Genetic diversity of chloroquine-resistant *Plasmodium vivax* parasites from the western Brazilian Amazon

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The molecular basis of Plasmodium vivax chloroquine (CQ) resistance is still unknown. Elucidating the molecular background of parasites that are sensitive or resistant to CQ will help to identify and monitor the spread of resistance. By genotyping a panel of molecular markers, we demonstrate a similar genetic variability between in vitro CQ-resistant and sensitive phenotypes of *P. vivax* parasites. However, our studies identified two loci (MS8 and MSP1-B10) that could be used to discriminate between both CQ-susceptible phenotypes among *P. vivax* isolates in vitro. These preliminary data suggest that microsatellites may be used to identify and to monitor the spread of *P. vivax*-resistance around the world.

Key words: *Plasmodium vivax* - chloroquine - resistance

*Plasmodium vivax*, although less pathogenic than *Plasmodium falciparum*, has a great socioeconomic impact. *P. vivax* has been associated with drug resistance, clinical severity and even fatality (Price et al. 2009). The first cases of *P. vivax* chloroquine-resistant (CQ-R) were reported in 1989 in Papua New Guinea (Rieckmann et al. 1989). Since then, reports of CQ-R have been published around the world. Some cases of CQ-R that presented adequate blood CQ levels had been confirmed in Brazil, Ethiopia, Indonesia, Malaysia, Myanmar, Papua New Guinea, Peru, the Solomon Islands and Thailand (WHO 2010). In Brazil, malaria is transmitted along the Amazon Basin. A total of 242,758 cases were reported in 2012, with *P. vivax* accounting for 85% of all cases (WHO 2013). The first reliable case of in vivo CQ-R in Brazil was reported in Manaus, state of Amazonas (AM) (Alecirim et al. 1999). In 2004/2005, 10% of *P. vivax* infections studied in Manaus showed an in vivo CQ-resistant (CQ-Rt) phenotype (Santana Filho et al. 2007). Recently, two studies evaluated *P. vivax* CQ-susceptibility by using short-term in vitro cultures of samples collected in 2004–2008 from AM and confirmed the same prevalence of CQ-R among *P. vivax* parasites (10%) (Chehuan et al. 2013, Pratt-Riccio et al. 2013). In other endemic regions of Brazil, such as in the state of Acre, no CQ-R has been reported (Orjuela-Sánchez et al. 2009) and no studies have been reported so far in other regions of Latin America (Gonçalves et al. 2014).

The genetic mechanism of *P. vivax* CQ-R has yet to be fully elucidated. While CQ-R in *P. falciparum* has been associated with particular point mutations in specific genes, such as *pfcrto* and *pfmdr-1* (Fidock et al. 2000, Duraisingh & Cowman 2005), this type of association is not very clear in the case of *P. vivax* (Gonçalves et al. 2014). The main difficulty in studying the mechanisms of *P. vivax* CQ-R is the absence of a continuous in vitro culture system, as studies utilising short-term in vitro cultures are useful for investigating drug resistance mechanisms and susceptibility analysis of therapeutic agents for *P. vivax* (Kerlin et al. 2012). Due to the absence of specific molecular markers of resistance, the characterisation of general molecular markers of *P. vivax* will not only help identify CQ-R parasites, but also define the geographical origins and dissemination of resistant isolates (Arnott et al. 2012). The genetic diversity of *P. vivax* has been studied using nearly neutral molecular markers, such as microsatellites (MS) and tandem repeats (TR), as well as with polymorphic antigens, including the PvMSP protein family members (MSP-1 and MSP-3α) [for a review see de Brito and Ferreira (2011)]. The present study focuses on the genetic diversity of *P. vivax* isolates from the Brazilian Amazon Basin with different CQ-susceptibility phenotypes in short-term in vitro cultures.

Patients with uncomplicated *P. vivax* malaria, as confirmed by a thick blood smear, were randomly selected in the outpatient clinics Dr Heitor Vieira Dourado Tropical Medicine Foundation (Manaus) between December 2007–July 2008. Written informed consent was obtained from all participating patients. The major exclusion criterion was the use of antimalarials within the previous 60 days. Blood was collected at the day of diagnosis and before the start of antimalarial treatment. The patients were treated according to the Brazilian Ministry of Health standards for malaria therapy (Chehuan et al. 2013). Eleven isolates from the Amazon Basin were evaluated: nine from AM, one from the state...
of Rondônia (RO) (sample I) and one from the state of Roraima (RR) (sample H). Due to the selective action of CQ on the young trophozoite (ring) stage (Chotivanich et al. 2001), only samples that contained between 50-70% of the total parasite forms at this stage using short-term in vitro culture were evaluated. The CQ-response of the isolates was measured as released plasmidic lactate dehydrogenase using the DELI test and the 50% inhibitory concentration (IC_{50}) values (Chehuan et al. 2013). Samples with IC_{50} values greater than 100 nM were considered to be CQ-Rt. This study was approved by the Ethical Committee of Clementino Fraga Filho University Hospital, Federal University of Rio de Janeiro, state of Rio de Janeiro (approval 42746/2012).

Nine neutral markers (Table I), two TRs (MN21 and MN23), five MS (MS2, MS5, MS6, MS7, MS8) and two highly polymorphic blocks of MSP-1 (MSP1-B2 and MSP1-B10) were evaluated by using primers, as described previously (Putaporntip et al. 2002, Feng et al. 2003, Rezende et al. 2009, 2010). These markers have been used previously in studies on the P. vivax population structure and diversity in the Brazil endemic area (Rezende et al. 2009, 2010) and in other endemic areas, showing the extent of their polymorphisms (Imwong et al. 2005, Koepfli et al. 2009, 2011).

Plasmodial DNA was extracted from dried blood spots using the QIAamp DNA Micro kit (Qiagen, USA). To increase the quantity of the DNA, parasite DNAs were subjected to whole-genome amplification using a REP-Li-g Mini kit (Qiagen) according to the manufacturer’s instructions. The markers were amplified using polymerase chain reaction (PCR), according to a previously described methodology (Putaporntip et al. 2002, Feng et al. 2003, Rezende et al. 2009, 2010). The amplified sequences were separated using capillary electrophoresis in an automatic DNA sequencer (MegaBACE, Amersham Biosciences, USA) and the lengths of the products were determined with reference to internal size standards (MegaBACETM ET550-R, Amersham Biosciences) using MegaBACETM Fragment Profiler v.1.2 software (Amersham Biosciences). The highest peak in the electropherogram was defined as the predominant allele and additional alleles were considered when the corresponding peak heights were at least 1/3 of the height of the predominant allele in the same sample. In all cases, the minimal peak height for an allele to be considered was set to 100 arbitrary fluorescence units. The predominant allele in each locus per isolate was used to determine gene diversity using the Arlequin 3.0 software that calculated the expected heterozygosity (H_s), i.e., the probability that a pair of alleles randomly obtained from the population differs from each other.

The six CQ-sensitive (CQ-S) isolates had IC_{50} medians of 26.35 ng/mL (7.3-66.4) and 152.8 ng/mL (108.8-211.8), respectively. The use of multiple molecular markers showed the usefulness and reproducibility of parasite genotyping, as it could be compared with the population studies performed in Brazil using the same panel of markers (Rezende et al. 2009, 2010, Araujo et al. 2012). The H_s average of CQ-Rt samples (H_s = 0.528) and CQ-S samples (H_s = 0.594) were comparable because the difference was not statistically significant (Wilcoxon rank sum test, p = 0.57). Forty-nine alleles were detected among the samples genotyped using nine loci: the more diverse (> 6 alleles) were MSP1-B10, MS7 and MS5, while the more conserved (< 4 alleles) were MS2 and MN23 (Table II). Allelic frequencies ranged from 8.3-80% among the studied samples. For some samples, two alleles were detected at a particular locus: CQ-S F (MSP-1 blocks 2 and 10), CQ-S 85 (MS5).

| Marker | First position | Chromosome | Repeating unit | Size fragments |
|--------|---------------|------------|----------------|---------------|
| MN21   | 1567132       | 8          | CC ACT         | 254-290       |
| MN23   | 1565219       | 8          | CAC C          | 219-302       |
| MS2    | 782947        | 3          | CA12           | 290-312       |
| MS5    | 30333         | 3          | CAT10          | 173-215       |
| MS6    | 2835596       | 14         | TGA19          | 194-298       |
| MS7    | 152239        | 2          | TAA22          | 349-388       |
| MS8    | 1561308       | 13         | TGT A7         | 284-560       |
| MSP1-B2| 1157742       | 7          | Putapornit     | 352-460       |
| MSP1-B10|             |            | et al. (2002)  | 226-329       |

a: GenBank accession NC_009913.1 (Rezende et al. 2009), chromosome location determined at ncbi.nlm.nih.gov.
CQ-S C (MS7 and MS8), CQ-S A (MS6) and CQ-R H (MSP1-B10) (Table III). Interestingly, despite similar genetic variability between CQ-S and CQ-Rt isolates, multiple alleles per locus were identified in four out six CQ-S isolates, whereas this was only found in one CQ-Rt sample, suggesting a higher variability among CQ-S parasites. Of note, the different alleles of MS8 and MSP1-B10 were not shared by the two phenotypes (Figure). Particularly for the MS8 locus, allele 317 was detected in 4/6 (67%) CQ-S phenotype samples and allele 313 in 3/4 (75%) CQ-Rt phenotype samples. Of note, the different alleles of MS8 and MSP1-B10 loci were not shared by the two phenotypes (Figure). Particularly for the MS8 locus, allele 317 was detected in 4/6 (67%) CQ-S phenotype samples and allele 313 in 3/4 (75%) CQ-Rt phenotype samples: all of which were from different states of the Amazon Basin (RO, AM and RR). This locus is located on chromosome 13 at 166 bp from the 5’ end of a gene encoding a hypothetical conserved protein with phosphatidylinositol phosphate kinase activity and at 1,942 bp from the 3’ end of a gene encoding nuclear transport factor 2 (Rezende et al. 2010). CQ-Rt phenotype exclusive alleles found in this study have also been described in other states of the Amazon Basin, such as Amapá, Pará and Mato Grosso (Rezende et al. 2010), suggesting that the CQ-Rt parasite population could be distributed in other regions of the Amazon Basin.

In conclusion, we found that CQ-S and CQ-Rt P. vivax parasites are genetically different. Although the level of genetic diversity was similar in the two phenotypes, both phenotypes could be differentiated by at least two loci. Furthermore, these phenotype-specific alleles were identified in different endemic areas of Brazil, suggesting the potential of resistance spreading across the whole Amazon Basin. Further studies should be performed with a larger number of samples to confirm these findings. Whether these loci are under linkage disequilibrium and involve their flanking genes to impart resistance warrants further investigation.

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CQ-Rt: chloroquine resistant; CQ-S: CQ-sensitive.
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