LACK OF AN ASSOCIATION BETWEEN THE ASN-108 MUTATION IN THE DIHYDROFOLATE REDUCTASE GENE AND IN VIVO RESISTANCE TO SULFADOXINE/PYRIMETHAMINE IN PLASMODIUM FALCIPARUM

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Abstract. Sulfadoxine/pyrimethamine (SP) is considered an alternative treatment for acute uncomplicated malaria caused by Plasmodium falciparum resistant to chloroquine. However, the appearance of resistance to this drug has been reported since its initial use in Colombia. Molecular analysis of the dihydrofolate reductase gene indicates a correlation between in vitro resistance to SP and the Asn-108 point mutation. Little is known about the association of this point mutation and in vivo resistance to SP. We used a mutation-specific polymerase chain reaction strategy to analyze the presence of the Asn-108 point mutation in 48 clinical samples with adequate clinical response (ACR), 2 early treatment failures (ETF), and 1 late treatment failure (LTF). The Asn-108 mutation was detected in 36 of the ACR samples and in all of the ETF and LTF samples. Eleven ACR samples amplified with the wild-type–specific primer and one amplified with the primer for the Thr-108 mutation described for cycloguanil. These results suggest that the Asn-108 marker may not be useful in predicting SP treatment failure.

Malaria has become a major public health problem in Colombia. In 1997, the Ministry of Health reported 180,910 cases of malaria, of which 36.3% were caused by Plasmodium falciparum. In Chocó, area of this study, the number of cases increased from 5,182 in 1996 to 25,236 in 1997 (Colombian Ministry of Health, National Malaria Control and Prevention Plan, 1997, unpublished data). However, the real magnitude of the problem is not well known due to inadequate notification. Furthermore, the appearance of resistance to chloroquine (CQ) has severely affected the National Malaria Control Program.

The urgent need for an alternative treatment due to CQ resistance and the scanty information on the magnitude of CQ resistance and its distribution has lead to the use of sulfadoxine/pyrimethamine (SP) alone or in combination with CQ. Sulfadoxine/pyrimethamine is a dihydrofolate reductase (DHFR) inhibitor affecting DNA synthesis. Unfortunately, since its introduction in Colombia in 1981, resistant cases have been reported. Molecular analysis of the DHFR gene has shown an association between in vitro resistance and the Asn-108 point mutation. This mutation produces an amino acid substitution at position 108 of the protein sequence. A serine (wild type) is changed to an asparagine, apparently modifying the active site of the enzyme. Although other point mutations have been detected, the Asn-108 mutation has been consistently associated with in vitro resistance. A mutation-specific polymerase chain reaction (PCR) strategy has been developed for use with small amounts of blood (100 µl). Using this method, we previously showed that in southwestern Colombia the Asn-108 point mutation was present in 51.6% of clinical samples. Our objective was to determine the possibility to use the Asn-108 mutation as a molecular marker for clinical SP resistance.

MATERIALS AND METHODS

The study was conducted at the Centro Internacional de Entrenamiento e Investigaciones Médicas (CIDEIM) in Cali, Colombia. This project was reviewed and approved by the Institutional Board of Ethic for Research on Human Subjects from CIDEIM. Informed consent was obtained from patients or their guardians if they were children. Fifty-one clinical blood samples were collected onto filter paper (Schleicher and Schuell, Keene, NH) by fingerprick from patients with malaria due to P. falciparum who were treated with SP (25 mg/kg of sulfadoxine plus 1.25 mg/kg of pyrimethamine), and air-dried. Samples were stored in separate plastic bags at room temperature. Extraction of DNA and a nested PCR were performed as described previously. The outer primer reaction was performed in a total volume of 50 µl containing 2.5 units of Taq DNA polymerase (Gibco-BRL, Gaithersburg, MD), 200 mM Tris-HCl (pH 8.4), 500 mM KCl, 1.5 mM MgCl2, 200 µM of each dNTP (Gibco-BRL), 1 µM of primers, and 10 µl of the sample. The nested (inner) reaction was performed as described above using 10 µl of a 1:10 dilution of the first PCR. Table 1 shows the primer sequences, product sizes, and reaction conditions used. The PCR products were visualized by electrophoresis in a 1% agarose gel stained with ethidium bromide. Double-distilled water was used as negative control and DNA extracted from laboratory culture strains HB3 (Asn-108) and 3D7 (wild type) was used as positive controls.

RESULTS

Clinical outcome data was obtained from an in vivo efficacy test with SP with supervised treatment and 14 days of follow-up performed in Quibdó, Chocó (Figure 1) (Osorio L., unpublished data). Of the 51 samples collected, 48 had an adequate clinical response (ACR), 2 an early treatment failure (ETF), and 1 had a late treatment failure (LTF). Results are summarized in Table 2. All samples from patients that had treatment failures (3) amplified with the Asn-108 point mutation–specific primer. Of the 48 ACR samples, 36 (75%) amplified with the Asn-108 point mutation–specific primer. Eleven ACR samples amplified with the wild-type–specific primer and one with the primer for the Thr-108 point mutation. Statistical analysis of the data indicated a lack of concordance (kappa = 0.04) between clinical results and PCR results.
<table>
<thead>
<tr>
<th>Reactions*</th>
<th>Primer sequence†</th>
<th>Product‡</th>
<th>Reaction conditions§</th>
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<tr>
<td>First reaction</td>
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| Amp 1’ | 5’-TTTATATTTTCTCCTTTTTA-3’ | 720 bp | 94°C for 3 min
| | | | 94°C for 94 sec
| | | | 45°C for 45 sec 45 cycles
| | | | 72°C for 45 sec
| Amp 2’ | 5’-CATTTTATTATGCCTTTTCT-3’ | | 72°C for 5 min (final extension)
| Second reaction | | | |
| SPI1 | 5’-ATGATGGAAAGCTGCTGAC-3’ | 339 bp | 94°C for 3 min
| DIA-3’ | 5’-GAATGCTTCCAGC-3’ | | 94°C for 30 sec
| | | | 55°C for 45 sec 20 cycles
| | | | 74°C for 45 sec
| DIA-12’ | 5’-GGAATGCTTTCCAGT-3’ | | 74°C for 5 min (final extension)
| DIA-9’ | 5’-GAATGCTTTCCAGG-3’ | | |

* f = forward primer; r = reverse primer.
† bp = basepairs.

FIGURE 1. Geographic location of the in vivo study site (square) and origin of the clinical samples used in the polymerase chain reaction study.
with low resistance. We have concluded that the Asn-108 point mutation is correlated with clinical therapeutic failure; ETF and LTF samples 48 DIA-3† 11 ETF and LTF samples 3 DIA-12‡ 3

**DISCUSSION**

Several studies on the molecular basis for SP resistance have concluded that the Asn-108 point mutation is correlated with *in vitro* resistance. Similar to what Plowe and others have suggested, Sirawaraporn and others showed that mutants with a synthetic *P. falciparum* dhfr gene with the Asn-108 point mutation were 10-fold less susceptible to pyrimethamine compared with the wild-type gene, but according to *in vivo* studies this decrease is not sufficient to generate clinical failure. Similar to what Plowe and others have suggested, Sirawaraporn and others proposed that the emergence of drug resistance involved sequential selection of single point mutations, starting from the least resistant single mutant that survived selection, Asn-108, and proceeding stepwise to the more resistant multiple mutants. Giraldo and others previously observed that in areas from southwestern Colombia with low *in vivo* treatment failure, there was high frequency of the Asn-108 mutation. Our study expands this observation to the northwestern region of Colombia by detecting the Asn-108 point mutation in 76.5% (39 of 51) of the samples from an area where the frequency of *in vivo* treatment failure was only 6% (3 of 51).

The discrepancy between our observation on the frequency of the Asn-108 mutation and clinical response may be due to several factors. Clinical response is the result of the interaction between the host, the parasite and the drug and includes immune status, parasite sensitivity, and pharmacologic characteristics of the drug used. Although a parasite may have a pyrimethamine-resistant phenotype, the clinical evaluation of SP efficacy provides information on the synergistic effect of sulfadoxine and pyrimethamine on folate metabolism. After an oral dose of 25 mg of pyrimethamine, the mean ± SD maximum concentration in blood plasma is 0.94 ± 0.08 μM. In *in vitro* studies have reported that pyrimethamine inhibits the Ser-108 wild-type allele at a mean ± SD concentration of 1.5 ± 0.2 nM and the Asn-108 mutant allele at a concentration of 13 ± 4 nM. Therefore, although the Asn-108 mutation confers a 10-fold reduction in sensitivity, the therapeutic levels obtained with the standard dosage used in our study (1.25 mg/kg) could inhibit these parasites. In addition, the parasites may still be sulfadoxine sensitive. Second, host immunity plays an important role in the resolution of infection and may be critical for control of low-level drug-resistant parasites. Finally, the number of point mutations and the patterns of these mutations may be important in the clinical presentation of resistance.

Our results indicate that the presence of the Asn-108 mutation is not sufficient to generate clinical failure; however, the 3 samples with the ETF and LTF outcomes could contain additional point mutations in the *dhfr* or dihydropteroate synthase (*dhps*) genes. Recently, Kublin and others have reported a genotypic marker in *P. falciparum* for *in vivo* resistance to SP that includes in addition to the Asn-108 mutation the Ile-51 and Leu-164 point mutations of the *dhfr* gene combined with the Gly-437, Glu-540, and Gly-581 point mutations of the *dhps* gene. The differences in the genotypic patterns of SP-resistant parasites observed in Africa and the Peruvian Amazon may reflect independent genetic changes in malaria parasites of different geographic sources. This is a very important observation since it is possible that more than one set of mutations exist that generate *in vivo* resistance to SP, but specific patterns may vary according to the geographic source of the parasite.

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