FREQUENCY OF THE ASN-108 AND THR-108 POINT MUTATIONS IN THE DIHYDROFOLATE REDUCTASE GENE IN Plasmodium falciparum FROM SOUTHWEST COLOMBIA

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Abstract. Several point mutations in the dihydrofolate reductase (DHFR) gene of Plasmodium falciparum have been correlated with in vitro anti-folate drug resistance of laboratory and field isolates. Furthermore, two different point mutations that generate amino acid substitutions at the same position of the enzyme have been observed in all the isolates studied to date. These point mutations change a serine (Ser-108) in the wild type to an asparagine (Asn-108 mutation) or to a threonine (Thr-108 mutation). Using the polymerase chain reaction (PCR), it is possible to identify isolates that present these mutations. We used a mutation-specific PCR to screen 71 samples from several geographic locations of Colombia for the Asn-108 mutation (pyrimethamine resistance). In this initial screening 53 of 71 yielded amplification product with the DHFR mutation-specific primers. We further analyzed the 18 samples that did not amplify using a mutation-specific nested PCR. Of those 18 samples, seven amplified with primers specific for the Thr-108 mutation (proguanil resistance), one with the wild type (Ser-108), and 10 did not amplify. Of these 18 samples, three were identified as P. falciparum using a species-specific diagnostic nested PCR base on sequences from the small ribosomal RNA subunit gene. Overall, 51.6% of the samples amplified for the Asn-108 mutation, 10.9% for the Thr-108 mutation, 35.9% with the wild type specific primer, and 4.8% did not amplify with any of the DHFR primers. We observed variability in the frequency of the mutation between the different geographic location. The frequency of the Asn-108 and Thr-108 mutations in the state of Nariño was 25% each, while in Valle del Cauca the frequencies were 59% and 11%, respectively. These results contrast with observations in Brazil in which the Asn-108 mutation was found in 90% of the blood samples screened.

Drug resistance has been considered one of the causes for the resurgence of malaria throughout the world.1 In a recent review, the World Health Organization reported for 1993 an estimate of 300–500 million clinical cases of malaria and 1.5–2.7 million deaths per year.2 In Colombia for 1995, the total number of cases reported by the Ministry of Health were 187,082 cases, of which 33.2% were produced by P. falciparum. Initial cases of chloroquine resistance were first detected in South America and Southeast Asia and this resistance has since spread around the world.3 The combination pyrimethamine/sulfadoxine (Fansidar®; Roche, Basel, Switzerland) has been proposed and is being used as an alternative medication for uncomplicated falciparum malaria resistant to chloroquine.4 In Colombia resistance to Fansidar® was first observed in three cases from the Amazon basin in 1981.5 Several studies conducted in the 1980s in Colombia reported treatment failure to pyrimethamine/sulfadoxine in 25–38% of the cases.6,7 A recent study has found a much smaller frequency (10%) of RI resistance,6 creating the need for studies that can clarify the anti-malarial drug resistance problem in Colombia.

Resistance to antifolate (pyrimethamine and proguanil) drugs is associated with point mutations in the dihydrofolate reductase–thymidylate synthase (DHFR-TS) gene.9,10 This gene codes for an enzyme essential for DNA synthesis by the malaria parasite. The substitution of a serine at position 108 (wild type) by an asparagine (Asn-108 mutation) or a threonine (Thr-108 mutation) have been correlated with in vitro resistance to pyrimethamine and cycloguanil, respectively.11,12 A mutation-specific polymerase chain reaction (PCR) selectively amplifies mutant and wild types by differential amplification of specific primer sequences that contain one nucleotide mismatch in the 3’ end.11-13

We analyzed a group of samples of P. falciparum from southwest Colombia to obtain current information on the frequency of these mutations.

MATERIALS AND METHODS

Study area and sample collection. The study was conducted at the Centro Internacional de Entrenamiento e Investigacione Medicas (CIDEIM) in Cali, Colombia. This project was reviewed and approved by the Committee on Use of Human Subjects (Tulane University) and the Institutional Review Board of CIDEIM. Blood samples were obtained from patients seeking medical attention for malaria symptoms at the three Malaria Control Service clinics in Cali and Buenaventura (Valle del Cauca), Florencia (Caquetà), and the San Andres Hospital, Tumaco in Nariño. Criteria to participate in the study included a positive diagnosis of P. falciparum malaria by thick smear, voluntary participation, and written consent. Demographic data was obtained from the registry form from the malaria clinics. Fifty to 100 microliters of blood obtained by fingerprick were blotted onto filter paper (S&S®, #903; Schleicher and Schuell, Keene, NH) and air-dried. Each sample was individually wrapped in a plastic bag to prevent cross-contamination and stored at room temperature. Additionally, 22 samples previously collected and stored in liquid nitrogen in 50% glycerol were included in the study. A total of 49 filter paper and 22 frozen samples were obtained from different geographic locations. Parasitemia was assessed by counting the number of parasite forms per field in 100 fields and reported as < + = < 2, + = 2–20, ++ + = 20–200, and +++ + = > 200 parasites per field. Laboratory strains of P. falciparum
DHFR POINT MUTATIONS IN P. FALCIPARUM

Polymerase chain reaction (PCR) conditions, primers, and product sizes used in the analysis of 18 samples that did not yield amplification products with the Ser-108 and Asn-108 specific primers*

<table>
<thead>
<tr>
<th>Species-specific nested PCR</th>
<th>Primer sequence</th>
<th>Product size</th>
<th>Reaction conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genus (first reaction)</td>
<td>5'-CTGTGTGTGGCTATAAAGGCTT-3'</td>
<td>1.2 kb</td>
<td>95°C for 5 min, followed by 30 cycles of 94°C for 45 sec, 58°C for 1.5 min, and 72°C for 1.5 min, with a final extension at 72°C for 4 min</td>
</tr>
<tr>
<td>P. falciparum (second reaction)</td>
<td>5'-TTAAATCTGCTGCTGAGAATTATTT-3'</td>
<td>205 bp</td>
<td>The same thermocycler program conditions were used for the second reaction</td>
</tr>
<tr>
<td>P. vivax (second reaction)</td>
<td>5'-ACCAAATGAATCTAATGTGACTCGGCT-3'</td>
<td>120 bp</td>
<td></td>
</tr>
<tr>
<td>P. malariae (second reaction)</td>
<td>5'-AAAATTCCATGATAAAAATAATTACAAA-3'</td>
<td>144 bp</td>
<td></td>
</tr>
<tr>
<td>Mutation-specific nested PCR</td>
<td>Primer sequence</td>
<td>Product size</td>
<td>Reaction conditions</td>
</tr>
<tr>
<td>DHFR gene (first reaction)</td>
<td>5'-TTTATATTCTCTCCCTTTTA-3'</td>
<td>720 bp</td>
<td>94°C for 3 min, followed by 45 cycles of 94°C for 30 sec, 45°C for 45 sec, and 72°C for 45 sec with a final extension at 72°C for 5 min</td>
</tr>
<tr>
<td>Ser-108</td>
<td>5'-GAATGCTTTCCAGC-3'</td>
<td>339 bp</td>
<td>94°C for 3 min, followed by 20 cycles of 94°C for 30 sec, 55°C for 45 sec, and 74°C for 45 sec with a final extension at 74°C for 5 min</td>
</tr>
<tr>
<td>Thr-108</td>
<td>5'-GAATGCTTTCCAGG-3'</td>
<td>1.2 kb</td>
<td>94°C for 5 min, followed by 30 cycles of 94°C for 45 sec, 58°C for 1.5 min, and 72°C for 1.5 min, with a final extension at 72°C for 4 min</td>
</tr>
<tr>
<td>Asn-108</td>
<td>5'-GAATGCTTTCCAGG-3'</td>
<td>205 bp</td>
<td>The same thermocycler program conditions were used for the second reaction</td>
</tr>
<tr>
<td>Common primer (second reaction)</td>
<td>5'-ATGATGGAACAGTGCTGCGC-3'</td>
<td>144 bp</td>
<td></td>
</tr>
</tbody>
</table>

* kb = kilobases; bp = basepair; 5'- five prime end; 3'- three prime end; P. = Plasmodium; DHFR = dihydrofolate reductase.

3D7 (wild type), HB3 (pyrimethamine resistant), and ItG2F6 (proguanil resistant) were used as positive controls.

Extraction of DNA. The DNA from filter papers and frozen blood samples was extracted using a modified saponin/Chlex-100 method. Briefly, half of a 1.2-cm circle of filter paper containing the dried blood sample was cut into small pieces and incubated in 1 ml of 1% saponin (Sigma, St. Louis, MO) on ice for 1–2 hr. Alternatively, 1 ml of a frozen 20% hematocrit blood sample was washed twice with phosphate-buffered saline (PBS), pH 7.2, resuspended in 1 ml of 1% saponin, and incubated on ice for 30 min. After the saponin incubation, all samples were washed twice with PBS and resuspended in 200 µl of 5% Chelex-100 (Sigma). The samples were incubated at 56°C for 15 min and then at 100°C for an additional 10 min. The filter paper was washed with a pipette tip and centrifuged at 12,000 × g for 3 min. The supernatant was transferred into a clean 1.5-ml tube and stored at -20°C. Five to 10 microliters of sample were used for PCR.

Mutation-specific PCR. The mutation-specific PCR was performed according to Peterson and others. Specific reverse primers for the Ser-108 (DIA-3) 5'-GAATGCTTTCCAGC-3' and Asn-108 mutation (DIA-12) 5'-GAATGCTTTCCAGT-3' were used separately with a common forward primer SP1 (5'-ATGATGGAACAGTGCTGCGC-3'). The PCR used a standard 50-µl reaction volume containing 50 mM KCl, 10 mM Tris-HCl, pH 8.5, 2.5 mM MgCl₂, 200 µM dNTP mixture (Gibco, Grand Island, NY), 2.5 U of Taq polymerase (Promega, Madison, WI), and 100 ng of each oligonucleotide. The reaction was hot-started using wax beads. The DNA was amplified with an initial cycle at 94°C for 5 min and continued for 35 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 45 sec, with a final extension at 72°C for 10 min. The PCR products were size-separated by electrophoresis on a 1.5% agarose gel, stained with ethidium bromide (Sigma), and visualized by ultraviolet transillumination. Fragment sizes were determined by comparison with molecular size standards (φX174 digested with Hae III; Stratagene, La Jolla, CA). The quality of the DNA and presence of inhibitors were assessed by the amplification of human β-actin or β-globin DNA.

Eighteen samples that did not amplify with the DIA-3 and DIA-12 primers were further studied using a mutation-specific nested PCR developed by Plowe and others. The first PCR amplified a 720 basepair (bp) fragment from the DHFR gene. An aliquot of this initial reaction was used for the second amplification, which included primers for the Ser-108 (DIA-3), Asn-108 mutation (DIA-12), and Thr-108 (DIA-9) 5'-GAATGCTTTCCAGG-3'. Additionally, these samples were characterized using a species-specific diagnostic nested PCR for Plasmodium species, which included primer sets for P. falciparum, P. vivax, and P. malariae. The reactions were performed as described in Table 1, with the following modifications to the original protocols: reaction mixtures were adjusted to a final volume of 12.5 µl and TaqStart (Clonetech, Palo Alto, CA) was used for the hot-start procedure. The PCR products were electrophoresed and visualized as described above.

RESULTS

A total of 71 samples were analyzed. The patient ages ranged from one to 57 years with a mean of 28 years; there were 49 males and 19 females (three were unknown). In the initial screening, 53 of the 71 samples yielded DHFR amplification products. A typical amplification result is shown in Figure 1. Twenty samples amplified with the primer specific for Ser-108 (wild type) and 31 for Asn-108 (mutant) and two with both primers, indicating a mixed infection (wild type/mutant). A total of 18 samples did not amplify with either of the Ser-108 or Asn-108 primers used. Because these negative results could indicate the presence of the Thr-
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FIGURE 1. Typical amplification result with the mutation-specific polymerase chain reaction. The DNA was extracted using a modified saponin/Chelex method. Amplification products were visualized by gel electrophoresis and stained with ethidium bromide. Samples were run in duplicate with specific primers for the wild type strain (Ser-108) and the Asn-108 mutation. \textbf{Top,} Ser-108 (wild type); \textbf{bottom,} Asn-108 (mutant). MW = molecular size markers; H = negative control (distilled water); 9 = sample that amplify with both primers (wild type and mutant); 3D7 = wild-type positive control; HB3 = Asn-108 mutant positive control. Values on the left are in basepairs.

108 point mutation or a diagnostic error (a different species or a negative sample), they were further analyzed using a more sensitive PCR technique to detect the DHFR mutations and a diagnostic nested PCR to determine the \textit{Plasmodium} species.\textsuperscript{17,18} Seven of the 18 samples yielded amplification product for Thr-108 mutation, one amplified with the Ser-108 primer (wild type), and 10 did not amplify with the DHFR primers. Of these 10 samples, three amplified with specific diagnostic primers for \textit{P. falciparum} (Table 2). The seven samples that did not amplify with the DHFR and species-specific primers, did yield amplification products for human \textit{β}-actin or \textit{β}-globin. There was no relationship between the parasitemia measured by number of parasite forms per microscopic field (100 fields) and the lack of amplification with the specific primers for the DHFR gene or for species.

Of these seven samples, three had between 20 and 200 forms per field, two between 0 and 20, and the other two samples had occasional parasites reported. These seven samples were not included when calculating the frequency of the mutations. Combining all the results (Table 2), the Asn-108 mutation was present in 51.6\% (33 of 64) of the samples, the Thr-108 mutation in 10.9\% (7 of 64), and no mutation was detected in 35.9\% (23 of 64).

Samples were obtained from areas of high-risk malaria transmission in which resistance to chloroquine and pyrimethamine-sulfadoxine has been previously documented.\textsuperscript{5-7} The majority of the samples (27) came from the state of Valle del Cauca, where the research center is located. Fourteen samples came from the state of Nariño, eight from Cauca, seven from Caquetá, seven from four other states, and

\begin{table}[h]
\centering
\begin{tabular}{|l|c|c|c|c|}
\hline
\textbf{Primer} & \textbf{Method DHFR\textsuperscript{14}} & \textbf{Method DHFR\textsuperscript{15}} & \textbf{Species identification method\textsuperscript{16}} & \textbf{\%} \\
 & 71 samples & 181 samples & 101 samples & \\
\hline
\textit{β}-actin/\textit{β}-globin§ & 71 & – & – & 100 \\
Ser-108 & 22\textdagger & 1 & – & 35.9 (23/64) \\
Thr-108 & ND & 7 & – & 10.9 (7/64) \\
Asn-108 & 33\textdagger & 0 & – & 51.6 (33/64) \\
Negative for the DHFR primers & 18 & 10 & – & – \\
Diagnostic primers for \textit{Plasmodium} spp & – & – & 3 (Pl) & 3/10 \\
Negative for the diagnostic primers & – & – & 7\# & – \\
\hline
\end{tabular}
\caption{Summary showing the frequency of the Ser-108 (wild type), Asn-108 mutation and the Thr-108 mutation from Colombian samples of \textit{Plasmodium falciparum} (Pl.) according to the different polymerase chain reaction methods used\textsuperscript{*}}
\end{table}

\textsuperscript{*} DHFR = dihydrofolate reductase; – = not applicable; ND = not done.
\textsuperscript{†} Negative samples from Peterson and others.\textsuperscript{11}
\textsuperscript{§} Internal controls for inhibition.
\textsuperscript{†} Includes two samples that contained wild-type and mutant parasites.
\textsuperscript{§} Were not included as part of the calculation of the frequency of the mutations.
four samples had no origin information. Valle de Cauca and Caquetá had the highest Asn-108 mutation frequency with 59% (16 of 27) and 57% (4 of 7), respectively. Cauca had 37.5% (3 of 8), and Nariño had 25% (3 of 12); the statistical significance was not calculated because of the sample size of some of the groups. Of the nine samples collected in the Municipality of Tumaco in Nariño, no Asn-108 mutation was detected. The Thr-108 mutation was detected in 25% (3 of 12) of the samples from Nariño (including Tumaco), 14% (1 of 7) from Caquetá, 11% (3 of 27) from Valle del Cauca, and none from Cauca.

**DISCUSSION**

Pyrimethamine and proguanil are the only anti-malarial drugs to which a specific mutation in the target enzyme has been associated with *in vitro* resistance, although a similar mechanism has been proposed for sulfadoxine resistance. Clinical resistance to pyrimethamine/sulfadoxine was observed soon after its introduction in 1981, and it is thought to be widespread in Colombia. These observations have limited the use of pyrimethamine/sulfadoxine as a second-level drug in cases of chloroquine-resistant *falciparum* malaria. However, this perception of the frequency of clinical resistance to pyrimethamine/sulfadoxine has been challenged by a recent study that found only 10% RI resistance.

We detected the presence of DHFR point mutations, which have been correlated with *in vitro* resistance to pyrimethamine and proguanil, in a group of *P. falciparum* samples from Colombia. This was possible even in samples with low parasitemia (less than two parasites per field), including samples with only occasional gametocytes. We did not find inhibition with the extraction method used and observed a 90% (64 of 71) agreement of the PCR result with the thick smear results. Similar correlation between PCR/thick smear results have been observed by others, who explained the PCR-negative, microscopy-positive samples as a problem of inaccuracy of the thick smear.

Overall, we detected 51.6% Asn-108 mutations and 10.9% Thr-108 mutations. Our results show a higher frequency of the mutation than the treatment failure reported in Colombia during the 1980s. A similar study in Brazil observed a 90% frequency of the Asn-108 mutation in *P. falciparum*. Although a direct correlation could not be made, the investigators suggested that their results were comparable with clinical resistance observed in previous clinical trials (63–92% treatment failures) performed in the same general geographic location. We observed variability in the frequency of the Asn-108 and Thr-108 mutations depending on the origin of the infection. The apparent variability of clinical resistance observed in the clinical trials conducted more than a decade ago, and in the results from our work with the mutation-specific PCR may be due to several factors including 1) a difference in sensitivity of the parasite population circulating in the different geographic locations, 2) the presence of other point mutations (Ile-51 and Arg-59) that modulated the level of *in vitro* resistance, and 3) variability of the exposure to the selective pressure of the drug. The results of previous clinical trials and the present study suggest that clinical resistance and the frequency of the point mutations in the DHFR gene may be a focal problem.
REFERENCES