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Article

Genetic polymorphisms of CYP2B6*6, CYP2C8*3 and CYP2D6*4 in vivax malaria patients from Brazilian Amazon

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Genetic variability in the host metabolism of antimalarial drugs influenced by the polymorphisms of cytochrome P450 (CYP) could lead to significant changes in antimalarial treatment response. However, little is known about the frequency of alleles CYP2B6, CYP2C8, and CYP2D6 in an Amazonian population, especially with vivax malaria. Therefore, this study aimed to determine the frequency of CYP alleles CYP2B6*6, CYP2C8*3, and CYP2D6*4 in patients with vivax malaria. The study included 231 patients with vivax malaria treated at a health care reference in Manaus, northern Brazil. A sample of peripheral blood from each subject was collected to perform DNA extraction and genotypic analysis. Genotyping of polymorphisms was performed by allelic discrimination using Real-time polymerase chain reaction. The CYP2D6*4 allele was the most prevalent among patients who developed severe malaria. The frequencies of the CYP2B6*6 and CYP2D6*4 were not different between the severe and uncomplicated malaria. There was a significant association between heterozygous CYP2D6*4 and severe cases of malaria. The results are in agreement with other reports described in the literature for different populations. Future studies are needed to understand the clinical implications of the polymorphisms in patients with vivax malaria.

Keywords: Malaria. Metabolism. CYP. Pharmacogenetics.

INTRODUCTION

Malaria is an infectious-parasitic disease that affects the health of different populations distributed in endemic areas around the world (Westenberger *et al*., 2010). The majority of reported cases of the disease in Latin America are concentrated in Brazil, and the agent with the highest prevalence is *Plasmodium vivax* (Oliveira-Ferreira *et al*., 2010; WHO, 2011).

In the past, vivax malaria was considered a benign and self-limiting disease. However, studies have demonstrated the importance of this species in the development of severe forms of the disease, previously attributed only to *P. falciparum* in different parts of the world, including deaths (Kochar *et al*., 2005).

According to World Health Organization (WHO) criteria (2010), severe malaria is defined as an infection with complications that are potentially fatal to humans. Among the main clinical manifestations described in the literature for severe *P. vivax* malaria, some follow:kell severe anemia, thrombocytopenia, respiratory distress syndrome, neurological syndrome, renal failure, pulmonary edema, spleen rupture, jaundice, metabolic acidosis, and hypoglycemia (Alexandre *et al*., 2010).

There is ample knowledge and publications on the type of clinical manifestations that have been commonly observed in severe vivax malaria. However, comparatively little is known about the pathophysiological mechanisms involved in severe *P. vivax* malaria, as well as the influence of genetic factors in increasing individual

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susceptibility of the host to the development of severe forms of the disease.

Adequate antimalarial therapy is employed in order to avoid the clinical progression of the infection. According to Mehlotra, Henry-Halldin and Zimmerman (2009), the response to antimalarial treatment may be virtually determined by the plasma concentration of active drug or metabolite in the blood. These authors also consider that the genetic variability of the host in the metabolism of antimalarial drugs could generate inadequate plasma concentrations, contributing to therapeutic inefficacy due to sub-therapeutic levels or even to the selection of resistant parasites.

Metabolism is an extremely important pharmacokinetic step for the drug to reach adequate plasma concentrations at its site of action. There is a large inter-individual variety in the pharmacokinetic profile of many antimalarial agents that may result in alteration in plasma drug concentration (Kerb *et al*., 2009). Cytochrome P450 (CYP) enzymes are mainly responsible for the metabolism of antimalarials in the body (Guzmán, Carmona-Fonseca, 2006). These enzymes are highly polymorphic and these differences, in isolation or associated with other factors, may contribute to the variability in the therapeutic response (cure, relapse, or resistance) or in the toxicity often reported with the use of antimalarials (Tomalik-Scharte *et al*., 2008; Kerb *et al*., 2009).

In addition, the CYP isoform genes have been frequently reported in the literature associated with changes in the metabolic profile of isoforms for various drugs, including antimalarials (Parikh *et al*., 2007, Tiong *et al*., 2010). Several CYP enzymes are involved in the metabolism of antimalarial drugs (Guzmán, Carmona-Fonseca, 2006). Among the main antimalarials currently used, some are listed: chloroquine (CYP2C8, CYP3A4, CYP2D6) (Projean *et al*., 2003); primaquine (CYP1A2, CYP2B6, CYP2D6, CYP3A4) (Li *et al*., 2003; Ganesan *et al*., 2009); mefloquine (CYP3A4) (Li *et al*., 2003); artemether (CYP2B6, CYP3A4) (Honda *et al*., 2011); (CYP2A6) (Yusof, Hua, 2012), and amodiaquine (CYP2C8) (Honda *et al*., 2011).

Studies that describe the prevalence of polymorphisms in areas endemic to malaria or even that relate antimalarial therapeutic efficacy to the phenotypic and genotypic characteristics of CYP are scarce. Studies in patients with malaria classified some individuals as poor metabolizers when they had certain polymorphisms in the isoforms CYP2C8 and CYP2C19, responsible for the metabolism of the drugs amodiaquine and proguanil, respectively (Cavaco *et al.* 2006, Parikh *et al*., 2007). More recently, Honda *et al.* (2011) reported the participation of different alleles of CYP2B6 involved in the demethylation of artesunate. However, it is necessary to carry out other studies to better understand the clinical significance of the influence of polymorphisms in these enzymes on the therapeutic efficacy of antimalarial drugs.

CYP2B6 is expressed primarily in the liver, comprising about 2 to 10 % of the total hepatic CYP content, and in some extrahepatic tissues including the kidney, brain, intestine and skin (Wang, Tompkins, 2008). This isoform plays an important role in the metabolism of drugs such as antineoplastic agents, anticonvulsants, benzodiazepines, and antimalarials such as those derived from artemisinin and primaquine (Ganesan *et al*., 2009; Honda *et al*., 2011).

Polymorphisms in CYP2B6*6, predominantly CYP2B6: 516GT, are associated with pharmacokinetic changes with important clinical repercussions for some substrates. This variant has often been associated with reduced CYP2B6 enzyme activity, elevated plasma concentration, reduced clearance and consequent increased neurotoxicity of efavirenz. In addition is the increased risk of developing drug resistance with efavirenz due to discontinuation of treatment after experiencing adverse effects in patients (Haas *et al*., 2004; Gounden *et al*., 2010).

CYP2C9 is responsible for the oxidative metabolism of important pharmacological classes including some antimalarials (chloroquine, amodiaquine, dapsone), hypoglycemic agents (repaglinide, rosiglitazone), chemotherapeutics (paclitaxolpaclitaxel), and hypolipemics (simvastatin) (Kim *et al*., 2003; Ingelman-Sundberg *et al*., 2007).

The CYP2C8*3 allele is characterized by the presence of two variants, CYP2C8 416G>A (rs11572080, Arg139Lys) and CYP2C8 1196A> G (rs10509681, Lys399Arg), exons 3 and 8, respectively (Dai *et al*., 2001).

It occurs more frequently in Caucasians and rarely in Africans and Asians (Dai *et al*., 2001; Bahadur *et al*., 2002; Wu *et al*., 2013). In a study by Cavaco *et al.* (2006), the frequency of the CYP2C8*3 allele was 19.8 % among Portuguese Caucasians.

The enzyme CYP2D6 is responsible for the metabolism of approximately 25 % of the drugs used in the clinical practice, with the dose required to achieve the same plasma concentration ranging from 10 to 30 times between the individuals (Ingelman-Sundberg, Rodriguez-Antona, 2005). Among the main classes of drugs metabolized by this enzyme are antimalarials such as chloroquine and primaquine, antidepressants, antipsychotics, antiarrhythmics, beta-blockers, antiemetics, and opioids (Projean *et al*., 2003; Pybus *et al*., 2013).

Studies have shown the influence of genetic polymorphisms of CYP2D6 on the therapeutic outcome of central nervous system and cardiovascular disorders, cancer, as well as drug interactions (Rodriguez-Antona *et al*., 2010; Madlensky *et al*., 2011). The polymorphism of this enzyme has often been implicated in the ML phenotype (Sachse *et al*., 1997). However, the role of polymorphisms in this enzyme in CYP2D6 is not known, as well as its influence on clinical response.

However, studies that describe the prevalence of polymorphisms in malaria endemic areas or relate the antimalarial therapeutic response and phenotypic and genotypic characteristics of CYP, or that describe the prevalence of polymorphisms in malaria endemic areas are scarce. There are no descriptions in the literature regarding polymorphisms in CYP genes and a possible association with the severity of *P. vivax* infection.

Studies of this nature could contribute to the genotypic characterization of individuals from areas endemic to malaria for CYP (the major CYP isoforms involved in antimalarial metabolism), as well as for the understanding of determinants of severe malaria as a function of metabolic alterations, and still allow the discovery of possible molecular markers of risk for the development of severe clinical forms of vivax malaria.

Therefore, the aim of this study was to carry out the molecular characterization of the main cytochrome P450 isoforms involved in the metabolism of antimalarials in patients with *Plasmodium vivax* malaria.

MATERIAL AND METHODS

Setting and study design

This was an observational, retrospective, case-control study, and samples from the proposal "Clinical characterization of malaria complicated by Plasmodium vivax", developed at the Tropical Medicine Foundation Dr. Heitor Vieira Dourado - FMT-HVD were used.

Patients with malaria caused by *Plasmodium vivax* treated at FMT-HDV from March 2012 to April 2015 were included in the study. The diagnosis of malaria was performed by the thick drop method and confirmation of monoinfection by this species was performed by molecular diagnosis - PCR. Clinical-laboratory information was obtained from all individuals, and this information was extracted from the medical records and standardized questionnaires. The study comprised 231 patients. Patients were classified as uncomplicated or severe malaria as described previously, according to WHO recommendations (WHO, 2015).

The study protocol was previously approved by the National Commission for Research Ethics (CONEP), in June 2009, decision no. 343/2009.

Molecular analysis

Genomic DNA was extracted from 300 µL of blood using the commercial Wizard® Genomic DNA Purification Kit, according to the manufacturer's protocol. After extraction, the DNA was stored at -70 °C until the time of molecular analysis.

Each patient's DNA was subjected to real-time PCR amplification through StepOnePlus ™ v. 2.0 (Applied Biosystems) using 96-well optical plates and the TaqMan® system for allelic discrimination (Table I).

TABLE I - Probes performed in the Real Time PCR technique

Polimorfism	Allele	Ref. SNP ID	Sequence
15631 G>T	*CYP2B6*6*	rs37455274	TCATGGACCCCACCTTCCTCTTCCA[G/T] TCCATTACCGCCAACATCATCTGCT
2130 G>A	*CYP2C8*3*	rs11572080	CTCTTGAACACGGTCCTCAATGCTC[C/T] TCTTCCCCATCCCAAAATTCCGCAA
1846 G>A	*CYP2D6*4*	rs3892097	AGACCGTTGGGGCGAAAGGGGCGTC[C/T] TGGGGGTGGGAGATGCGGGTAAGGG

RefSNP accession ID (rs number): https://www.ncbi.nlm.nih.gov/snp/

Statistical Analysis

Results were presented as mean, standard deviation, and percentages. The 1-sided Fisher´s exact test was applied to verify the association of variables and the level of significance was set to $P < 0.05$. All analyses were performed using SPSS statistical software (version 18.0).

RESULTS AND DISCUSSION

Of the 231 patients with vivax malaria included in the study, 85 (36.8 %) had severe malaria, 136 (58.9 %) of uncomplicated malaria, and 10 (4.3 %) could not be classified as severity due to the absence of clinical-laboratory data, which were evaluated only in relation to allelic frequency.

The mean age was similar between the two groups (31.8 ±24.9 vs 33.5 ± 19.9 - p=0.611), uncomplicated and severe, respectively. The occurrence of severe malaria was higher in male patients (45.5 %) than female (31.2 %) (p=0.020). Interestingly, we found that twice as many (36.1 % versus 63.9 %) patients who reported having been previously infected with *Plasmodium* did not develop severe disease. Although this relationship was not statistically significant, it is believed that there is clinical protection in patients frequently exposed to malarial infections (Alves *et al.*, 2002; Costa *et al.*, 2011).

The genotypic and allelic frequencies of CYP2B6*6, CYP2C8*3, and CYP2D6*4 are summarized in Table II. The frequency of mutated patients to CYP2B6*6 was 42.2 %, being 27.8 % in heterozygosity (G/T) and 14.3 % in homozygous (T/T). To the CYP2C8*3, only 13.5 %, were found, being all heterozygous patients G/A. To the CYP2D6*4 allele, the frequency of mutated patients was 6.5 %, being 15.2 % heterozygosity (G/A) and 1.3 % homozygous (A/A).

Polymorphisms in CYP enzymes may contribute to variations in efficacy and safety of antimalarial drugs (Tomalik-Scharte *et al.*, 2008). Both efficacy and safety are dependent on plasma concentrations and adequate exposures, since insufficient exposures to the drug are associated with a greater risk of failure or therapeutic resistance while longer exposure is related to a greater chance of developing adverse reactions (Guzmán, Carmona-Fonseca, 2006).

In Brazil, chloroquine and primaquine are still the basis of malaria vivax complications treatment. Antiparasitic activity of these antimalarial drugs is dependent on metabolism (Projean *et al.*, 2003; Ganesan *et al.*, 2009; Pybus *et al.*, 2013) and therefore, polymorphisms in these isoforms may alter the catalytic activity of the enzyme. Studies suggest that the CYP2D6*4 and CYP2C8*3 alleles reduce the catalytic activity of CYP2D6 and CYP2C8 enzymes involved in the metabolism of drugs (Sachse *et al.*, 1997; Paganotti *et al.*, 2011; Stage *et al.*, 2013). Thus, the metabolism of these antimalarial drugs would be involved in the presence of these alleles. Therefore, the hypothesis of the conversion of primaquine and chloroquine in their respective active metabolites would be lower, resulting consequently, in a

lower pharmacological effect of antimalarials in patients of these alleles being suggested.

CYP2B6*6 (T), CYP2C8*3 (A) and CYP2D6*4 (A) were 28.26 %, 6.7 %, and 8.87 %, respectively, in the study population. The frequency of these polymorphisms specifically in patients with vivax malaria was not found in the literature. However, the allelic frequencies found in this study were very similar to the data published on the NCBI home page. According to Table III, in general the allelic frequencies found in this study were similar to other reports described in the literature.

Genotyping studies conducted in Colombians and Africans for the CYP2B6*6 allele reported a frequency of 36.5 % and 35 %, respectively (Restrepo *et al.*, 2011, Hodel *et al.*, 2013). For CYP2C8*3, a frequency of 0 % among Africans and 2.94 % among Chinese was reported (Hodel *et al.*, 2013, Wu *et al.*, 2013). In the present study, we compared the prevalence of CYP2D6*4 with a frequency of 14.5 % among Brazilians and 4.1 % in Africans (Maciel *et al.*, 2009; Hodel *et al.*, 2013).

Although this study was not designed with the objective of evaluating the impact of the polymorphisms found in the metabolism of antimalarials, some inferences can be made from the pharmacological point of view. Previous studies have reported increased *in vivo* and *in vitro* metabolic activity of the CYP2B6 enzyme associated with the CYP2B6*6 variant (Nakajima *et al.*, 2007; Ariyoshi *et al.*, 2011; Honda *et al.*, 2011). It is assumed that the main antimalarial activity of artemisinin derivatives is carried out by dihydroartemisinin (DHA), which is the main active metabolite of this pharmacological group. Therefore, individuals carrying this variant could have higher concentrations of DHA, which, theoretically, would increase the pharmacological effect, thus justifying the higher frequency of carriers of this polymorphism found in the non-severe group.

Likewise, primaquine's parasitic anti-infectious activity is biotransformation dependent (Ganesan *et al.*, 2009). Heterozygous carriers of this allele tend to have a lower enzymatic activity when compared to those carrying the two functional alleles (Zanger, Schwab, 2013). Thus, primaquine metabolism would be compromised in the presence of the CYP2D6*4 allele, therefore it can be suggested that the conversion of this drug into the active metabolite would be lower, and consequently, result in a lower pharmacological effect of the antimalarial. This would explain the higher frequency of individuals with this polymorphism in the group with severe malaria.

However, we know that such data are not sufficient to evaluate the impact of these polymorphisms on the outcome of the malaria disease, since other factors, not only parasitemia, are necessary to characterize the severity of the disease. In addition, the complexity involving the genotype-metabolism relationship *in vivo* requires a study designed specifically for this purpose.

Polymorphisms in CYP isoforms have been considered as risk factors for development or may interfere with the clinical evolution of diseases. These studies reveal that the importance of CYP may be beyond pharmacogenetics, since polymorphisms in these enzymes can act as adjuvants in the pathogenesis or even interfere with the prognosis of many diseases, regardless of the introduction of drug therapy.

In the specific case of malaria, it is already known that some genetic factors linked to the host, e.g. hemoglobinopathies, offer differential risks to the development of infection with *Plasmodium spp.* There were no significant differences between the distribution of genotypes, the number of CYP2C8*3 allele carriers, and the severity of malaria. In contrast, for the CYP2D6*4 allele, it was verified that the frequency of polymorphism was higher in the severe group, and there was an association between the heterozygous genotype and the severity of the disease. Yet the chance of severity development among the patients with this polymorphism in this study was 2,647 times higher when compared to non-carriers. However, because it is a pioneering and innovative association of CYP2B6*6, CYP2C8*3, and CYP2D6*4 polymorphisms for the severity of malaria, it was not possible to establish whether these were random or if in fact there was a significant relationship in the evolution of malaria.

Hematological, biochemical and parasitemia data of patients included in this study before or after starting pharmacological treatment were not available, together with the scarcity of data in the literature on the influence of these polymorphisms in current-day

antimalarial metabolism in our region, were limiting factors to evaluate the outcome of the polymorphisms studied in the treatment of patients with vivax malaria. This study attempted to describe the frequency of CYP2B6*6, CYP2C8*3, and CYP2D6*4 alleles in patient carriers of vivax malaria and attempt to associate such polymorphisms with the severity of the disease, However, it is important to mention the importance of developing future studies focused on the evaluation of the impact of the pharmacogenetic profile on the metabolism of antimalarials, especially primaquine, the only available drug used in the treatment and prevention of relapses by *P. vivax*, and also for the confirmation and clarification of the associations found in this study, since only then will it be possible to predict of these genetic factors in the outcome of malaria.

TABLE II – Genotypic and allelic frequency of CYP2B6*6, CYP2C8*3 and CYP2D6*4 in uncomplicated and severe *P. vivax* malaria patients from Manaus, Amazonas state

Allele	Profile	Genotype	Total		Severe		Uncomplicated		p-value	p-value RP(IC95%)
			(N)	% allele	(N)	% allele	(N)	% allele		
CYP2B6*6	Wild	G/G	133		54		75			
	Mutated	G/T	64	28.26	25	21.76	35	31.48	0.059[b]	0.152[b] 0.728 (0.411-1.253)
		T/T	33		6		25			
	Total		230		85		135			
CYP2C8*3	Wild	G/G	192		73		111			
	Mutated	G/A	30	6.76	8	9.87	20	15.27	0.180[a]	0.180[a] 0.608 (0.254-1.454)
		A/A	0		0		0			
	Total		222		81		131			
CYP2D6*4	Wild	G/G	193		64		121			
	Mutated	G/A	35	8.87	19	13.52	14	5.88	0.026[b]	0.007[a] 2.647 (1.277- 5.485)
		A/A	3		2		1			
	Total		231		85		136			

N - Total number of patients; PR - prevalence ratio; CI - confidence interval; p[a] - p-value based on Fisher's exact tests; p[b] X^2- Yates´s corrections; %allele - mutant allele.

TABLE III - Allelic frequencies in patients with vivax malaria compared to other studies published in the literature

Population	Number of patients included	Percentage			Reference
		CYP2B6*6	CYP2C8*3	CYP2D6*4	
Brazilians	231	28.2	6.7	8.8	This study
Brazilians	1,034	36.9	9.8	11.7	Suárez-Kurtz *et al.*, 2012
Brazilians	115	-	-	14.5	Maciel *et al.*, 2009

TABLE III - Allelic frequencies in patients with vivax malaria compared to other studies published in the literature

Population	Number of patients included	Percentage			Reference
		CYP2B6*6	CYP2C8*3	CYP2D6*4	
Colombians	152	36.5	-	-	Restrepo *et al*., 2011
Indians	123	-	12	-	Muthiah *et al*., 2005
Chinese	123	38	-	-	Hodel *et al*., 2013
Black Africans	148	34	0	4.1	Hodel *et al*., 2013
White Europeans	35	28.6	-	-	Lang *et al*., 2001
Black Europeans	146	34	-	-	Wyen *et al*., 2008

CONCLUSION

This study described the profile of allelic frequencies of some important polymorphisms from metabolizing enzymes in patients suffering from malaria. Based on the results presented in this study, it can be concluded that the frequencies of the CYP2B6*6, CYP2C8*3, and CYP2D6*4 alleles in patient carriers of vivax malaria are in agreement with other reports described in the literature for different populations. The CYP2D6*4 allele was the most prevalent among patients who developed serious malaria. The frequencies of CYP2B6*6 and CYP2D6*4 were not different between the severe and uncomplicated groups. There was a significant association between heterozygous CYP2D6*4 and severe cases of malaria. Further studies are needed to elucidate the role of these polymorphisms in the outcome of vivax malaria.

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COMPETING INTERESTS:

The authors have declared that no competing interests exist.

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