

# Status of *dhps* and *dhfr* genes of *Plasmodium falciparum* in Colombia before artemisinin based treatment policy

## Estado de los genes *dhps* y *dhfr* de *Plasmodium falciparum* en Colombia antes de la recomendación de tratamiento basado en artemisinina

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### Abstract

**Introduction:** Surveillance of the genetic characteristics of *dhps* and *dhfr* can be useful to outline guidelines for application of intermittent preventive therapy in Northwest Colombia and to define the future use of antifolates in artemisinin-based combination therapy schemes.

**Objective:** To evaluate the frequency of mutations in *dhps* and *dhfr* and to characterize parasite populations using *msp-1*, *msp-2* and *glurp* in historic samples before artemisinin-based therapy was implemented in the country.

**Methods:** A controlled clinical study was carried out on randomly selected *Plasmodium falciparum* infected volunteers of Northwest Colombia (Turbo and Zaragoza). A sample size of 25 subjects per region was calculated. Treatment efficacy to antifolates was assessed. Molecular analyses included *P. falciparum* genotypes by *msp-1*, *msp-2* and *glurp* and evaluation of the status of codons 16, 51, 59, 108 and 164 of *dhfr* and 436, 437, 540, 581 and 613 of *dhps*.

**Results:** In total 78 subjects were recruited. A maximum number of 4 genotypes were detected by *msp-1*, *msp-2* and *glurp*. Codons 16, 59 and 164 of the *dhfr* gene exhibited the wild-type form, while codons 51 and 108 were mutant. In the *dhps* gene, the mutant 437 glycine was detected in 85% on day 0, while codons 436, 540, 581 and 613 were wild-type.

**Conclusions:** *Plasmodium falciparum* populations were very homogeneous in this region of Colombia, and the triple mutants of *dhfr* and *dhps* Asn108, Ile51 and Gly437 were predominant in clinical isolates.

**Keywords:** *Plasmodium falciparum*, malaria, *dhps*, *dhfr*, resistance, sulphadoxine, Colombia

### Resumen

**Introducción.** La vigilancia de las características genéticas de *dhps* y *dhfr* puede utilizarse para delinear guías de aplicación de terapia preventiva intermitente en el nordeste de Colombia y para definir el uso futuro de los antifolatos en esquemas terapéuticos basados en artemisinina.

**Objetivo.** Evaluar la frecuencia de mutaciones en *dhps* y *dhfr*, y caracterizar las poblaciones parasitarias usando *msp-1*, *msp-2* y *glurp*, en muestras históricas obtenidas antes de la implementación en el país de la terapia basada en artemisinina.

**Métodos.** Se llevó a cabo un estudio clínico controlado en voluntarios infectados con *Plasmodium falciparum* seleccionados aleatoriamente y provenientes del nordeste de Colombia (Turbo y Zaragoza). Se calculó una muestra de 25 sujetos por región. Se evaluó la eficacia al tratamiento con antifolatos. Los análisis moleculares incluyeron la obtención de genotipos de *msp-1*, *msp-2* y *glurp* y el estado de los codones 16, 51, 59, 108 y 164 de *dhfr*, y 436, 437, 5540, 581 y 613 de *dhps*.

**Resultados.** Se estudiaron 78 sujetos. Se detectó un número máximo de 4 genotipos con *msp-1*, *msp-2* y *glurp*. Los codones 16, 59 y 164 del gen *dhfr* se encontraron en su forma silvestre, mientras que los codones 51 y 108 estaban mutados. En el gen *dhps*, la forma mutante (glicina) en el codón 437, se detectó en 85% el día 0, mientras que los codones 436, 540, 581 y 613 se encontraron silvestres.

**Conclusiones.** Las poblaciones de *P. falciparum* son muy homogéneas en esta región de Colombia y las triple mutantes de *dhfr* y *dhps* Asn108, Ile51 and Gly437, predominaron en los aislamientos clínicos.

**Palabras clave:** *Plasmodium falciparum*, malaria, *dhps*, *dhfr*, resistencia, sulfadoxina, Colombia.

### Introduction

During the past years, several studies in Colombia on the efficacy of different drugs against *Plasmodium falciparum* malaria, including sulphado-

xine/pyrimethamine (SP) have been published<sup>(1-4)</sup>. A review in 2005 of 15 studies in the country confirmed a mean frequency of treatment failure of 14.6%<sup>(2)</sup>. In the northwest region, in Urabá and Bajo Cauca, the reported frequency of

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treatment failure was 24%<sup>(5)</sup>. However, studies on the molecular epidemiology of antimalarial resistance are still scarce in the country<sup>(4, 6-11)</sup>. These studies constitute a useful tool to understand the events underlying treatment failure and resistance to antifolates<sup>(12)</sup>. The exploration of the genetic background of clinical isolates circulating in a specific region is of great relevance, since the efficacy and emergence of resistant phenotypes to new drug regimens might be affected by the baseline genetic characteristics of the local parasite strains<sup>(13)</sup>.

Currently, most intermittent preventive therapies (IPT) are based on the administration of antifolates, since they are relatively safe and exhibit good cost-efficacy<sup>(14)</sup>.

Resistance to antifolates has been attributed to mutations in the genes coding the enzymes involved in the metabolism of sulphadoxine and pyrimethamine: dihydrofolate reductase (*dhfr*) and dihydropteroate synthase (*dhps*), respectively. Point mutations at positions 51, 59, 108 and 164 in the *dhfr* gene<sup>(15-17)</sup> and mutations at positions 436, 437, 540, 581, and 613 in the *dhps* gene, have been directly implicated in such resistance<sup>(18, 19)</sup>.

We conducted a study in the northwest region of Colombia, aimed at understanding the genetic characteristics of SP resistance in field isolates and to complement the framework in which a new artemisinin-based combination therapy (ACT) is administered in the country. The study explored the frequency of mutations in the *dhps* and *dhfr* genes as well as the general constitution of parasite populations by assessing the *msp-1*, *msp-2* and *glurp* genes. Surveillance of the genetic characteristics of these genes can be useful to outline IPT guidelines for application in the region and to define the future use of SP in an ACT scheme.

## Materials and methods

We conducted a controlled clinical study on *P. falciparum* infected subjects. Randomly selected volunteers were part of a larger study which ai-

med at studying the efficacy of different antimalarial regimens.

### Study area

The study was carried out between October 2002 and July 2004 in two malaria endemic regions in Colombia: Turbo (8°5'42"N, 76°44'123"W) and Zaragoza (7°29'38"N, 74°52'15"W). Both regions have malaria transmission throughout the year with mean annual parasite indexes (API) (cases/1000 inhabitants) during 2002-2004 of 45.3 in Turbo, and 111.6 in Zaragoza (20, 21).

### Malaria patient enrollment and follow-up

The study included patients attending the local malaria clinics with acute symptomatic non-severe *P. falciparum* malaria. Unique infection by this species was confirmed by microscopy and by a semi-nested PCR<sup>(22, 23)</sup>. The study protocol was reviewed and approved by the Ethics Committee of the Facultad de Medicina, Universidad de Antioquia (Medellín, Colombia). Each participant gave a fully informed consent. The inclusion criteria for the original study were  $\geq 1$  years age, unique *P. falciparum* parasitaemia with  $\geq 1000$  asexual forms/ $\mu$ l and willingness to participate. Patients were excluded if consent was withdrawn.

A sample size for each municipality was calculated based on the number of *P. falciparum* malaria cases in a year: 1061<sup>(24)</sup>, 11% frequency of treatment failure<sup>(25)</sup>, a 95% confidence interval and sample error of 12%; this resulted in 25 subjects per region. Patients were randomly assigned one of various different antimalarial regimens. Subjects selected for this report were administered a single dose of sulfadoxine/pyrimethamine 25 mg/kg. All patients were treated at the local malaria clinic and the follow up period was at least 21 days. Treatment response was monitored according to the 1998 WHO criteria and classification into adequate treatment response (ATR) or treatment failure (early, ETF; and late, LTF) was established<sup>(26)</sup>.

### Sample collection

Whole blood for molecular analysis was taken from a peripheral vein on day 0 before treatment administration and when treatment failure was diagnosed. Samples were collected onto Whatman 3MM filter paper and stored at -20°C. DNA was extracted with Chelex<sup>®</sup>, according to standard procedures<sup>(27)</sup> and stored at -20°C until use.

### *Plasmodium falciparum* confirmation and allele genotyping

DNA templates were amplified to confirm the unique infection by *P. falciparum*. A semi-nested PCR was performed at the Laboratorio de Referencia de Malaria, Departamento de Parasitología, Centro Nacional de Microbiología, Instituto Nacional de Salud Carlos III in Madrid, according to Rubio, *et al.*<sup>(23)</sup>.

Genotyping of *msp-1*, *msp-2* and *glurp* genes was performed in order to establish the genotype and the presence of polyclonal infections. For this, a nested PCR was carried out according to published protocols<sup>(28, 29)</sup>. Products were resolved in 2% agarose and fragment size was determined under UV light after ethidium bromide staining.

### Identification of point mutations in *dhps* and *dhfr* genes

DNA templates were evaluated for the presence of point mutations in *dhfr* using a PCR-RFLP assay according to Duraisingh, *et al.*<sup>(30)</sup>. A first round of amplification was followed by two additional separate PCR reactions resulting in a 522bp segment flanking codons 16, 51 and 164, and a 326bp segment flanking codons 59 and 108. Further confirmation of the status of *dhfr* was performed in 50% of the samples following the protocol published, in which mutation-specific nested PCR and/or restriction digestions were applied, as described elsewhere<sup>(17, 31, 32)</sup>. Meanwhile, the *dhps* gene was assessed using a nested PCR approach. A detailed description of

these methods is available at <http://medschool.umaryland.edu/CVD/plowe.html>.

Laboratory maintained 3D7 and Dd2 strains of *P. falciparum* were used as controls. Products were resolved in 2-3% agarose, and fragment size was determined under UV light after ethidium bromide staining.

### Statistical analyses

The data were processed using EpiInfo 6.04. The frequency of each allele was calculated, based on *msp-1*, *msp-2* and *glurp* genes. In addition, frequencies of mutations in the different codons of *dhps* and *dhfr* were determined. Polyclonal samples were included in the analysis, since *in vivo* interactions of different clones might exhibit a trend or association. However, for statistical purposes, cases of polyclonal infections were considered each as individual samples according to the number of alleles detected.

### Results

A total 78 subjects were recruited, 44 in Turbo and 34 in Zaragoza. Baseline characteristics of the population were similar between the two municipalities. Among the variables associated to acquisition of previous immunity which might affect *in vivo* efficacy to treatment, we highlight the following: the mean age was 23; the mean number of malaria episodes within the previous year of recruitment was 2.5; the mean number of days with symptoms during the current episode was 4.5; and the mean parasitaemia was 4964 parasites/ $\mu$ l.

*In vivo* response to antifolates confirmed a pondered mean treatment failure frequency of 24% in the two localities. The frequency of treatment failure to sulphadoxine-pyrimetamine was 20% (9/44) in Turbo, and 26% (9/34) in Zaragoza. Among the cases diagnosed as clinical failure in Turbo, 44% (4/9) had early treatment failure (ETF) and 66% (5/9) were late treatment failure

(LTF). In Zaragoza, ETF cases were 77% (7/9), and LTF were 33% (2/9) of the total failure cases in the locality.

### *Plasmodium falciparum* genotyping

A predominance of the 150bp allele of the MAD20 family of *msp-1* both in Turbo and Zaragoza, was observed. Similar results were detected in samples obtained at treatment failure (ETF and LTF). For *msp-2*, the most frequent allele observed in the two localities was a 500bp corresponding to IC-1 (61% in Turbo, 71% in Zaragoza) followed by a 550bp (23% in Turbo, 15% in Zaragoza). The proportion of polyclonal infections according to this gene was 5% in Turbo.

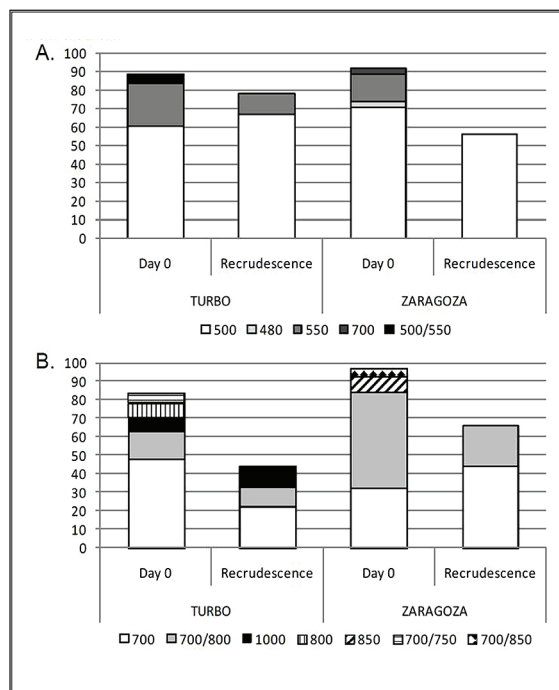
In samples obtained at treatment failure (ETF and LTF), the most commonly detected alleles also corresponded to the most common detected on day 0 (Figure 1).

Predominance of the 700bp allele of *glurp* was detected in both localities (48% in Turbo, 32% in Zaragoza) regardless of the time of collection of the sample (day 0 or recrudescence). However, the majority of infections in Zaragoza were polyclonal (700bp/800bp) on day 0 according to this marker (Figure 1). Details on the base pair size range of the different genes and the number of genotypes detected in the two localities according to the amplified genes are provided in Table 1.

No relationship could be confirmed between the degree of malarial endemicity reported at the time of sample collection in the two regions, and the number of alleles found ( $p \geq 0.05$ ,  $\chi^2$  test).

### *Analysis of dhfr and dhps*

In both localities, amplification of the *dhfr* gene confirmed the replacement of wild populations by mutant forms for codons 51 and 108, and the persistence of wild-type forms for codons 16, 59 and 164, in all isolates (Table 2).



**Figure 1.** Percentage of the different size fragments obtained after amplification of *msp-2* (IC family) (panel A) and *glurp* (panel B) in the two endemic localities according to time of collection of the sample: day 0 (in all samples), and day of recrudescence. A product of amplification was not observed in some cases. Polyclonal infections were detected in the two localities.

Codons 436, 540, 581, and 613 of the *dhps* gene were detected in the wild-type form in all samples (Table 2). As for codon 437, a high proportion (mean 85.2%) of the isolates exhibited the mutant glycine instead of alanine (wild-type) on day 0 and all isolates from recrudescence had the mutant form of the gene. Separate analysis of the distribution frequency of this mutant by locality confirmed similar proportions, with 81% in Turbo and 90% in Zaragoza.

## Discussion

Among the factors affecting the *in vivo* response to antimalarials, the status of the genes involved in their metabolism and the presence of antimalarial acquired immunity of the host, are of great importance<sup>(33)</sup>. The present study confirmed treatment failure in 23% of the subjects, regardless of the presence of more than one mutation in the genes involved in resistance to antimalarials. However, specifically designed studies should address the role of specific acquired immunity

**Table 1.** Distribution of frequencies of *Plasmodium falciparum* genotypes according to analysis of *msh-1*, *msh-2* and *glurp* and time of sample collection (day 0 and day of recrudescence).

|   | <i>msh-1</i> MAD-20 <sup>†</sup> |                      | <i>msh-2</i> IC <sup>†</sup> |                      | <i>glurp</i> |                      |
|---|----------------------------------|----------------------|------------------------------|----------------------|--------------|----------------------|
|   | Day 0                            | Day of recrudescence | Day 0                        | Day of recrudescence | Day 0        | Day of recrudescence |
| Base pair range                                 | 150                              | 150                  | 480-700                      | 500-550              | 700-1000     | 700-1000             |
| Number of genotypes*                            | 1/1                              | 1/1                  | 2/4                          | 2/4                  | 3/2          | 3/2                  |
| Total number of genotypes                       | 1                                | 1                    | 4                            | 2                    | 5            | 3                    |
| Proportion (%) of polyclonal infection*         | 0/0                              | 0/0                  | 0/5                          | 0/0                  | 20/56        | 11/22                |
| Proportion (%) of samples lacking amplification | 1,2                              | 6,25                 | 8,9                          | 18,7                 | 6,4          | 37,5                 |

\* Turbo/Zaragoza

† Frequencies of amplification for the *msh-1* K1 and RO33 allelic families and *msh-2* FC27 were null.

in clearance of parasites harboring multiple mutations in *dhps* and *dhfr* in Colombian isolates.

The present study confirmed the homogeneity of *P. falciparum* populations according to *msh-1*, *msh-2* and *glurp*, in these regions of Colombia. Previous studies carried out some years before (2000-2001) in the same localities reported similar findings<sup>(34)</sup>. However, some differences were observed in the number of alleles detected for *glurp* and, more significantly, in the frequency of polyclonal infections, which was substantially higher in Turbo (15% vs. 0.2%) and in Zaragoza (53% vs. 0%). Based on these results, we can conclude that the analysis of *glurp* might be useful for genotyping parasite populations in new studies in Colombia aimed at exploring parasite population dynamics and *in vivo* efficacy of antimalarials. Analysis of *msh-1* and *msh-2* can also be of value to compare with past studies, but not when the objective is to compare parasites obtained from the same patient, for instance, during treatment efficacy studies. Sequencing analysis of these genes in Colombian samples might prove useful to understand population dynamics in different regions of the country and to confirm the homogeneity of the populations. However, special care should be taken when selecting particular regions of the genes to be monitored in order to avoid lack of amplification (due to significant polymorphism), particularly in the case of *glurp*.

A small number of studies on the characterization of *dhps* and *dhfr* have been carried out in

Colombia (Table 2). A comparison of reports on mutations in *dhps* and *dhfr*, regardless of the region where the studies were carried out, revealed the increasing presence of mutant alleles for codons 51 and 108 of *dhfr*, and for codon 437 of *dhps*. Association of the triple mutants with treatment failure has been reported by several authors from different regions<sup>(25, 35-38)</sup>. This might explain the frequency of treatment failure to SP observed in this particular group of patients: 20% in Turbo and 26% in Zaragoza. The comparison of samples from day 0 and day of failure was not useful to confirm a difference in the *dhps* and *dhfr* genotypes, probably as a consequence of the reduced number of samples obtained at treatment failure and the high frequency of mutant codons within the population of parasites. However, selection of mutant strains was confirmed for codon 437 of *dhps* during recrudescence. Since treatment failure rates in the region have also been reported as very high by other authors<sup>(2)</sup>, clinical studies on the efficacy of SP are probably not worthwhile in these localities. On the contrary, following up the trend of mutations in these genes should be performed and must include more precise methods, *i.e.* quantitative PCR or sequencing, as to define the frequency of mutants within a mixture of clones in naturally infected individuals.

The change to artesunate plus mefloquine in Northwest Colombia, or arthemether plus lumefantrine in other regions<sup>(39, 40)</sup> as the first line choice between 2006-2009 and to arthemether plus lumefantrine in all the country after 2009,

**Table 2.** Status of the *dhps* and *dhfr* genes in Colombia and comparison between the different reports.

| Gene        | Codon          | Aminoacid<br>(wild/mutant) | Southwest <sup>[7]</sup> | West <sup>[8]</sup> | Northwest <sup>[10]</sup> | Northwest/<br>East <sup>[11]</sup> | Southwest <sup>[1]</sup> | Northwest/<br>East <sup>[44]</sup> | Northwest |
|-------------|----------------|----------------------------|--------------------------|---------------------|---------------------------|------------------------------------|--------------------------|------------------------------------|-----------|
|             |                |                            | (n=51)                   | (n=48)              | (n=13-19)                 | (n=123)                            | (n=153)                  | (n=40)                             | (n=78)    |
| <i>Dhfr</i> | 16             | Alanine (w)                |                          |                     | 100                       |                                    |                          |                                    | 100       |
|             | 51             | Asparagine (w)             |                          |                     | 79                        |                                    | 41                       | 0                                  | 0         |
|             |                | Isoleucine (m)             |                          |                     | 21                        |                                    | 59                       | 100                                | 100       |
|             | 59             | Cysteine (w)               |                          |                     | 84                        |                                    | 100                      | 100                                | 100       |
|             |                | Arginine (m)               |                          |                     | 16                        |                                    | 0                        | 0                                  | 0         |
|             | 108            | Serine (w)                 | 39                       | 23                  | 37                        | 34                                 | 14                       | 0                                  | 0         |
|             |                | Asparagine (m)             | 61                       | 75                  | 63                        | 100                                | 86                       | 100                                | 100       |
|             |                | Threonine (m)              |                          | 2                   |                           | 3                                  |                          | 0                                  | 0         |
| 164         | Isoleucine (w) |                            |                          | 100                 |                           | 100                                | 100                      | 100                                |           |
| <i>Dhps</i> | 436            | Serine (w)                 |                          |                     | 100                       |                                    |                          | 100                                | 100       |
|             | 437            | Alanine (w)                |                          |                     | 77                        |                                    | 90                       | 7                                  | 15        |
|             |                | Glycine (m)                |                          |                     | 23                        |                                    | 10                       | 92                                 | 85        |
|             | 540            | Lysine (w)                 |                          |                     | 100                       |                                    | 100                      | 95                                 | 100       |
|             |                | Glutamic acid (m)          |                          |                     |                           |                                    | 0                        | 5                                  | 0         |
|             | 581            | Alanine (w)                |                          |                     | 100                       |                                    | 100                      |                                    | 100       |
| 613         | Alanine (w)    |                            |                          | 100                 |                           |                                    |                          | 100                                |           |

Regions from which the samples were studied are detailed and the number (n) included.

Results represent percentage <sup>a</sup>.

Results for the current study after analysis on day of recruitment are presented in the last column.

<sup>a</sup> Mutant codons are presented in bold.

Blank cell means no information is available, w means wild type, m means mutant.

might provide a different scenario for the evolution of mutations in *dhps* and *dhfr*. However, regional health authorities have irregularly supplied ACT and, in some localities, patients are being administered amodiaquine plus SP, the regimen applied before 2006, or they self-medicate with other antimalarials, such as halofantrine, purchased in the black market. Future studies should address the significance of this lack of delivery of the recommended antimalarials on the presence of *dhps* and *dhfr* mutations and on other resistance associated genes.

Several authors have reported on the synergy of >1 mutations in *dhps* and *dhfr* with the *in vitro* efficacy of antifolates, whereby the number of mutations is directly proportional to *in vitro* resistance or early treatment failure <sup>(41-43)</sup>. However, such findings have been controversial, with some authors reporting lack of association <sup>(44, 45)</sup>. In any case, the

trend of accumulation of mutations in samples from Colombia associated to the lack of *in vivo* response reported by other authors can be useful to establish an association between these phenomena, at least for this part of the country.

In conclusion, the results suggested homogeneity of *P. falciparum* populations according to *msp-1*, *msp-2* and *glurp*, in these regions of Northwest Colombia and the triple mutants of *dhfr* and *dhps* Asn108, Ile51 and Gly437, are highly frequent in the clinical isolates of these endemic localities.

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