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

NORMA IBÓN RALLÓN, LYDA ELENA OSORIO, AND LUIS ERNESTO GIRALDO
Corporación Centro Internacional de Entrenamiento e Investigaciones Médicas, Cali, Colombia

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 resistance to 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.1 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from 5,182 in 1996 to 25,236 in 1997 (Colombian Ministry of Health, National Malaria Control and Prevention Plan, 1997, unpublished data).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.2 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.3 Unfortunately, since its introduction in Colombia in 1981, resistant cases have been reported.4 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.5 A serine (wild type) is changed to an asparagine, apparently modifying the active site of the enzyme.6 Although other point mutations have been detected, the Asn-108 mutation has been consistently associated with in vitro resistance.5 A mutation-specific polymerase chain reaction (PCR) strategy has been developed for use with small amounts of blood (100 μl).7 Using this method, we previously showed that in southwestern Colombia the Asn-108 point mutation was present in 51.6% of clinical samples.8 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.5,8 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 sequenc-

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 1**
Mutation-specific nested polymerase chain reaction conditions, primers, and product expected size

<table>
<thead>
<tr>
<th>Reactions*</th>
<th>Primer sequence†</th>
<th>Product‡</th>
<th>Reaction conditions§</th>
</tr>
</thead>
<tbody>
<tr>
<td>First reaction</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amp 1f</td>
<td>5'-TTTATATTTTCCTTTT-3'</td>
<td>720 bp</td>
<td>94°C for 3 min</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>94°C for 30 sec</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>45°C for 45 sec 45 cycles</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>72°C for 45 sec</td>
</tr>
<tr>
<td>Amp 2r</td>
<td>5'-CATTTTATTATCCTTTCTT-3'</td>
<td></td>
<td>72°C for 5 min (final extension)</td>
</tr>
<tr>
<td>Second reaction</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPI1f</td>
<td>5'-ATGATGGAACAAGTCTGCGAC-3'</td>
<td>339 bp</td>
<td>94°C for 3 min</td>
</tr>
<tr>
<td>DIA-3r</td>
<td>5'-GAATGCTTTCCCAGG-3'</td>
<td></td>
<td>94°C for 30 sec</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>55°C for 45 sec 20 cycles</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>74°C for 45 sec</td>
</tr>
<tr>
<td>DIA-12r</td>
<td>5'-GAATGCTTTCCCAGG-3'</td>
<td></td>
<td>74°C for 5 min (final extension)</td>
</tr>
<tr>
<td>DIA-9r</td>
<td>5'-GAATGCTTTCCCAGG-3'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* 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 

ously observed that in areas from southwestern Colombia 

60.94

more resistant multiple mutants.

Sirawaraporn and others proposed that the emergence of 

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accurate information on the syn-

particularly 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 gen-

erate in vivo resistance to SP, but specific patterns may vary 

according to the geographic source of the parasite.

Several studies on the molecular basis for SP resistance 

have concluded that the Asn-108 point mutation is correlated 

with in vitro resistance.3,5,7,9 This has led to the assumption 

that this mutation could be important in clinical resistance 

to SP.10 However, many of the studies have been carried out 

with laboratory-adapted strains that may not be representa-

tive of field strains.11 Although, the correlation between the 

Asn-108 mutation and resistance to SP has been observed 

in vitro, several in vivo studies have shown that this mutation 

by itself does not correlate with clinical therapeutic fail-

ure.10,12–15 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.12

Similar to what Plowe and others10 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.12 Giraldo and others previ-

ously observed that in areas from southwestern Colombia 

with low in vivo treatment failure, there was high frequency 

of the Asn-108 mutation.8 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 frequen-

cy 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 pharmaco-

logic characteristics of the drug used.16 Although, a parasite 

may have a pyrimethamine-resistant phenotype, the clinical 

evaluation of SP efficacy provides information on the syn-

ergistic effect of sulfadoxine and pyrimethamine on folate 

metabolism.13 After an oral dose of 25 mg of pyrimethamine, 

the mean ± SD maximum concentration in blood plasma is 

0.94 ± 0.08 μM.17 In vitro studies have reported that pyri-

methamine 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.13 Therefore, although 

the Asn-108 mutation confers a 10-fold reduction in sensi-

tivity, the therapeutic levels obtained with the standard dos-

age used in our study (1.25 mg/kg) could inhibit these par-

asites. In addition, the parasites may still be sulfadoxine sen-

sitive. 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 im-

portant in the clinical presentation of resistance.13

Our results indicate that the presence of the Asn-108 mu-

tation 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 re-

ported a genotypic marker in P. falciparum for in vivo re-

sistance to SP that includes in addition to the Asn-108 mu-

tation 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.14 The differences in the 

genotypic patterns of SP-resistant parasites observed in 

Africa and the Peruvian Amazon may reflect independent gen-

etic changes in malaria parasites of different geographic

sources.15 This is a very important observation since it is 

possible that more than one set of mutations exist that gen-

erate in vivo resistance to SP, but specific patterns may vary 

according to the geographic source of the parasite.

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Authors’ addresses: Norma Ibón Rallón, Lyda Elena Osorio, and 

Luis Ernesto Giraldo, Corporación Centro Internacional de Entren-

amiento e Investigaciones Médicas, Avenida 1 Norte No. 3-03, Cali, 

Colombia.

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