SHORT REPORT: PREVALENCE OF *PLASMODIUM FALCIPARUM* MUTATIONS ASSOCIATED WITH ANTIMALARIAL DRUG RESISTANCE DURING AN EPIDEMIC IN KUNA YALA, PANAMA, CENTRAL AMERICA

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Abstract. A molecular epidemiology study was conducted to determine the distribution of antimalarial drug resistance alleles among field isolates of *Plasmodium falciparum*. Samples were obtained during an epidemic affecting Kuna Amerindians in Panama. A high prevalence of mutations associated with chloroquine, pyrimethamine, and sulfadoxine was observed. Genotype analysis of msp2 revealed a low genetic diversity of *P. falciparum* parasites circulating in the studied area. The public health implications of these findings for the Central American region are discussed.

Malaria is now considered the most important reemerging disease and one of the major public health problems in Panama. During the past three decades, malaria infection in the country was virtually under control. A few small areas with malaria transmission remained in geographically isolated localities where control measures were difficult to implement and sustain. Almost all the indigenous cases reported in the country during that period were caused by *Plasmodium vivax*. However, each year a handful of imported *P. falciparum* cases were sporadically observed in Kuna Yala and Darien regions, near the Colombian border. These small numbers of imported cases were rapidly controlled, and thus *P. falciparum* transmission was not established in Panama. Nowadays, the epidemiologic scenario has completely changed. The number of malaria cases have strikingly increased to epidemic proportions, reaching 4,500 new cases during 2003 (3,873, 86% *P. vivax* and 627, 14% *P. falciparum*), the highest incidence since 1971. Furthermore, several deaths ($N = 5$) due to *P. falciparum* infection have been reported during the same period, a situation not observed in the country in the past 30 years.[1]

The majority of the *P. falciparum* cases ($N = 538$, 86%) and deaths ($N = 4$, 80%) reported during 2003 were from the Kuna Yala region on the Caribbean coastline, revealing a geographic clustering among the Amerindian population that lives in this area. At the time of the outbreak chloroquine (CQ) plus primaquine were the first-line drugs used to treat uncomplicated *P. falciparum* malaria. Nowadays, sulfadoxine-pyrimethamine (SP) is currently used. Although there is no official description of resistance to these drugs, clinicians from the area insist in declaring treatment failures even after the second choice regimen. Thus, there is an urgent need to determine whether the current drug treatment policy is still adequate to control *P. falciparum* infections in the country.

Mutations in selected *P. falciparum* genes have been associated in several studies with resistance to antimalarial drugs. The K76T mutation at the pfcrt gene is considered a reliable genetic marker for CQ resistance, particularly in non-immune populations.[2,3] Some studies have suggested that pfmdr 1 mutations, most notably at codon 86, might be involved in the modulation of resistance to CQ and mefloquine.[4-6] However, several field studies have failed to confirm this finding.[7,8] In relation to antifolate drugs, point mutations at the dihydro-

folate reductase (dhfr) and dihydropteroate synthetase (dhps) genes have shown a good correlation with *in vitro* resistance to pyrimethamine and sulfadoxine, respectively.[9-11] In the clinical setting, however, this association may be confounded by many factors such as transmission intensity and host immunity. The objective of this study was to rapidly detect through molecular techniques the prevalence of *P. falciparum* mutations associated with resistance to the antimalarial drugs currently used in Panama. Alleles of the msp2 polymorphic genetic marker were analyzed to asses the genetic diversity of field isolates.

A molecular epidemiology study was conducted in two mainland localities (Carreto and Playon Grande) in the Kuna Yala Amerindian territory (Figure 1). Both localities have each less than 300 inhabitants and presented the highest malaria transmission rates during the 2003 epidemic. Kuna Yala is the name of an autonomous territory geographically isolated from the rest of Panama. At present, the only access to this region is by plane or boat, both very expensive routes. An active search for malaria symptomatic patients who had not previously received antimalarial treatment was performed at the beginning of the epidemic in June 2003 ($N = 60$). Finger-prick blood was collected and blotted onto filter paper for assessment of specific molecular markers. DNA was extracted by the Chelex method,[3] and positive *P. falciparum*
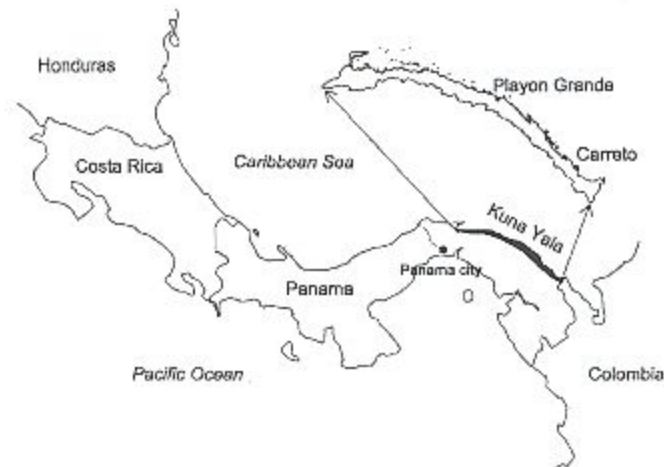

FIGURE 1. Map of Panama showing the location of the studied sites in Kuna Yala.

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patients ($N = 50$) were detected by PCR using 5 μL of the above DNA preparation as previously described.[12] Nine mutations in *P. falciparum* genes closely related with resistance to antimalarial drugs were analyzed using published PCR-based methods for pfcrt K76T,[3] pfmdr1 N86Y,[5] dhfr (Ile-51, Arg-59, Asn-108, Leu-164),[11] and dhps (Gly-437, Glu-540, Gly-581).[9] Genotyping of the merozoite surface protein-2 gene (msp2) was performed in 26 samples using nested PCR and Hinf I digestion as previously described.[13]

PCR-based mutation analysis was possible in the 50 *P. falciparum* positive samples. The pfcrt 76T mutation critical to cloroquine resistance was detected in all field isolates. We also found a 100% fixation of the mutation pfmdr1 86Y associated in some studies with an increased resistance to CQ. Mutations dhfr Ile-51 and dhfr Asn-108 associated with pyrimethamine resistance were present in all the samples, and mutations at codons 59 or 164 were not detected. Concerning dhps genotypes, all samples had the wild-type Lys-540 and Ala-581; however, 33 (66%) samples had the sulfadoxine resistance–associated mutation Gly-437. No mixed infections containing both wild type and mutant alleles at any dhfr or dhps genes were detected. The msp2 genotyping revealed the presence of two different alleles circulating in the studied sites. One allele from the allelic family 3D7 was predominant, found in 88.8% (23/26) of the field isolates. The other allele (WOS 3) belongs to the FC27 allelic family and was observed in three samples (11.5%). Three patients had mixed infections with both alleles.

As in many Latin American countries, in Panama malaria control operations deteriorated when the program was integrated into the existing health services. It is clear now that despite efforts to control the epidemic, *P. falciparum* endemic transmission has been reestablished in the country. Logistical problems, as well as insufficient funds to initiate and maintain malaria control activities in areas of difficult access, such as the Kuna Yala region, have been held partially responsible for this epidemic. However, other factors such as resistance of the parasite to the antimalarial drugs may also be implicated.

In the current study we have found a 100% fixation of the Pfcrt K76T mutation, which is considered a reliable means to predict CQ therapeutic failure particularly in populations with low or no acquired immunity, presumably like the one evaluated in this study. The presence of two dhfr mutations (Ile-51 and Asn-108) in all the field samples coupled with the presence of the mutation dhps Gly-437 in one third of the isolates suggests that that resistance to SP already exists or may develop soon in this region of Panama. However, our findings need to be complemented with *in vivo* tests as these will reflect more closely the therapeutic efficacy of CQ and SP in this epidemiologic scenario.

Before the epidemic, SP was not used to treat *P. falciparum* malaria in Panama, thus the high prevalence of mutations at dhfr and dhps genes could be explained by the recent introduction in Kuna Yala of parasite populations containing these mutations. In this region, there is an intense transitory migration associated with commercial exchange from the Atlantic coast of Colombia, where similar mutation patterns in these genes have been previously reported.[14,15] This fact further enhances this possibility.

Results from msp2 analysis suggest that *P. falciparum* population circulating in the studied site was genetically homogenous as evidenced by the fact that only two alleles could be demonstrated, one of which was predominant. Furthermore, with the exception of a single mutation on dhps observed in 33% of the field isolates, only one genotypic pattern was observed in the other studied genes related to drug resistance. Contrary to reports from several malaria-endemic African countries, limited genetic diversity is common in Central and South American countries with similar *P. falciparum* transmission intensity.[16–18] On the other hand, the relatively small number of samples analyzed and the fact that they were obtained from symptomatic individuals during a cross-sectional survey should also be considered an alternative explanation for the limited diversity observed. It is possible that the evaluation of other polymorphic markers such as Msp 1, glurp, and microsatellites markers would have detected a higher genetic diversity in these field samples. Studies are in progress to further evaluate the genotype of the parasites circulating in this area.

During 2004, the number of *P. falciparum* cases and deaths in the country continued to increase, and the epidemic spread to other Kuna Amerindian communities located in Eastern Panama, 100 km away from Panama City. This epidemiologic situation together with our molecular findings prompted the Ministry of Health to change the first-line drug to mefloquine, until further clinical studies could demonstrate the efficacy of SP to treat *P. falciparum* cases.

Although preliminary, our results show evidence that potentially drug-resistant *P. falciparum* populations are circulating in Panama. This data can be used as an early warning for the Central America region where according to several reports, *P. falciparum* from this region is still sensitive to CQ and SP.[19,20] It also highlights the importance of establishing sentinel sites in the region for monitoring by molecular methods the propagation of this probable resistant genotype. The introduction of resistant *P. falciparum* populations can cause a serious public health problem for the rest of Panama and the Central American region, where most of its population has low or no acquired immunity and thus is susceptible to severe *P. falciparum* malaria.

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REFERENCES

1. Ministerio de Salud, Panamá, República de Panamá, Departamento de Vigilancia de los Factores Protectores y de Riesgo a la Salud y Enfermedad. Situación epidemiológica de la malaria en Panamá. Año 2003.

2. Djimde A, Doumbo OK, Cortese JF, Kayentao K, Doumbo S,

Diourte Y, Dicko A, Su XZ, Nomura T, Fidock DA, Wellems TE, Plowe CV, 2001. A molecular marker for chloroquine-resistant *falciparum* malaria. *N Engl J Med 344*: 257–263.

3. Schneider AG, Premji Z, Felger I, Smith T, Abdulla S, Beck HP, Mshinda H, 2002. A point mutation in codon 76 of pfcrt of *P. falciparum* is positively selected for by chloroquine treatment in Tanzania. *Infect Genet Evol 1*: 183–189.

4. Foote SJ, Kyle DJ, Martin SK, Oduola AMJ, Forsyth K, Kemp DJ, Cowman AF, 1990. Several alleles of the multi-drug resistance gene are closely linked to chloroquine resistance in *Plasmodium falciparum*. *Nature 345*: 255–258.

5. Price RN, Cassar C, Brockman A, Duraisingh M, Vugt M, White NJ, Nosten F, Krishna S, 1999. The pfmdr1 gene is associated with a multidrug-resistant phenotype in *Plasmodium falciparum* from the western border of Thailand. *Antimicrob Agents Chemother 43*: 2943–2949.

6. Reed MB, Saliba KJ, Caruana SR, Kirk K, Cowman AF, 2000. Pgh 1 modulates sensitivity and resistance to multiple antimalarials in *Plasmodium falciparum*. *Nature 403*: 906–909.

7. Bhattacharya PR, Biswas S, Kabilan L, 1997. Alleles of the *Plasmodium falciparum* pfmdr 1 gene appear not to be associated with chloroquine resistance in India. *Trans R Soc Trop Med Hyg 91*: 454–455.

8. Basco LK, Ringwald P, 1998. Molecular epidemiology of malaria in Yaounde, Cameroon. Analysis of chloroquine resistance and point mutations in the multidrug resistance (pfmdr 1) gene of *Plasmodium falciparum*. *Am J Trop Med Hyg 59*: 577–581.

9. Plowe CV, Cortese JF, Djimde A, Nwanyanwu OC, Watkins WM, Winstanley PA, Estrada-Franco JG, Mollinedo RE, Avila JC, Cespedes JL, Carter D, Doumbo OK, 1997. Mutations in *Plasmodium falciparum* dihydrofolate reductase and dihydropteroate synthase and epidemiologic patterns of pyrimethamine-sulfadoxine use and resistance. *J Infect Dis 176*: 1590–1596.

10. Basco LK, Tahar R, Ringwald P, 1998. Molecular basis of in vivo resistance to sulphadoxine-pyrimethamine in African adult patients infected with *Plasmodium falciparum* malaria parasites. *Antimicrob Agents Chemother 42*: 1811–1814.

11. Nzila-Mounda A, Mberu EK, Sibley CH, Plowe CV, Winstanley PA, Watkins WM, 1998. Kenyan *Plasmodium falciparum* field isolates: correlation between pyrimethamine and Chlorcycloguanil activity *in vitro* and point mutations in the dihydrofolate reductase domain. *Antimicrob Agents Chemother 42*: 164–169.

12. Tirasophon W, Rajkulchai P, Ponglikitmongkol M, Wilairat P, Boonsaeng V, Panyim S, 1994. A highly sensitive, rapid, and simple polymerase chain reaction-based method to detect human malaria (*Plasmodium falciparum* and *Plasmodium vivax*) in blood samples. *Am J Trop Med Hyg 51*: 308–313.

13. Felger I, Irion A, Steiger S, Beck HP, 1999. Genotypes of merozoite surface protein of *Plasmodium falciparum* in Tanzania. *Trans R Trop Med Hyg 93 (Suppl 1)*: 3–9.

14. Schmider N, Peyerl-Hoffmann G, Restrepo M, Jelinek T, 2003. Point mutations in the dihydrofolate reductase and dihydropteroate synthase genes of *Plasmodium falciparum* isolates from Colombia. *Trop Med Int Health 8*: 129–132.

15. Méndez F, Muñoz A, Carrasquilla G, Jurado D, Herrera-Arévalo M, Cortese J, Plowe CV, 2002. Determinants of treatment response to sulfadoxine-pyrimethamine and subsequent transmission potential in *falciparum* malaria. *Am J Epidemiol 156*: 230–238.

16. Montoya L, Maetre J, Carmona J, Lopes D, Rosario Do V, Blair S, 2003. *Plasmodium falciparum*: diversity studies of isolates from two Colombian regions with different endemicity. *Exp Parasitol 104*: 14–19.

17. Haddad D, Snounou G, Mattei D, Enamorado I, Figueroa J, Stahl S, Berzins K, 1999. Limited genetic diversity of *Plasmodium falciparum* in field isolates from Honduras. *Am J Trop Med Hyg 60*: 30–34.

18. Gómez D, Chaparro J, Rubiano C, Rojas MO, Wasseman M, 2002. Genetic diversity of *Plasmodium falciparum* field isolates from an isolated Colombian village. *Am J Trop Med Hyg 67*: 611–616.

19. Roll Back Malaria/World Health Organization (WHO), 2001. The use of antimalarial drugs. Report of a WHO informal consultation. Geneva: WHO.

20. Talisuna AO, Bloland P, D́ Alessandro U, 2004. History, dynamics, and public health importance of malaria parasite resistance. *Clin Microbiol Rev 17*: 235–254.