasites with the most mutant dhfr/dhps allelotype (RF) carried a single resistant pfcr1 allele.
(DIV) in 94% of the infections assayed. In contrast, malaria with the least mutant antifolate resistance allelotype (RA) presented with one of four resistant pfcr alleles in roughly equal proportions, as well as the single sensitive pfcr allele detected in this study. Two intermediate dhfr/dhps allelotypes showed intermediate distributions of the resistant pfcr alleles. Nearly all parasites harboring dhfr C50R and/or dhps K540E and A581G carried DIV or 7G8 pfcr. The DIV pfcr allele has been detected in all or nearly all infections in Brazil and Bolivia, where dhfr C50R and I164L are highly prevalent, and in most Peruvian parasites carrying dhfr I164L; the exceptions harbor the 7G8 allele. In contrast, genotypically antifolate-sensitive parasites in Peru and Colombia bear other resistant pfcr alleles. The prevalence of pfcr DIV parallels both the level of antifolate resistance and the prevalence of dhfr C50R and I164L, as well as dhps K540E and A581G, mutations that microsatellite analysis of diverse parasite isolates has shown to have had a single origin in South America. Thus, Bolivar appears to be experiencing a migration of antifolate-resistant P. falciparum malaria from Brazil, and the detection of intermediate dhfr/dhps allelotypes, such as the rare RB and RD forms, which carry dhfr C50R in the absence of dhps K540E, is suggestive of at least some degree of recombination between the invading and resident populations. The absence of any localized preponderance of particular allelotypes in our regional analysis of Bolivar and the paucity of polyclonal infections detected in this survey are consistent with an itinerant host population disseminating parasites whose clonality is predicated on the region’s low and/or unstable vector transmission intensity. The emigration from Brazil of the antifolate-resistant pathogens is likely due to migratory labor practices in the midst of antifolate drug pressure, although our limited analysis cannot exclude other selective forces. For example, both the DIV and 7G8 pfcr alleles harbor C72S in addition to K76T, but as a consequence of different nucleotide substitutions, and recent work has demonstrated a unique in vitro chloroquine-resistant phenotype of parasites harboring these pfcr alleles. The DIV and 7G8 mutations are also the pfcr alleles most often present with the P. falciparum multiple drug resistance 1 (pfmdr1) gene mutation D1246Y in South America, a mutation shown to enhance in vitro chloroquine resistance in mutant pfcr parasites. Other advantageous genetics may also be carried by the antifolate-resistant parasites that have made their way into Venezuela.

The ultimate goal of geographic molecular surveys for P. falciparum drug resistance is to reliably quantify antimalarial drug efficacy to benefit regional malaria control policies. Although this has yet to come to fruition, since the relationships between parasite drug-resistant allelotype and drug failure in the field are more complex than was anticipated, preliminary statistical models for the estimation of drug efficacy based on parasite allelotype have been developed both for chloroquine in Mali and the SP in Malawi. The refinement and validation of such models may have application for malaria control in South America, although the continued paucity of South American drug efficacy studies complemented by relevant genetic analysis will remain an impediment. Even our more modest epidemiologic survey has implications for antimalarial drug policy in Bolivar. First, the almost complete prevalence of pfcr K76T, despite the reported restricted use of chloroquine in Venezuela for almost 15 years, necessitates that chloroquine continue to be used cautiously in Bolivar, if at all. Second, the persistent absence of dhfr I164L suggests that SP failure rates should remain relatively low, although this therapy should also be used with caution, and ideally in combination with an additional antimalarial, since dhfr C50R and the extensively mutated dhps in the Bolivar samples will likely confer some degree of SP failure. The continued absence of dhfr I164L also makes the antifolate combination chlorproguanil-dapsone an attractive alternative to SP, preferably in combination with another drug such as artesunate, to prevent emergence of resistance, since chlorproguanil is active against the RF dhfr allele in vitro, and has proven in vivo efficacy against mid-level antifolate-resistant malaria in Africa. However, a more systematic survey for dhfr I164L and drug inhibition assays of culture-adapted parasites harboring the Bolivar dhfr/dhps allelotypes should precede any implementation of this promising antifolate combination.

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