INTRODUCTION

Plasmodium vivax, the most widespread human malaria parasite, causes up to 390 million episodes of disease each year in Central and South America; the Middle East; central, southern, and Southeast Asia; Oceania; and eastern Africa, where 2.85 billion persons are currently at risk for infection.1,2 Plasmodium vivax is the main cause of malaria-related morbidity outside Africa, and the recent emergence of chloroquine (CQ)–resistant P. vivax strains further complicates current malaria control efforts.3

Plasmodium vivax resistance to CQ in vivo, defined as the persistence of parasites in the blood despite adequate blood levels of CQ and its main metabolite desethylchloroquine,4 has already reached alarming prevalence rates in Indonesia, East Timor, and Papua New Guinea, and is currently emerging in several countries across southern and Southeast Asia, the Middle East, and the Americas.3,5 However, only a small proportion of clinical trials of antimalarial drugs worldwide have assessed their efficacy against P. vivax.3 Accordingly, whether CQ-resistant P. vivax represents a major reason for concern in Brazil remains unclear,6 but parasite recrudescences despite adequate CQ levels were recently described in 11 of 109 P. vivax malaria patients treated in Manaus, Amazonas State.7

The molecular mechanisms of CQ resistance in P. vivax are elusive, but genetic variation at loci encoding digestive-vacuole membrane proteins may contribute to this phenotype. One such locus is the multidrug resistance 1 gene of P. vivax (pvmdr-1), which encodes a P-glycoprotein of the family of ATP binding cassette transporters. The Y976F mutation (TAC→TTC) in pvmdr-1 has been associated with CQ resistance in Southeast Asia8 and Papua New Guinea,9 but whether this polymorphism can be used as a molecular marker of CQ resistance in parasites collected worldwide remains unclear.3,10–12 Interestingly, the Y976F change has been reported to be rare in alleles that do not carry the F1076L (TTT→CTT) change, consistent with a two-step mutation pathway (F1076L followed by Y976F) putatively leading to CQ resistance.13,14

Similar to P. falciparum,15 susceptibility to CQ in P. vivax appears to be also modulated by pvmdr-1 copy number. Amplification of the pvmdr-1 gene correlates with increased susceptibility to CQ and decreased susceptibility to amodiaquine, artesunate and mefloquine in vitro.16 Because parasites are exposed to different drug treatment regimens in each country, local adaptation may theoretically favor parasites with increased pvmdr-1 copy number or select for Y976F alleles in different malaria-endemic sites.

We examined the prevalence of five common non-synonymous single-nucleotide polymorphism (SNPs) at the pvmdr-1 locus, including Y976F and F1076L, and the number of pvmdr-1 gene copies in > 200 P. vivax isolates from northwestern Brazil, where CQ and mefloquine are commonly used in malaria treatment. We also analyze patterns of genetic variation at the pvmdr-1 locus previously reported in other malaria-endemic regions and discuss the potential bases for the striking differences observed worldwide.

MATERIALS AND METHODS

Study area. New parasite samples analyzed in this study were collected in the states of Acre and Amazonas, in the Western Amazon Basin of Brazil, near the borders with Peru, Bolivia, and the Brazilian state of Rondônia (Figure 1). The area is characterized by a humid equatorial climate and receives most rainfall during December–March. Blood samples from P. vivax-infected persons were collected in the farming settlement of Granada and the towns of Acrelândia (40 km south of Granada) and Plácido de Castro (35 km south of Acrelândia), all in Acre State, and in the farming settlement of Remansinho (140 km east of Granada), in Amazonas State.

Samples from Granada (n = 139) were collected from symptomatic and asymptomatic persons enrolled into prospective cohort studies conducted in this site during 2004–2006.17–19 and those from Acrelândia (n = 15) and Plácido de Castro (n = 61) were collected from febrile patients attending the...
Malaria clinics in these towns during 2008–2011. Samples from Remansinho (n = 105) were collected from asymptomatic and symptomatic persons participating in an ongoing prospective cohort study of malaria risk factors.

These sites are characterized by year-round, hypoendemic malaria transmission, with *P. vivax* prevalence rates of 2–10% in Remansinho (Ferreira MU, unpublished data) and typically < 1% in the other sites. *P. vivax* accounts for 80–90% of all malaria infections diagnosed in this area, which is located more than 1,000 km south of Manaus, the capital of Amazonas State, where CQ-resistant *P. vivax* malaria has been recently characterized. Parasite samples described in this study were collected under protocols approved by the Institutional Review Board of the Institute of Biomedical Sciences, University of São Paulo, Brazil (936/CEP, 2010). Written informed consent was obtained from adult patients and parents or guardians of minors.

**Laboratory diagnosis of malaria.** Giemsa-stained thick blood smears had at least 100 fields examined for malaria parasites under 1,000× magnification by two experienced microscopists. Blood samples were further examined for malaria parasites by a quantitative real-time polymerase chain reaction (PCR) specific for the 18S ribosomal RNA gene. DNA templates for PCR amplification were isolated from 200 µL of whole venous blood using QIAamp DNA blood kits (QIAGEN, Hilden, Germany). Each 15-µL reaction mixture contained 2 µL of sample DNA, 7.5 µL of 2× Maxima SYBR Green qPCR master mixture (Fermentas, Vilnius, Lithuania), and 0.5 µM of each primer. We used the genus-specific primer P1 (5′-ACG ATC AGA TAC CGT CGT AAT CTT-3′) with either of the species-specific primers, V1 (5′-CAA TCT AAG AAT AAA CTG GA A-3′) or F2 (5′-CAA TCT AAA CTC GAA AGA TG-3′), for *P. vivax* and *P. falciparum*, respectively. These primers enable amplification of a species-specific approximately 100 bp fragment of the 18S ribosomal RNA gene.

Standard curves were prepared with serial 10-fold dilutions of the target sequence, cloned into pGEM-T Easy vectors (Promega, Madison, WI), to enable species-specific quantification of parasite loads (number of parasites/microliter of blood). We used a Mastercycler reaoplex S real-time thermal cycler (Eppendorf, Hamburg, Germany) for PCR amplification with an initial step at for 2 minutes at 50°C, followed by template denaturation for 10 minutes at 95°C, and 40 cycles for 15 seconds at 95°C and 1 minute at 60°C, with fluorescence acquisition at the end of each extension step. Amplification was immediately followed by a melting program consisting of 15 seconds at 95°C, 15 seconds at 60°C, and a stepwise temperature increase of 0.03°C/second until 95°C, with fluorescence acquisition at each temperature transition.
Antimalarial treatment. *Plasmodium vivax* episodes in Brazil are treated with chloroquine (25 mg/kg over three days; adult dose = 1.5 g over three days) plus primaquine (0.5 mg/kg/day for seven days; adult dose = 30 mg/day). Until 2007, *P. falciparum* infections were treated with mefloquine (15 mg/kg; single dose; adult dose = 1,000 mg). Although mefloquine has not been used to treat laboratory-confirmed single-species *P. vivax* infections in Brazil, local *P. vivax* strains were likely to be exposed to mefloquine used to treat previous episodes of *P. falciparum* in the same patient because of the long half-life of this drug. Starting in 2007, fixed-dose combinations of either artesunate plus mefloquine (in Acre) or artemether plus lumefantrine (in Amazonas) have been gradually introduced as the first-line therapy for *P. falciparum* infections. There is no evidence of CQ resistance (defined as parasite recrudescence observed amplifluor assay).

**Analysis of single-nucleotide polymorphism in *P. vivax mdr-1*.** We typed five common nonsynonymous SNPs (A266G, A1498G, A2722C, A2927T, and T3226C) described in the *pvmdr-1* gene of field isolates collected worldwide, which correspond to the amino acid changes N89S, N500D, M908L, Y976F, and F1076L. These polymorphisms were genotyped under contract with the following oligonucleotide primer pairs: PvmdrF (5'-CTG ATA CAA GTG AGG AAG AAC ACT TAC G-3') and PvmdrR (5'-GTC CAC CTG ACA ACT TAG ATG C-3') for *pvmdr-1* and PvaldoF (5'-GAC AGT GCC ACC ATC CTT ACC-3') and PvaldoR (5'-CTT CTC CAA CAT TCT CTC TCT C-3') for *pvaldolase*. Each 15-µL reaction mixture contained 2 µL of sample DNA (prepared as described above), 7.5 µL of 2× Maxima SYBR Green qPCR master mixture (Fermentas), and 0.3 µM of each oligonucleotide primer. Amplification included a template denaturation step for 10 minutes at 95°C, followed by 40 cycles of at 95°C for 15 seconds and 60°C for 1 minute, with fluorescence acquisition at the end of each extension step. Amplification was immediately followed by a melting program consisting of 95°C for 15 seconds and 60°C for 1 minute, and a stepwise temperature increase of 0.3°C/second until 95°C, with fluorescence acquisition at each temperature transition.

**Sources of additional data.** For global comparisons of the prevalence of the Y976F and F1076L mutations, we collated data from the following publications: Brega and others, 2005 (4 isolates from French Guiana, 4 from Azerbaijan, 3 from Turkey, 9 from Thailand, and 3 from Indonesia); Suwanarusk and others, 2007 (7 isolates from Thailand and 24 from Indonesia); Marfurt and others, 2008 (80 isolates from Madagascar); Imwong and others, 2008 (5 isolates from Indonesia and 24 from Indonesia); Barnadas and others, 2008 (4 isolates from Malaysia); Orjuela-Sánchez and others, 2010 (6 isolates from Brazil, 20 from Sri Lanka, 69 from Cambodia, and 9 from Vietnam); Jovel and others, 2011 (37 isolates from Brazil, 24 from Thailand, 11 from Myanmar, 5 from Laos, and 11 from Thailand); Marfurt and others, 2008 (94 isolates from Papua New Guinea); Gama and others, 2009 (28 isolates from Brazil); Orjuela-Sánchez and others, 2010 (6 isolates from Brazil, 20 from Sri Lanka, 69 from Cambodia, and 9 from Vietnam).

**Analysis of copy number variation in *P. vivax mdr-1*.** We used real-time quantitative SYBR green PCR, with the single-copy gene coding for *P. vivax* aldolase as a reference, to estimate *pvmdr-1* copy number. Plasmids containing amplified fragments of the *pvmdr-1* and *pvaldolase* genes cloned into pGEM-T Easy vectors (Promega) were used as calibrators. We thus estimated the number of *pvmdr-1* copies by using a comparative threshold method, with the formula

\[
Ct_{\text{pvmdr-1}} - Ct_{\text{pvaldolase}} = \text{sample} - \text{calibrator}
\]

Where *Ct* is the cycle threshold for each gene. A copy number > 1.6 was defined as a duplication of the *pvmdr-1* gene; assays were repeated if the following results were obtained: copy number between 1.3 and 1.6, Ct value > 35 or standard deviation of Ct (for either *pvmdr-1* or *pvaldolase* replicates) > 0.24.
South Korea, 27 from Thailand, and 11 from Myanmar27; and Mint Lekweiry and others, 2011 (105 isolates from Mauritania).28 The complete dataset comprising 917 samples is shown in Supplemental Table 2.

RESULTS

Single-nucleotide polymorphism at the mdr-1 locus in *P. vivax* isolates from northwestern Brazil. We analyzed newly obtained SNP data for 13 isolates collected in Acrelândia, 21 in Plácido de Castro and 46 in Remansinho, and data previously obtained with the same typing strategy, for 37 isolates from Plácido de Castro and 111 from Granada.14 Of five SNPs assayed, two were monomorphic in the Western Amazon Basin of Brazil: all 228 isolates examined had the amino acid replacements (relative to the *pvmdr-1* sequence of Sal-1 strain, which is defined as wild type) N89S and M908L (Table 1). Although these changes are not putatively associated with CQ resistance, they can be useful in tracing the geographic origin of *pvmdr-1* haplotypes carrying mutations Y976F and F1076L, which in turn might underlie CQ resistance. The changes N500D, Y976F and F1076L were found in 64 (28.1%), 4 (1.8%) and 37 (16.2%) isolates from the Western Amazon Basin of Brazil, respectively.

Only a single isolate from the Western Amazon Basin of Brazil (which was collected in the Granada settlement) had the Y976F change but did not carry the F1076L mutation (complete haplotype = SNLFF). We conclude that the Y976F mutation at the *pvmdr-1* locus remains relatively rare among *P. vivax* isolates from the study sites in the states of Acre, Rondônia and Amazonas, Western Brazilian Amazon.

Single-nucleotide polymorphism at the mdr-1 locus in worldwide *P. vivax* isolates. The prevalence of the Y976F and F1076L mutations in 883 isolates of *P. vivax* collected in the Americas (Brazil, French Guiana, and Honduras), Africa (Mauritania and Madagascar), Asia (Azerbaijan, Turkey, Sri Lanka, Cambodia, Vietnam, Myanmar, Laos, Thailand, Indonesia, and South Korea), and the Southwest Pacific region (Papua New Guinea) is shown in Supplemental Table 1. Overall, 304 (34.5%) and 479 (56.2%) isolates carried the Y976F and F1076L changes, respectively. Wild-type (YF) haplotypes were defined as those not carrying the Y976F or the F1076L mutation, regardless of other amino acid replacements (relative to the *pvmdr-1* sequence of Sal-1 strain, which is defined as wild type) N89S and M908L (Table 1).

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Table 1

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<td>46</td>
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*Values are no. (%) Haplotypes were defined as unique combinations of the following polymorphisms: N89S, N500D, M908L, Y976F, and F1076L (Figure 2). Note that the N906S and M908L changes were found in all isolates.
Figure 2. Distribution of *Plasmodium vivax* multidrug resistance 1 (*pvmdr-1*) haplotypes YF (wild type), YL (single mutant), FF (single mutant), and FL (double mutant) in worldwide isolates. Upper panel, Latin America; central panel, Africa; lower panel, Southeast Asia and Southeastern Pacific region. Sources of data, number of isolates analyzed in each country, and genotyping methods used are described in Supplemental Table 1.
Figure 3. Distribution of the number of Plasmodium vivax multidrug resistance 1 (pvmdr-1) gene copies in worldwide isolates. Upper panel, Latin America; central panel, Africa; lower panel, Southeast Asia. Sources of data, number of isolates analyzed in each country, and genotyping methods used are described in Supplemental Table 2.
nonsynonymous nucleotide substitutions and gene amplification may coexist at the **pvmdr-1** locus.

**Copy number variation of the *mdr-1* gene in worldwide *P. vivax* isolates.** Data shown in Figure 3 indicate that **pvmdr-1** amplification remains relatively rare worldwide, with the exception of Tak Province, Thailand (Supplemental Table 2).\(^{16,25}\) The **pvmdr-1** duplication has not reached prevalence rates more than 3% in any location outside Tak, and the only two isolates with three copies of **pvmdr-1** so far characterized also came from Tak.\(^{16}\) Interestingly, *P. vivax* isolates have been exposed to antimalarial drugs such as mefloquine (either alone or in combination with artesunate) in many other malaria-endemic sites, such as Brazil\(^6\) and most of Southeast Asia, and to artesunate plus amodiaquine in Mauritania,\(^{28}\) but **pvmdr-1** gene amplification does not seem to have been positively selected by these antimalarial drug regimens in most of these malaria-endemic areas.

In addition, we found no clear negative association between the prevalence of the **pvmdr-1** mutations Y976F and F1076L (that are putatively associated with CQ resistance) and **pvmdr-1** gene amplification (that has been associated with increased susceptibility to CQ). Accordingly, Y976F and F1076L mutations co-occur in 15 (29.4%) of 51 isolates from Tak Province, where **pvmdr-1** amplification affects more than 10% of isolates examined. Furthermore, **pvmdr-1** amplification remains uncommon in Brazil and Honduras (Figure 3), where more than two-thirds of local isolates carry the wild-type YF allele (Figure 2).

**DISCUSSION**

Surveillance of emerging drug resistance in *P. vivax* remains technically challenging. Most currently available data have been obtained from *in vivo* assays,\(^5\) but recurrent *P. vivax* parasitemias in clinical trials carried out in malaria-endemic areas may indicate true recrudescences caused by treatment failure, relapses caused by reactivation of liver-stage hypnozoites, or new infections. Molecular genotyping of recurrences does distinguish true recrudescences from relapses with the same strain, and interpreting results from *in vivo* assays is further complicated by confounding factors such as host immunity and previous use of antimalarial drugs.

*In vitro* assays can theoretically provide drug susceptibility estimates free from the effects of most confounding factors that affect *in vivo* assays. However, the lack of a robust, well-standardized and widely applicable protocol for long-term continuous *in vitro* culture remains a major gap in *P. vivax* malaria research.\(^{29}\) As an alternative, short-term *ex vivo* assays have been successfully used to monitor *P. vivax* resistance to CQ across Southeast Asia,\(^8,16,25,27\) but so far there is no published report of *in vitro* antimalarial resistance patterns of Latin American strains of *P. vivax*. Moreover, comparing 50% inhibitory concentration (IC\(_{50}\)) values across different malaria-endemic sites and establishing cut-off IC\(_{50}\) values for defining resistance to CQ and other antimalarial drugs is complicated by factors such as the varying proportion of early trophozoites in the initial blood sample (late trophozoites of *P. vivax* are usually CQ tolerant),\(^{30}\) differences in the time delay between sample collection and *in vitro* analysis, and variation in the duration of the *ex vivo* assay.\(^{31}\)

Molecular markers can represent a more practical tool for monitoring introduction and spread of drug resistance in *P. vivax* populations. The ATP binding cassette transporters such as P-glycoprotein, which is encoded by the **pvmdr-1** gene, have recently been shown to modulate *P. vivax* responses to CQ and other antimalarial drugs.\(^{8,10,13,16,25}\) Further work is needed to assess the predictive value of assays focused on **pvmdr-1** SNPs and copy number variation as resistance monitoring tools.

We showed that more than one-third of all *P. vivax* isolates so far examined worldwide carry the Y976F mutation in the **pvmdr-1** gene, which is putatively associated with CQ resistance. Overall, high Y976F allele frequencies are found in areas where treatment failure with CQ monotherapy became commonplace, although available data remains fragmentary.\(^3\) For example, treatment failure rates have reached 70% in Papua, Indonesia, where 93% of the local *P. vivax* isolates carry the Y976F change, but remain relatively low in Thailand,\(^3\) Brazil,\(^6,7\) and South Korea,\(^32\) where the Y976F change is much less frequent (Figure 2). The present study confirms the relative rarity of the Y976F change in isolates of *P. vivax* from Brazil.

Amplification of the **pvmdr-1** gene, which modulates the *P. vivax* response to CQ, mefloquine, amodiaquine and artesunate,\(^8,16,25\) remains infrequent in Brazil and in most other sites worldwide (Figure 3). The only exception is the Tak Province of Thailand, where > 10% of local *P. vivax* strains carry two or three copies of **pvmdr-1**. The IC\(_{50}\) estimates for mefloquine, amodiaquine and artesunate in *P. vivax* isolates from Tak are significantly higher than those obtained with the same *ex vivo* assay for parasites from Papua, where **pvmdr-1** amplification has not been documented.\(^{16}\) Furthermore, parasites carrying **pvmdr-1** amplification have higher IC\(_{50}\) values for mefloquine when compared with those with a single copy of this gene.\(^{36}\) However, because **pvmdr-1** amplification is mostly restricted to a single geographic location, more *ex vivo*, *in vivo*, and molecular data are required to validate the association between decreased response to mefloquine and the number of **pvmdr-1** gene copies in parasites from other malaria-endemic areas.

In conclusion, we have evaluated nucleotide replacements and copy number variation in the **pvmdr-1** gene, a potential molecular marker of drug-resistant *P. vivax* malaria, in *P. vivax* isolates from northwestern Brazil. We also assembled and analyzed a global database of **pvmdr-1** polymorphism that comprises isolates from all continents and regions where *P. vivax* still poses a major public health challenge. Our data provide a baseline for future studies of *P. vivax* drug resistance and associated molecular markers in Brazil and worldwide. Gathering more *in vivo*, *in vitro*, and molecular data in a standardized way is expected to aid in the process of validating these and other potential markers of *P. vivax* drug resistance.\(^{33}\)

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