Biomédica 2008;28:523-30

ARTÍCULO ORIGINAL

Plasmodium falciparum: high frequency of *pfcrt* point mutations and emergence of new mutant haplotypes in Colombia

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Introduction. Studies on the molecular epidemiology of antimalarial resistance constitute a useful tool to understand the events underlying treatment failure and resistance in falciparum malaria in Colombia. Several authors have reported on the efficacy of some molecular markers to predict drug resistance *in Plasmodium falciparum*. The *P. falciparum pfcrt* gene has been widely characterized in this context.
Objective. The frequency of *pfcrt* gene mutations in *P. falciparum* were associated with treatment failure to the antimalarials chloroquine, mefloquine, amodiaquine and sulfadoxine/pyrimethamine.
Materials and methods. A representative sample of 172 patients with non-complicated falciparum malaria was selected from two highly malaria-endemic areas of northeastern Colombia, the Turbo and Bajo Cauca regions. These patients were assessed for treatment response together with the status of codons 72, 74, 75 and 76 in the *pfcrt* gene using a PCR-RFLP approach.
Results. A high frequency of treatment failure to chloroquine (82%) and to amodiaquine (29%) was confirmed, whereas mefloquine and combined therapy remained effective. The presence of the T76 mutation in *pfcrt* was confirmed in all samples. The most common haplotype was CMNT (67%).
Conclusions. No significant association was confirmed between specific haplotypes and the treatment response in any of the treatment groups. Two haplotypes, SMET and SMNT, were reported for the first time in Colombia. Twelve percent of the samples carried both mixed mutant and wild-type alleles.

Key words: *Plasmodium falciparum*, malaria, antimalarials, chloroquine, mutation, Colombia.

Alta frecuencia de mutaciones puntuales en *pfcrt* de *Plasmodium falciparum* y emergencia de nuevos haplotipos mutantes en Colombia

Introducción. Los estudios en epidemiología molecular de resistencia a antipalúdicos constituyen una herramienta útil para comprender eventos involucrados en la falla al tratamiento y la resistencia en paludismo por *Plasmodium falciparum* en Colombia. Diversos autores han informado sobre la eficacia de algunos marcadores moleculares para predecir resistencia a fármacos en *P. falciparum* y el gen *pfcrt* ha sido ampliamente caracterizado en este contexto.
Objetivo. Estudiar la frecuencia de mutaciones en el gen *pfcrt* de *P. falciparum* y su asociación con falla al tratamiento con cloroquina, mefloquina, amodiaquina y sulfadoxina/pirimetamina, en dos regiones muy endémicas para paludismo del noroeste de Colombia: Turbo y Bajo Cauca.
Materiales y métodos. Una muestra representativa de pacientes con paludismo por *P. falciparum* no complicado fue seleccionada de cada localidad para la evaluación de la respuesta al tratamiento y la determinación del estado de los codones 72, 74, 75 y 76 de *pfcrt*, usando una aproximación basada en PCR-RFLP.
Resultados. Se confirmó una alta frecuencia de falla al tratamiento con cloroquina (82%) y amodiaquina (29%), mientras que la mefloquina y la terapia combinada fueron eficaces para eliminar la infección. La presencia de la mutación T76 en *pfcrt* fue confirmada en todas las 172 muestras; el haplotipo más común fue CMNT (67%).

Conclusiones. No se observó asociación significativa entre un haplotipo particular y la respuesta al tratamiento en cualquiera de los grupos. Se reporta por primera vez en Colombia la presencia de dos haplotipos, SMET y SMNT; se encontraron alelos mutantes y silvestres simultáneamente en 12% de las muestras.

Palabras clave: *Plasmodium falciparum*, malaria, antimaláricos, cloroquina, Colombia, mutación.

Plasmodium falciparum malaria affects approximately 300 million people worldwide and claims an estimated 1.5 million lives every year. Without a fully protective vaccine, chemotherapy is the only effective treatment of the disease. In many parts of the world, the *P. falciparum* parasite has become resistant to most drugs (1,2), undermining the efforts for malaria control.

Traditionally, chloroquine (CQ) has been the drug of choice for the treatment of malaria. However, CQ-resistant parasites are now present in most areas where malaria is endemic (3). In Colombia, treatment failure to CQ was detected as early as 1960, and the number of resistant cases continued to rise. Consequently, the decline in the efficacy of CQ as monotherapy led to the use of combined therapy with other antimalarials, such as antifolates; however, parasite resistance to these regimens was also appearing. Studies on efficacy of amodiaquine (AQ) have discovered associated parasite mutations, such as the CQ resistance transporter *(pfcrt*) gene. They concluded that selection by drugs on the parasite population was the probable explanation for the presence of mutations in parasites of recurrent cases after treatment with the antimalarial (4,5). In the case of mefloquine (MQ), the evidence suggested an association between mutations on the codon 76 of the *pfcrt* gene and reduced susceptibility to MQ (6,7).

Currently, the national Colombian health authorities are monitoring the efficacy of alternative drugs, either administered alone or as combined therapy, to develop policy recommendations for adequate treatment regimens. Studies on the molecular epidemiology of antimalarial resistance constitute a useful tool to understand the events underlying treatment failure and resistance in falciparum malaria in Colombia.

Certain molecular markers predict drug resistance in *P. falciparum*. CQ resistance is conferred by mutations in the *pfcrt* gene; this gene encodes a putative transporter localized in the digestive vacuole (8,9). The substitution of lysine (K) for threonine (T) at amino acid 76 (K76T) in the pfcrt protein appears to be a primary genetic mechanism conferring resistance to CQ (10). This codon has been considered as a highly reliable genetic marker for the monitoring CQ resistance (11). In African or Southeast Asian isolates, the K76T mutation in *pfcrt* does not occur alone, but is accompanied by mutations in other codons (74, 75, 220, 271, 326, 356 and 371). South American isolates may carry, besides K76T, mutations in codons 72, 74, 75, and 220 (12,13).

In the current study, the frequency of mutations in the *pfcrt* gene was determined. The presence of each mutation was associated with treatment failure to the antimalarials CQ, MQ, AQ as monotherapy as well as AQ plus sulfadoxine/pyrimethamine (SP) therapy.

Materials and methods

Patients and blood samples

The study was conducted from 2002 to 2004 in patients living in three counties: Turbo (8°5'42" N, 76°44'123" W), El Bagre (7°35'25" N, 74°48'27" W), Zaragoza (7°29'24"N, 74°51'35"W) in two regions of northwestern Colombia where malaria is highly endemic: Urabá and Bajo Cauca which are mainly inhabited by descendants from African and indigenous ancestors, as well as mixed indigenous and Spanish ancestry. Subjects were residents in townships located within a 40

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Recibido: 18/10/07; aceptado:14/08/08

km radius from the urban area of the county. The economy of Bajo Cauca is based on gold mining, whereas Urabá is a banana-producing region. Both regions have high migration rates and malaria transmission is perennial and unstable with mean annual parasites indices of 19.6 per 1,000 in Turbo and 37.4 per 1,000 in El Bagre (data of 2002-2004). Because the research reported herein was conducted before 2007, it was not recorded in the WHO and ICMJE (International Committee of Medical Journal Editors) databases for clinical studies.

The reference population consisted of patients aged ≥1 year, with a history of fever and symptoms compatible with malaria. A single infection with *P. falciparum* was confirmed by thick smear. The patients had no treatment with CQ, SP or AQ treatment within the past 3 days and no history of MQ intake during the previous 4 weeks. The patients were visitors at the malaria diagnosis clinic at a local hospital. The minimum required sample size (n) was calculated by the following equation: n=NZ2p(1-p)/(Ne2)+{Z2p(1-p)}, where N was the number of *P. falciparum* malaria cases in each locality, Z was the confidence level (for 0.95, Z = 1.96), p was the proportion or probability of *P. falciparum* genetic variation in the region, 1-p was the probability that the genetic variation was not real, and e was the sample error (13.3%). Based on this calculation, a minimum sample of 50 patients was chosen from each community.

Patients were included in the study if they were males or non-pregnant females (confirmed by a rapid pregnancy test), living at their normal residence in the rural or urban zone of their municipality. The inclusion criteria were the following: (a) to have non-complicated malaria (confirmed by clinical and microscopy findings); (b) to harbor between 250 and 50,000 asexual parasites/ml; (c) to be free of a concomitant disease (based on a clinical interview and examination by the team's physician); (d) to voluntarily participate in the study; and (e) to commit to attendance at the follow-up examinations. The exclusion criteria were the following: (a) evidence of severe malaria or other disease; (b) indication of adverse effect of antimalarial drugs or other medicationss; or (c) failure to attend any of the first three follow-up examinations (days 1, 2, 3). Evidence of treatment failure to due to antimalarial therapy resulted in withdrawal of the patient from the study. Recommendations for a rescue treatment was formulated according to the guidelines for malaria treatment provided by the Colombian Ministry of Health (i.e., quinine and clyndamycin treatment).

Each patient (or their parents) gave fully informed written consent. Ethical clearance was granted by the Ethics Committee of the Centro de Investigaciones Medicas, Facultad de Medicina, Universidad de Antioquia (Medellin, Colombia).

Patients were randomly assigned to one of the following treatments: (1) chloroquine, 25 mg/kg body weight for 3 days; (2) amodiaquine, 25 mg/kg body weight for 3 days; (3) mefloquine, 15 mg/kg body weight for 1 day; or(4) amodiaquine, 25 mg/kg body weight for 3 days, plus a single dose of sulfadoxine/pyrimethamine 25/1 mg/kg. All patients were administered treatment at the local malaria clinic; the follow up period was at least 21 days. Treatment response was assessed according to the 1998 WHO protocol for *in vivo* efficacy studies of antimalarial drugs, and the treatment outcomes were classified either as an adequate response to treatment or as treatment failure (14).

Peripheral blood parasitaemia was determined from fingerprick blood. Giemsa-stained thick/thin blood films were examined microscopically to identify the *Plasmodium* species, parasite density, and schizontaemia. Parasite density was measured by counting the number of asexual parasites per 200 leukocytes, based on a mean count of 8,000 leukocytes per microliter of blood. A slide was considered negative after examination of microscopic fields corresponding to at least 500 leukocytes.

Molecular analyses

Blood for molecular analyses was taken on day 0 and on the day of treatment failure. Whole blood was collected on Whatman 3M filter paper and stored at -20° C until DNA extraction with Chelex®, according to standard procedures (15). DNA templates were stored at -20° C until use. Polymorphisms of codons 72, 74, 75 and 76 of the *pfcrt* gene were analysed by nested PCR as

described by Djimde *et al.* (11). The product size was assessed by polyacrylamide (13%) gel electrophoresis. The results were interpreted as follows: *pfcrt* allele 72 (C72 wild, S72 mutant); 74 (M74 wild, I74 mutant); 75 (N75 wild, E75 mutant); and 76 (K76 wild, T76 mutant). Controls included the reference *P. falciparum* strains 3D7, Dd2 and Hb3.

Statistical analysis

The data obtained were processed using EpiInfo 6.04. The frequency of each variable was calculated, and the association between treatment response and the presence of mutant alleles was tested by j^2 test. The level of significance was set at $p<0.05$.

Results

In vivo evaluation of the efficacy of antimalarials

In total, 172 patients were included in the study—91 from Turbo and 81 from Bajo Cauca (Table 1). The geometric mean parasite density was 38,858 asexual parasites/ml. A high number of patients evidenced signs of severe malaria after being allocated to groups 1 or 2. In such cases, patints were excluded from the study and offered antimalarial treatment for severe malaria.

The overall proportion of treatment failure was 30%, with CQ showing the highest proportion (82%). The distribution of patients according to the respective treatment and the rates of success are shown in Table 1.

pfcrt genotypes

Genetic characterization of *pfcrt* was performed on 153 blood samples collected on the day before treatment and on 19 post-treatment samples from patients who presented with treatment failure. The 19 post-treatment failures were distributed among the treatment groups as follows: 6 in the CQ group, 8 in AQ , 4 in MQ and 1 in AQ-SP. The key codons of the *pfcrt* gene were analyzed in 172 DNA samples.

The mutant allele T76 in the *pfcrt* gene was found in all samples (100%). Infection with a single haplotype was observed in 88% of the samples; infections with two haplotypes was detected in 12% of the samples. No significant association was detected between a particular haplotype and the treatment response in any of the groups. Similarly, selection for a specific haplotype with any of the antimalarials was not observed (Table 2).

The most common haplotype was CMNT, observed in 67% of the samples. The distribution of the haplotype patterns is shown in table 2. No significant association between a particular haplotype and the origin of the sample was observed.

Discussion

In Colombia, the recommended treatment regimen for uncomplicated falciparum malaria has been AQ (25 mg/kg in 3 days) and SP (25 mg/kg–1 mg/kg. This scheme was used throughout the country fpr the past decade, until 2006. However, the AQ-SP regime had been adopted much earlier in the Turbo-

Table 1. Distribution of patients according to treatment group, place of residence (locality) and percentages of patients with treatment failure regardless of the locality.

Treatment	Locality	Number of patients recruited	Number of patients with treatment failure	Patients with treatment failure (%)
Chloroquine	Bajo Cauca	9	7	82
	Turbo	8	7	
Amodiaquine	Bajo Cauca	13	5	29
	Turbo	21	5	
Mefloquine	Bajo Cauca	33	3	5
	Turbo	40	1	
Amodiaquine-sulfadoxine-pyrimethamine	Bajo Cauca	26	1	2
	Turbo	22	0	

Table 2. Distribution of haplotypes of *pfcrt* codons 72, 74, 75 and 76 according to treatment group and treatment response in 153 isolates from day 0 (D.0) and in 19 isolates from day of failure (D.F.). CQ, chloroquine; AQ, amodiaquine; MQ, mefloquine; SP, sulfadoxine/pyrimethamine.

Allele	Haplotype	Percentage (n)	CQ (n) D.0/D.F.	AQ (n) D.0/D.F.	MQ (n) D.0/D.F.	AQ+S-P (n) D.0/D.F.
Wild	C M N K	0% (0)	0/0	0/0	0/0	0/0
Mutant 1	C M N T	67% (115)	9/5	8/8	41/3	40/1
Mutant 2	C M E T	9% (16)	0/0	0/0	14/1	1/0
Mutant 3	S M N/E T	9% (15)	0/0	0/0	9/0	6/0
Mutant 4	S M N T	12% (20)	11/1	3/0	5/0	0/0
Mutant 5	C/S M N T	3% (6)	1/0	1/0	2/0	2/0
TOTAL		100% (172)	21/6	12/8	71/4	49/1

Bajo Cauca region, because of clinical evidence confirming an increasing treatment failure by that time (16).In addition, other antimalarials, such as mefloquine and quinine, are available for resistant or complicated malaria. Furthermore, self medication with halofantrine is known to occur in some localities. At present, artemisinin derivates are included as first-line medication. The current study confirmed the high frequency of treatment failure to all the antimalarials historically used for monotherapy in the country (CQ, AQ). However, high efficacy of treatment with MQ and AQ-SP was observed.

The purpose of the present study was to characterize the treatment failure phenomenon at the molecular level by identification of mutations in the *pfcrt* gene. The presence of *pfcrt* T76 was confirmed in each of the 172 samples studied, indicating complete selection of this mutation by the antimalarial drugs and explaining the high treatment failure rates for CQ and, probably, to AQ.

This concurs with reports of the distribution of this gene in other geographic regions worldwide (1,17,18).

In addition to *pfcrt*, another extensively investigated gene is the *P. falciparum* multiple drug resistance 1 (*pfmdr1*), encoding a P-glycoprotein 1 (Pgh-1). Mutations at the 86, 184, 1034, 1042, and 1246 positions have been strongly linked to CQ-resistance *in vitro* (19-23) and *in vivo* (24,25). These mutations of the *pfmdr1* gene were thought to play a role in the enhancement of resistance levels in CQ-resistant parasites (26). However, recently Mita *et al.* (27) observed a significant non-random association between the *pfcrt* T76 and *pfmdr1* Y86 alleles. They concluded that under CQ selection pressure the Y86 mutation in *pfmdr1* may augment the level of CQ resistance in the T76 alleles.

Analysis by PCR-RFLP of the *pfmdr1* gene on the samples reported here confirmed the presence of pfmdr1 N86 (wild) in 97% of the samples, and the 1246Y mutant allele was detected on 92% of them (28). The significance of these associations remains to be determined.

Previous studies have reported the reversal of the wild type (K76) genotype after cessation of CQ use (29,30). Although the current study was not aimed at exploring this phenomenon, the persistence of the T76 mutant phenotype was detected in a geographic region where use of CQ for falciparum malaria (but not vivax malaria) was abolished 21 years ago. Because vivax malaria accounts for a high proportion of the total malaria cases and because CQ remains for use in vivax treatment, undoubtedly, some falciparum parasites continue be exposed to CQ and pressure selection for mutant alleles continues to be applied in the Turbo-Bajo Cauca region. This exposure will likely occur in malaria patients who had been treated previously for vivax malaria and shortly thereafter undergo treatment for a new falciparum infection. Possibly the previous treatment had occurred in a case of undetected mixed malaria infections or alternatively, low CQ plasma levels remain frp, the previous treatment. The significance of this hypothesis in explaining the persistence of the T76 mutant phenotype remains to be explored.

The most common *pfcrt* haplotype was CMNT. This confirmed previous reports of the presence of codon 76 (31), but contrasts with others that reported on samples from the Colombian Pacific Coast (32).The SMNT haplotype was the second in frequency (24%) and was observed in combination with the SMET haplotype in 9% of the samples. SMNT has been reported in Brazil and Peru (33); however, the SMET mutant haplotype reported herein has never been reported previously in South America. The presence of African, South American, Southeast Asian, and of the SMET and SMNT *pfcrt* haplotypes are reported here in Colombia for the first time. The questions remains as to whether these five haplotypes have evolved by sequential mutations of the *pfcrt* gene, or whether parasites with these haplotypes have been imported into the region. The latter alternative is a feasible explanation for the presence of SMNT in Colombia, since this haplotype is widely distributed throughout the continent. E75 (as in SMET) is very common in Asia and has been reported in Brazil and Peru (34). Its presence in Colombia may have resulted from recombination of several parasite populations carrying SMNT or CMET. Analysis directed toward haplotype origin in the genomes of *P. falciparum* samples remains to be undertaken.

Mixed mutant and wild-type alleles were detected in 12% of the 176 samples. In addition, polyclonal infections were present, at least for the *pfcrt* codon,, in all samples obtained on the day of treatment failure. This was possibly the consequence of a clearance of susceptible phenotypes. However, the effect of drug pressure on selecting mutant alleles, at least for codons 72 and 75, was not statistically confirmed. Further analysis of other genes such as MSP-1, MSP-2 and GLURP is necessary to better address the issue of single or polyclonal infection.

In conclusion, the treatment of *P. falciparum* patients with either CQ or AQ as monotherapy, is not effective in the Turbo-Bajo Cauca regions, whereas MQ and the combined AQ-SP therapy remain effective in clearing the infection. The most common *pfcrt* haplotype was CMNT (67%), followed in frequency by the SMNT haplotype (12%). However the SMNT haplotype was detected in association with a second haplotype in an additional 12% of the samples. No significant association was confirmed between a particular haplotype and the treatment response in any of the treatment groups, or the place of residence of the patient. With a greater sample size, positive relationships may yet be detected. Alternatively, existing relationships may have been swamped by the overwhelming presence of the CMNT haplotype.

Conflicts of interest

The authors have no conflicts of interest concerning the work reported herein.

Funding

This work was supported by Universidad de Antioquia, Colciencias contract RC 156-2002, code 1115-04-11948 and Dirección Seccional de Salud de Antioquia.

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