POINT MUTATIONS IN DIHYDROFOLATE REDUCTASE AND DIHYDROPTEROATE
SYNTHASE GENES OF PLASMODIUM FALCIPARUM ISOLATES FROM VENEZUELA

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Abstract. The present study was designed to characterize mutations in dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) genes of Plasmodium falciparum in the Bolivar region of Venezuela, where high levels of clinical resistance to sulfadoxine-pyrimethamine (SP; Fansidar®, F Hoffmann-La Roche, Basel, Switzerland) have been documented. We used a nested mutation-specific polymerase chain reaction and restriction digestion methods to measure 1) the prevalence of DHFR mutations at 16, 50, 51, 59, 108, and 164 codon positions, and 2) the prevalence of mutations in the 436, 437, 581, and 613 codon sites in DHPS gene. In the case of the DHFR gene, of the 54 parasite isolates analyzed, we detected the presence of Asn-108 and Ile-51 in 96% of the isolates and Arg-50 mutation in 64% of the isolates. Each of these mutations has been associated with high level of resistance to pyrimethamine. Only 2 samples (4%) showed the wild type Ser-108 mutation and none showed Thr-108 and Val-16 mutations that are specific for resistance to cycloguanil. In the case of DHPS gene, we found a mutation at position 437 (Gly) in 100% of the isolates and Gly-581 in 96% of the isolates. The simultaneous presence of mutations Asn-108 and Ile-51 in the DHFR gene and Gly-437 and Gly-581 in the DHPS gene in 96% of the samples tested suggested that a cumulative effect of mutations could be the major mechanism conferring high SP resistance in this area.

Morbidity and mortality due to Plasmodium falciparum malaria has continued to increase in the past few years. While there are several factors that have contributed to the increase in malaria globally, the failure to control the disease in many parts of the world is partially due to decreased susceptibility of the malaria parasites to once effective antimalarial drugs, such as chloroquine. Numerous documented reports of high-grade chloroquine resistance have prompted governments in sub-Saharan Africa to reconsider their antimalarial treatment guidelines. The requirement for alternative drugs has led to the extensive use of the sulfadoxine-pyrimethamine (SP) combination drug in the treatment of uncomplicated malaria.

One of the concerns with the use of SP is the rapid development of resistance. In Thailand, SP replaced chloroquine in the early 1970s, but within 5 years, radical cure rates by day 28 had decreased from 83% in 1975 to 22% in 1979. The long half-life of sulfadoxine and pyrimethamine (180 and 95 hr) favor the selection of resistant parasites, and there is a general concern that in Africa, where the intensity of malaria transmission is considerably higher, the effective life of SP may be even shorter.

Molecular characterization of the DHFR and DHPS genes has revealed a correlation between pyrimethamine resistance and the following point mutations: an amino acid substitution at residue 108 from Ser to Asn, mutations at codon 51 that changes Asn to Ile, and a mutation at codon 59 that changes Cys to Arg. A threonine at position 108 paired with an alanine to valine change at position 16 is associated with resistance to cycloguanil. Previous studies have demonstrated that a change from Ile to Leu at position 164, coupled with Asn-108 plus Ile-51 and/or Arg-59 confers high-level resistance to both pyrimethamine and cycloguanin. New mutations in the DHFR gene at position 50 (Cys to Arg) and a five amino acid repetitive insert between positions 30 and 31 have been found to be highly prevalent in Bolivia, where SP resistance is high. Recently, a novel leucine mutation at position 140 in the DHFR gene was found in the isolate VP8, which also has the characteristic changes at positions Val-16 and Thr-108, which confer cycloguanil resistance. In the case of the DHPS gene from P. falciparum lines resistant to the most commonly used sulpha drug (sulfadoxine), amino acid changes at the 436, 437, 581, and 613 codons have been shown to be associated with resistance. A recent study has shown association between mutation at codon 540 with sulfadoxine resistance.

The sulpho and pyrimethamine drugs have been used in the treatment of uncomplicated malaria in Venezuela over the past 20 years. Several in vitro and in vivo studies have documented failures of SP in the treatment of uncomplicated malaria. Recent drug efficacy studies in the malaria-endemic regions of Bolivar and Amazonas States have revealed 100% resistance to several drugs such as chloroquine, amodiaquine, and SP (Riggione F, unpublished data). During the period of sample collection, SP was the first-line treatment used by the Malaria Program in uncomplicated P. falciparum malaria in Venezuela, where chloroquine-resistance predominates. The clinical and parasitologic evidence of a high degree of resistance to SP led us to initiate a molecular epidemiologic study to determine the prevalence of mutations in DHFR and DHPS that have been associated with resistance to sulfadoxine-pyrimethamine in the Bolivar region of Venezuela.

MATERIALS AND METHODS

Study area. The sample collection was conducted during the rainy season in 1995 in several communities of the malariaous region of Bolivar State in a tropical, humid forest in southeastern Venezuela. Sixty-seven fresh isolates of P. falciparum were collected from malaria-infected miners and Amerindians, who had moderate parasitemia detected by microscopy and had not received drugs during the previous 14 days. The parasites were cultured for 24–36 hr to increase
the number of parasites, and they were immediately cryopreserved. Clones with known mutations in the DHFR and DHPS genes were used as controls for polymerase chain reaction (PCR) assays and digestion with restriction enzymes.

The study protocols were approved by the Biomedical Committee of Malariology of the Ministry of Health of Venezuela.

**Extraction of genomic DNA and mutation-specific PCR assays.** The parasite genomic DNA was extracted from infected red blood cells using proteinase K, followed by extraction with phenol-chloroform and ethanol precipitation. Analysis of mutations at the DHFR 16 and 108 sites was done using the PCR method followed by restriction enzyme digestion as previously described, with the following modifications: 50–100 ng of genomic DNA, 1 μM of each primer, 200 μM of each dNTP buffer (50 mM KCl, 10 mM Tris, pH 8.4, 1.5 mM MgCl₂, 0.1 mg/ml of gelatin), and 2.5 units of Taq polymerase were used in a 100-μl reaction. The denaturation was performed at 94°C for 5 min, the samples were then incubated at 94°C for 45 sec, 50°C for 1 min, 72°C for 2 min for 40 cycles, and a final extension was done at 72°C for 3 min. The PCR product was purified with Wizard PCR prep (Promega, Madison, WI) and resuspended in TE buffer (0.1 M Tris, 0.1 M EDTA, pH 8.0). The restriction endonucleases *Alu I*, *Bsr I*, and *Scrf I*, which exclusively cleave the Ser-108 (wild), the Asn-108 (mutant), and the Thr-108 (mutant), respectively, were used to digest the amplified DHFR fragment. The enzyme *Nla III* was used to digest Ala-16 (wild) or Val-16 (mutant), showing a pattern characteristic for each one as described. Digestions were done in 40-μl reactions containing 10 μl of purified PCR fragments according to the manufacturer’s (New England Biolabs, Inc., Beverly, MA) specifications. The digested fragments were separated by electrophoresis on a 1.5% agarose gel and stained with ethidium bromide for ultraviolet visualization. The codon sites 50, 51, 59, and 164 in the DHFR gene were studied using methods previously described. The DHFR domain was first amplified in a primary round of PCR using the primers AMPI/AMP2 as described. An aliquot of 0.1–0.5 μl of the first amplification was used in a mutation-specific second round PCR to detect the DHFR mutation sites 51, 59, and 164, as described. For the analysis of the DHFR 50 codon, we used mutation-specific sense primers FR50w (5’-GGAGTATTACCATGGAAAT-3’), which specifically amplifies the sequence containing the wild type TGT (Cys) codon, and FR50m (5’-GGAGTATTACATGGAAAC-3’), which is specific for the mutant codon CGT (Arg); these primers were paired with the common antisense primer SP2 to give a final product of 570 basepairs. The cycling parameters for second round were initial denaturation at 95°C for 3 min, followed by 15 cycles of denaturation at 92°C for 30 sec, annealing at 55°C for 45 sec, and extension at 72°C for 45 sec, and a final extension at 72°C for 3 min. For this assay, the MgCl₂ was present in the reaction at a concentration of 1.5 mM; the other reaction conditions were as described. The PCR products were analyzed by electrophoresis on a 2% agarose gel and stained with ethidium bromide for ultraviolet visualization.

The prevalence of DHPS mutations at residues 436, 581, and 613 was detected using a set of specific primers previously published. Genomic DNA (10–20 ng) was amplified following conditions for a nested PCR as described. The mutations at codon position 436 and 437 in DHPS gene were studied for enzymatic digestion of the DHPS domain amplified with the 185 and 218 primers. We used *Msp* AI, which exclusively cleaves the Ala-436 mutant, and *Ava* II, which exclusively cleaves the Gly-437 mutant type; the digestions were done in 40-μl reactions according to manufacturer’s (New England Biolabs, Inc.) specifications. All the DHPS samples were analyzed by electrophoresis on a 1.5% agarose gel stained with ethidium bromide.

**RESULTS**

From the 67 *P. falciparum* isolates collected, we were able to amplify the DHFR and DHPS genes from 54 parasite isolates. Allelic frequencies of DHFR and DHPS mutations were determined for these 54 samples.

The screening of 16 and 108 codon sites in the DHFR gene was done by enzymatic digestion of the amplified DHFR domain with *Alu I* (for Ser-108), *Bsr I* (for Asn-108), *Scrf I* (for Thr-108), and *Nla III* (for Ala-16 or Val-16). The digestion of the 708 basepair (bp) of the DHFR domain produced the characteristic restriction patterns of 322 and 386 bp for *Alu I*, *Bsr I*, and *Scrf I*. The 108-Asn mutant type was detected by digestion with *Bsr I* on Venezuelan *P. falciparum isolates* (Figure 1a; lanes 3, 7, and 11), and the wild type 108-Ser was revealed by digestion with *Alu I* on a parasite isolate and the 3D7 reference clone (Figure 1b; lanes 1 and 5). The 108-Thr and 16-Val mutant types present in the FCR3 reference clone were revealed by digestion with *Scrf I* (Figure 1b; lane 10) and *Nla III*, respectively (Figure 1b; lane 12 producing the characteristic 568- and 140-bp fragments). From 54 DNA samples of *P. falciparum* tested, 96% of the isolates contained DHFR genes with the Asn-108 mutation and 4% showed the wild type Ser-108 residue. No sample showed the Thr-108 or Val-16 mutations, which have been linked with cycloguanil resistance. The prevalence of mutations at 50, 51, 59, and 164 codon sites in the DHFR gene was investigated using a nested mutation-specific PCR. The Arg-50 mutation (a new mutation recently reported from Bolivian malaria-endemic areas with high rates of SP resistance) was present in 64% (34 samples), showing a specific band of 570 bp (Figure 2a). The Ile-51 mutation was found in 96% (52 samples), giving the characteristic positive signal (574 hp) (Figure 2b). The Arg-59- and Leu-164-specific mutations were not found in any samples tested.

In the case of the DHPS gene, the prevalence of Phe-436, Gly-581, Thr-613, and Ser-613 was studied by a nested mutation-specific PCR. We found that 52 (96%) of the 54 samples carried Gly-581 mutation; a typical signal for this mutant type is shown in Figure 3. None of the other mutations studied was present. The prevalence of Ala-436 and Gly-437 mutations was tested by specific-mutation enzyme analysis; the digestion of the amplified DHPS domain (Figure 4a) by *Ava* II shows that Gly-437 was present in all isolates, showing the same profile as that of the 3D7 mutant reference clone (Figure 4b, lane 11). The DHFR and DHPS mutations prevalent in Venezuela, in comparison with previous reports
POINT MUTATIONS IN DHFR AND DHPS GENES OF *P. FALCIPARUM*

**Figure 1.** Restriction enzyme digestion of the amplified dihydrofolate reductase domain (708 basepairs [bps]) at codons 108 and 16, using restriction enzymes *Alu* I (lanes 1, 5, and 9), *Scf* I (lanes 2, 6, and 10), *Brs* I (lanes 3, 7, and 11), and *Nla* III (lanes 4, 8, and 12). A, Venezuelan samples (lanes 1–12) show the mutant type Asn-108 digested with *Brs* I; absence of the wild type is revealed by no digestion with *Alu* I; absence of mutant type Thr-108 is shown by no digestion with *Scf* I; and the wild type Ala-16 was digested with *Nla* III (568- and 93-bp [bp] fragments). B, Venezuelan isolate (lanes 1–4) and 3D7 clone (lanes 5–8) were used as controls for the wild type; *Alu* I digested the DNA in lanes 1 and 5. The FCR3 clone (lanes 9–12) was used as a control for the mutant types Thr-108 and Val-16 (568- and 140-bp fragments). *Scf* I digested the DNA in lane 10 and *Nla* III digested the DNA in lane 12. DNA size markers are shown in lanes M. Values on the left are in basepairs.

From areas with a high rate of SP failure in Bolivia, Mali, Kenya, Malawi, Brazil, Papua New Guinea, and Cameroon are shown in Table 1.

**Discussion**

*Plasmodium falciparum* resistance to SP is conferred by point mutations in parasite DHFR and DHPS, the enzymes targeted by these drugs. This allows the use of standard PCR techniques for the *in vitro* monitoring of SP efficacy. In these assays, either point mutation–specific primers are used to amplify the gene or point mutation–specific enzymes are used to specifically digest a product of amplification.

Previous studies that used reference clones and clinical isolates from different parts of the world have revealed that the *in vitro* antifolate resistance is associated with point mutations in the DHFR domain of the DHFR–thymidylate synthetase gene. Among the point mutations in the DHFR gene, a Ser to Asn-108 point mutation is considered the key mutation that confers resistance to antifolate drugs. Other mutations associated with high levels of pyrimethamine resistance are codons 50, 51, 59, and 164. In the case of *in vitro* resistance to sulfadoxine, moderate resistance is defined by a single Ala to Gly mutation in codon 437. High levels of sulfadoxine resistance are associated with double (Ser-436, Gly-437, Gly-581, Ala-613) or triple (Phe-436, Gly-437, Ala-581, Ser-613) DHPS mutations. Although in most of the studies, confirmatory clinical data are not available, the results point to the association of select point mutations in these two genes with resistance.

In the present study, we have characterized point mutations in the DHFR and DHPS genes in *P. falciparum* isolates from the Bolivar region of Venezuela, where a high level of clinical resistance to SP has been reported. Studies conducted over the past several years in the Bolivar region have revealed high (up to 100% in some studies) level of *in vitro* resistance to SP. At the time parasites were collected for this study, treatment failure of SP was also frequently observed. The high levels of clinical failure of SP in the treatment of malaria in this region provided us an opportunity to characterize mutations in the DHFR and DHPS genes that have previously been associated with resistance in areas of less resistance.

Characterization of the DHFR gene of 54 samples showed that 96% had Asn-108 and Ile-51 mutations and 64% had an Arg-50 mutation. The prevalence of mutations in the
FIGURE 2. a, Nested mutation-specific polymerase chain reaction (PCR) of the dihydrofolate reductase gene showing the Arg-50 mutation in 3 Venezuelan samples (lanes 1–3). A *Plasmodium falciparum* isolate containing the wild type Cys-50 (lane 4); the positive control containing Arg-50 (lane 5); and the negative control (without DNA) for the PCR (lane 6) are shown. b, Nested mutation-specific PCR showing the Ile-51 mutation from *P. falciparum* isolates (lanes 1, 2, and 3). The positive control for the mutation was V/1 S (lane 4), a sample containing the wild type Asn-51 is showed in lane 5, and the negative control (without DNA) for the PCR is in lane 6. DNA size markers are shown lanes M. Amplification primers used in this experiment are described in the Methods and Material section. Values are in basepairs.

DHPS gene from 54 samples studied showed 100% for Gly-437 and 96% for Gly-581. Similar prevalence rates of these mutations have been recently reported from Bolivia (Table 1), where the prevalences of the new mutation Arg-50 in the DHFR gene and Gly-437 and Gly-581 in the DHPS gene correlate with increasing use of SP use and in vivo resistance. The prevalence of mutations in the DHFR and DHPS genes detected in this study (Table 1) suggests a strong association with high levels of in vitro resistance and high clinical resistance present in this area. The simultaneous presence of mutations Asn-108, Ile-51, and Arg-50 in the DHFR gene and Gly-437 and Gly-581 in the DHPS gene suggests that this cumulative effect of mutations is a possible

FIGURE 3. Nested mutation-specific polymerase chain reaction (PCR) (a 756-basepair [bp] fragment) in the dihydropteroate synthase gene showing the Gly-581 mutant from 4 *Plasmodium falciparum* isolates (lanes 1–4) and V/1 S clone, (control for the mutant type, lane 8). Two Venezuelan samples with Ala-581 wild type (lanes 5 and 6) and 3D7 clone (control for the wild type, lane 7) showed the specific band of 441 basepairs. The negative control (without DNA) for the PCR is shown in lane 9. DNA size markers are shown in lanes M. Values on the left are in basepairs.

FIGURE 4. a, Amplification of the dihydropteroate synthase (DHPS) domain (1,330 basepairs) of *Plasmodium falciparum* from Venezuelan samples (lanes 1–10) and the 3D7 clone (lane 11). b, Restriction enzyme digestion of the DHPS domain at the codon 437 site shows digestion by *Ava* II of the mutant type Gly-437 (lanes 1–10) and the 3D7 clone (mutant control) (lane 11). The digestion of 1,330 basepairs of the DHPS domain generates 849- and 303-basepair bands. DNA size markers are shown lanes M. Values on the left are in basepairs.
Table 1
Prevalence of dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) mutations of Venezuelan Plasmodium falciparum isolates in comparison with previous reports*

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* No. = total number of samples tested.

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