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Single-Nucleotide Polymorphism and Copy Number Variation of the Multidrug Resistance-1 Locus of *Plasmodium vivax*: Local and Global Patterns

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Abstract. Emerging resistance to chloroquine (CQ) poses a major challenge for *Plasmodium vivax* malaria control, and nucleotide substitutions and copy number variation in the *P. vivax* multidrug resistance 1 (*pvmdr-1*) locus, which encodes a digestive vacuole membrane transporter, may modulate this phenotype. We describe patterns of genetic variation in *pvmdr-1* alleles from Acre and Amazonas in northwestern Brazil, and compare then with those reported in other malaria-endemic regions. The *pvmdr-1* mutation Y976F, which is associated with CQ resistance in Southeast Asia and Oceania, remains rare in northwestern Brazil (1.8%) and its prevalence mirrors that of CQ resistance worldwide. Gene amplification of *pvmdr-1*, which is associated with mefloquine resistance but increased susceptibility to CQ, remains relatively rare in northwestern Brazil (0.9%) and globally (< 4%), but became common (> 10%) in Tak Province, Thailand, possibly because of drug-mediated selection. The global database we have assembled provides a baseline for further studies of genetic variation in *pvmdr-1* and drug resistance in *P. vivax* malaria.

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 Asia[8] 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

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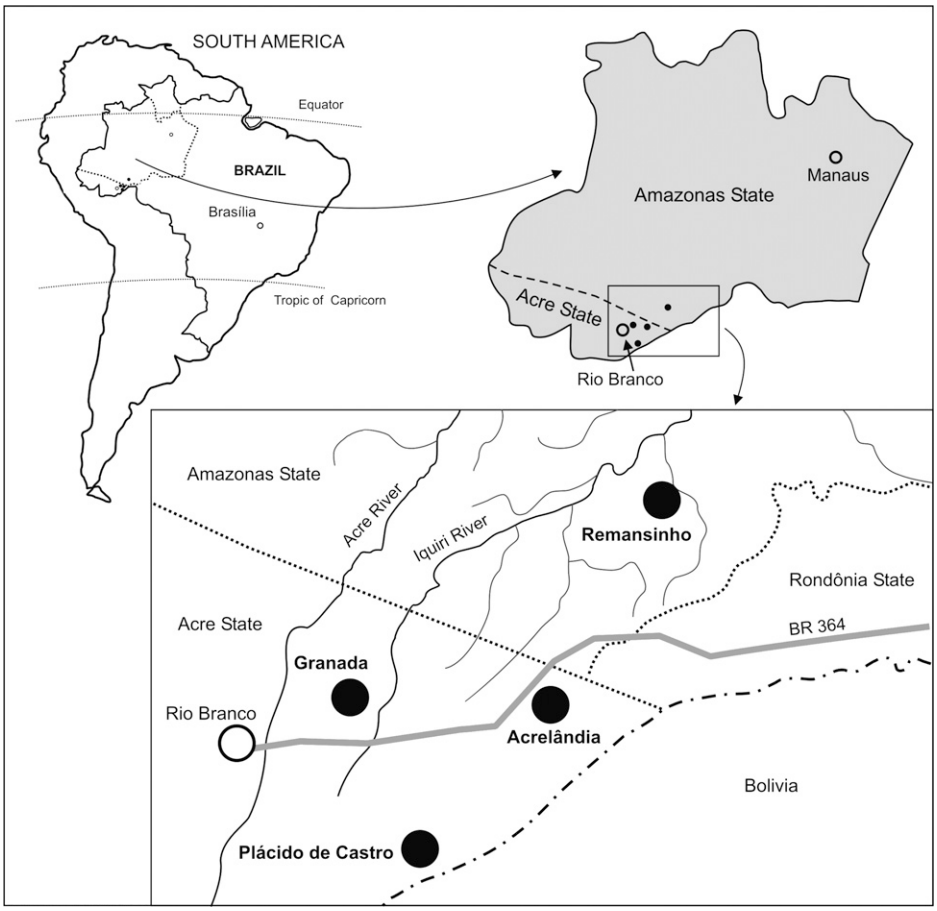

FIGURE 1. Map of northwestern Brazil, showing the three field sites in the Acre State: the farming settlement of Granada and the towns of Acrelândia (40 km south of Granada) and Plácido de Castro (35 km south of Acrelândia), and the one in Amazonas State, the farming settlement of Remansinho (140 km east of Granada). BR 364 is the paved highway that connects Rio Branco (capital of Acre) to the central and southeastern regions of Brazil.

malaria clinics in these towns during 2008–2011.[20] Samples from Remansinho (n = 105) were collected from asymptomatic and symptomatic persons participating in an ongoing prospective cohort study of malaria risk factors.[21]

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.[17,18] *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.[6,7] 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 CTC CGA AGA GAA A-3′) or F2 (5′-CAA TCT AAA AGT CAC 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.[22]

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 quantitation of parasite loads (number of parasites/microliter of blood). We used a Mastercycler realplex 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.[6] There is no evidence of CQ resistance (defined as parasite recrudescence observed despite therapeutic whole-blood CQ levels) in *P. vivax* populations from this region.[19]

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,[8,10,12,13] which correspond to the amino acid changes N89S, N500D, M908L, Y976F, and F1076L. These polymorphisms were genotyped under contract by K-Biosciences (Cambridge, United Kingdom) with an amplifluor assay.[23]

The nucleotide sequences of oligonucleotide primers used to amplify the SNPs were N89S, primer for allele 1 (G): 5′-GAA GGT GAC CAA GTT CAT GCT ATC ATT TAC ATT TTC TCC CAA GTT CAT AC-3′; primer for allele 2 (A): 5′-GAA GGT CGG AGT CAA CGG ATT AAT ATC ATT TAC ATT TTC TCC CAA GTT CAT AT-3′; common primer: 5′-TTC GTA TCC GTT TTT GGG GTC ATT ATG AA-3′; N500D, primer for allele 1 (G): 5′-GAA GGT GAC CAA GTT CAT GCT TAT CGG AGG AGT CGA ACG AAG-3′; primer for allele 2 (A): 5′-GAA GGT CGG AGT CAA CGG ATT CTT ATC GGA GGA GTC GAA CGA AA-3′; common primer: 5′-GCG GCT GTT GGA ATC ACT TTG AGA A-3′; M908L, primer for allele 1 (A): 5′-GAA GGT GAC CAA GTT CAT GCT GGT CAA AAA AGC TAA TTT CTT GGT ACA T-3′; primer for allele 2 (C): 5′-GAA GGT CGG AGT CAA CGG ATT GGT CAA AAA AGC TAA TTT CTT GGT ACA G-3′; common primer: 5′-GAA GAC CAT GAA ACT TAG ACT GTT CGA AA-3′; Y976F, primer for allele 1 (T): 5′-GAA GGT GAC CAA GTT CAT GCT GGC TGT ACT GAC CGG AAC GTT-3′; primer for allele 2 (A): 5′-GAA GGT CGG AGT CAA CGG ATT GGC TGT ACT GAC CGG AAC GTA-3′; common primer: 5′-CGC TCT GAT GGC AAA CAC TCT CAT A-3′; and F1076L, primer for allele 1 (C): 5′-GAA GGT GAC CAA GTT CAT GCT GGT TCA GTC AGA GTG CCC AAC-3′; primer for allele 2 (T): 5′-GAA GGT CGG AGT CAA CGG ATT GGG TTC AGT CAG AGT GCC CAA T-3′; common primer: 5′-CCA AAC CAG TAG GCA AAA CTG TTA ATG AA-3′.

The annealing temperature for all primer pairs was 60°C. Accuracy of genotyping was empirically assessed as 99.8% in blind replicate analyses.[14] Counting all alleles when multiple clones co-occur within infections would result in an overestimation of frequencies of rare alleles and underestimation of common alleles. To minimize this bias, we excluded infections in which > 1 allele was observed in any of the SNPs tested. We also excluded infections with one or more SNPs without allele calls.

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.[8] Plasmids containing amplified fragments of the *pvmdr-1* and *pvaldolase* genes cloned into pGEM-T Easy vectors (Promega) were used as calibrators. In brief, PCR was performed in triplicate in a StepOnePlus real-time thermal cycler (Applied Biosystems, Foster City, CA), with the following oligonucleotide primer pairs: PvmdrF (5′-CTG ATA CAA GTG AGG AAG AAC 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′-CCT TCT CAA CAT TCT CCT TCT TTC 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.

The PCR efficiency of primer pairs for *pvmdr-1* and *pvaldolase* was evaluated using six serial dilutions of the plasmids containing the respective targets and was found to be sufficiently similar to obviate the need for any correction factor. We thus estimated the number of *pvmdr-1* copies by using a comparative threshold method, with the formula $\Delta\Delta Ct = (Ct_{pvmdr\text{-}1} - Ct_{aldolase})_{sample} - (Ct_{pvmdr\text{-}1} - Ct_{aldolase})_{calibrator}$, where Ct is the cycle threshold for each gene.[8] 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) > 0.2.[24]

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),[13] Suwaranusk and others, 2007 (7 isolates from Thailand and 24 from Indonesia)[8]; Barnadas and others, 2008 (80 isolates from Madagascar)[11]; Imwong and others, 2008 (5 isolates from Myanmar, 5 from Laos, and 11 from Thailand)[25]; Marfurt and others, 2008 (94 isolates from Papua New Guinea)[9]; Gama and others, 2009 (28 isolates from Brazil)[26]; Orjuela-Sánchez and others, 2010 (8 isolates from Brazil, 20 from Sri Lanka, 69 from Cambodia, and 9 from Vietnam)[14]; Jovel and others, 2011 (37 isolates from Honduras)[24]; Lu and others, 2011 (96 isolates from South Korea, 24 from Thailand, 11 from Myanmar, 24 from Thailand, and 1 from Papua New Guinea)[27]; and Mint Lekweiry and others, 2012 (103 isolates from Mauritania).[28] Further details about this dataset, which comprises 883 samples, are shown in Supplemental Table 1.

We also collated data for *pvmdr-1* copy number from the following publications: Suwanarusk and others, 2008 (71 isolates from Thailand and 114 from Indonesia)[16]; Imwong and others, 2008 (49 isolates from Myanmar, 50 from Laos, and 116 from Thailand)[25]; Jovel and others, 2011 (63 isolates from Honduras)[24]; Lu and others, 2011 (96 isolates from

South Korea, 27 from Thailand, and 11 from Myanmar)[27]; 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 such as N89S, N500D, and M908L (that were commonly found, for example, in the Western Amazon Basin of Brazil). Worldwide distribution of the YF, YL, FF, and FL *pvmdr-1* haplotypes is shown in Figure 2; only countries and regions with > 15 isolates analyzed were represented. Wild-type YF haplotypes predominated in the Americas and the Southwest Pacific region, but already became rare in Africa and South Korea.

The YF haplotypes are heterogeneously distributed in Brazil. Most (82.9%) of 228 samples from the Western Amazon, but none of the 28 isolates from Paragominas, in Pará State (Eastern Brazilian Amazon),[26] had the YF haplotype. The distance between Paragominas and the other four sites in Brazil is > 2,300 km. The substantial geographic variation in *pvmdr-1* allele frequencies that has been documented in Brazil contrasts with the fact that parasites are exposed to essentially the same antimalarial drugs countrywide. Double-mutant FL haplotypes, which are rare in Brazil, were found in 88–100% of isolates from Madagascar, Cambodia, Vietnam, and Indonesian Papua, and single-mutant YL haplotypes, which predominated in Brazil, were also common in Mauritania and some locations in Asia, especially in South Korea.

Single-mutant FF haplotype (in which the Y976F mutation does not co-occur with the F1076L mutation) was not exceedingly rare, but was heterogeneously distributed. Sixty-five (7.4%) of the 883 *P. vivax* isolates analyzed worldwide had the FF haplotype, but they originated from only three countries: Brazil (Paragominas and Granada),[14,26] Honduras,[24] and Papua New Guinea.[9] More extensive genotyping at and surrounding the *pvmdr-1* locus might indicate whether these haplotypes share a common ancestor. The relatively high prevalence of the FF haplotype in some sites undermines the hypothesis of a two-step mutation pathway underlying CQ resistance.[13] Thus, we cannot interpret detection of the first mutation (F1076L) as an early warning of the imminent emergence of CQ resistance.[14]

Copy number variation of the *mdr-1* gene in *P. vivax* isolates from northwestern Brazil. Of 217 samples from northwestern Brazil available for analysis, we obtained a *pvmdr-1* copy number estimate for 215 isolates (10 collected in Acrelândia, 54 in Plácido de Castro, 97 in Granada, and 54 in Remansinho); only two samples (both from Granada) yielded no amplification. Although local *P. vivax* strains are potentially exposed to mefloquine, which is commonly used to treat *P. falciparum* malaria, we found only two isolates with a duplication of the *pvmdr-1* gene. Samples with two copies of *pvmdr-1* were collected at different times in Acre State: one was from Plácido de Castro (collected in 2008) and other was from Granada (collected in 2004). These sites are located 75 km apart. In both cases, parasites carried the wild-type YF haplotype. Nevertheless, isolates with *pvmdr-1* gene amplification from Mauritania[28] and Thailand[25] also carried mutations in residue 1076 or in residues 976 and 1076, which suggested that

TABLE 1

Frequencies of *Plasmodium vivax* multidrug resistance 1 gene haplotypes among isolates from four locations in northwestern Brazil*

Haplotype	Location (date) of isolate collection				
	Acrelândia, Acre (2008–2010)	Plácido de Castro, Acre (2008–2010)	Remansinho, Amazonas (2008–2010)	Granada, Acre (2004–2006)	All locations (2004–2010)
SDLYF	4 (30.8)	5 (8.5)	31 (67.4)	1 (0.9)	41 (18.0)
SDLYL	1 (7.9)	11 (19.0)	0	11 (9.9)	23 (10.1)
SNLYF	4 (30.8)	39 (67.2)	15 (32.6)	90 (81.1)	148 64.9)
SNLYL	4 (30.8)	2 (3.4)	0	6 (5.4)	12 (5.3)
SNLFL	0	0	0	2 (1.8)	2 (0.9)
SNLFF	0	0	0	1 (0.9)	1 (0.4)
SDLFL	0	1 (1.7)	0	0	1 (0.4)
No. samples	13	58	46	111	228

*Values are no. (%). Haplotypes were defined as unique combinations of the following polymorphisms: N89S, N500D, M908L, Y976F, and F1076L (Figure 2). Note that the N89S and M908L changes were found in all isolates.

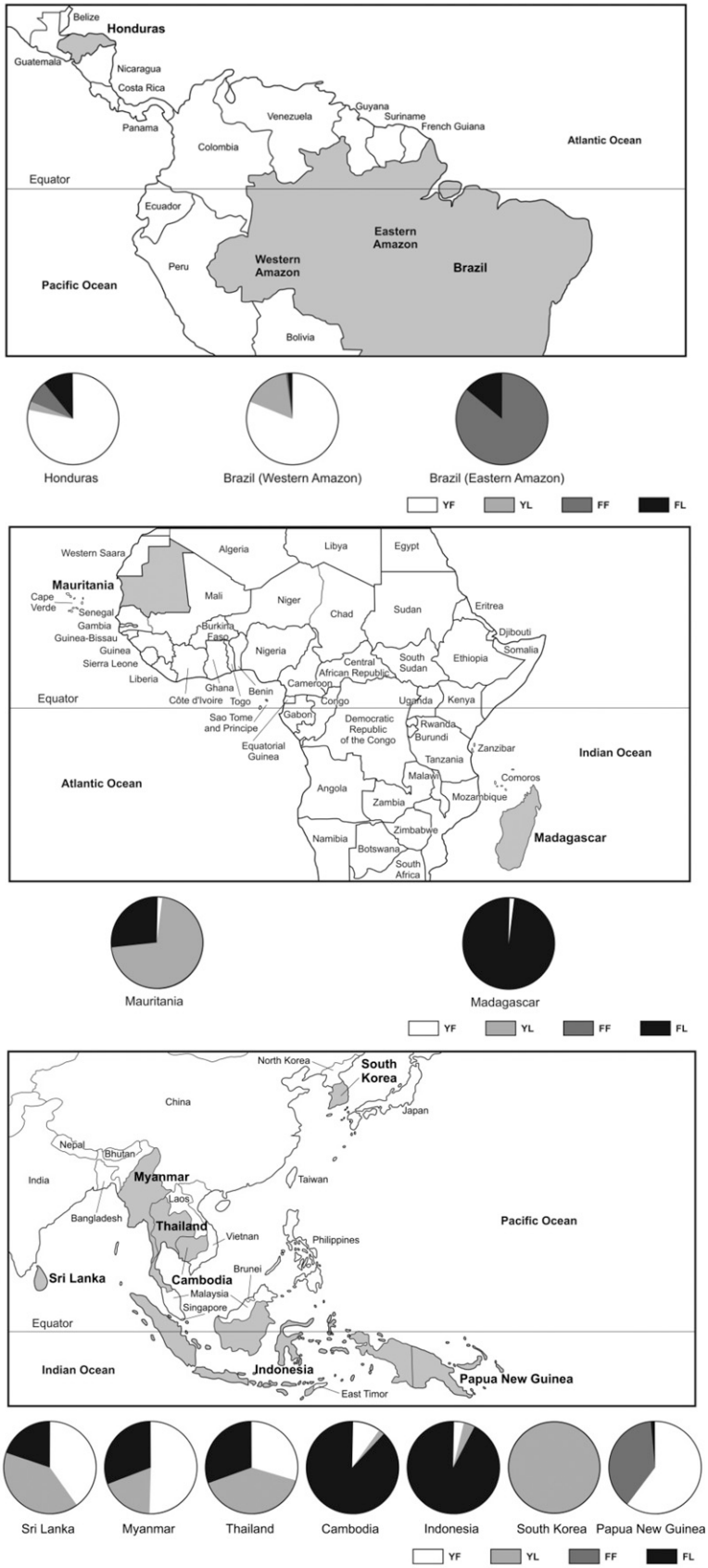

FIGURE 2. Distribution of *Plasmodium vivax* multidrug resistance 1 (*pvmdr-1*) haplotypes YF (wild type), YL (single mutant), FF (single mutant), and FL (double mutant) in worldwide isolates. Upper panel, Latin America; central panel, Africa; lower panel, Southeast Asia and Southeastern Pacific region. Sources of data, number of isolates analyzed in each country, and genotyping methods used are described in Supplemental Table 1.

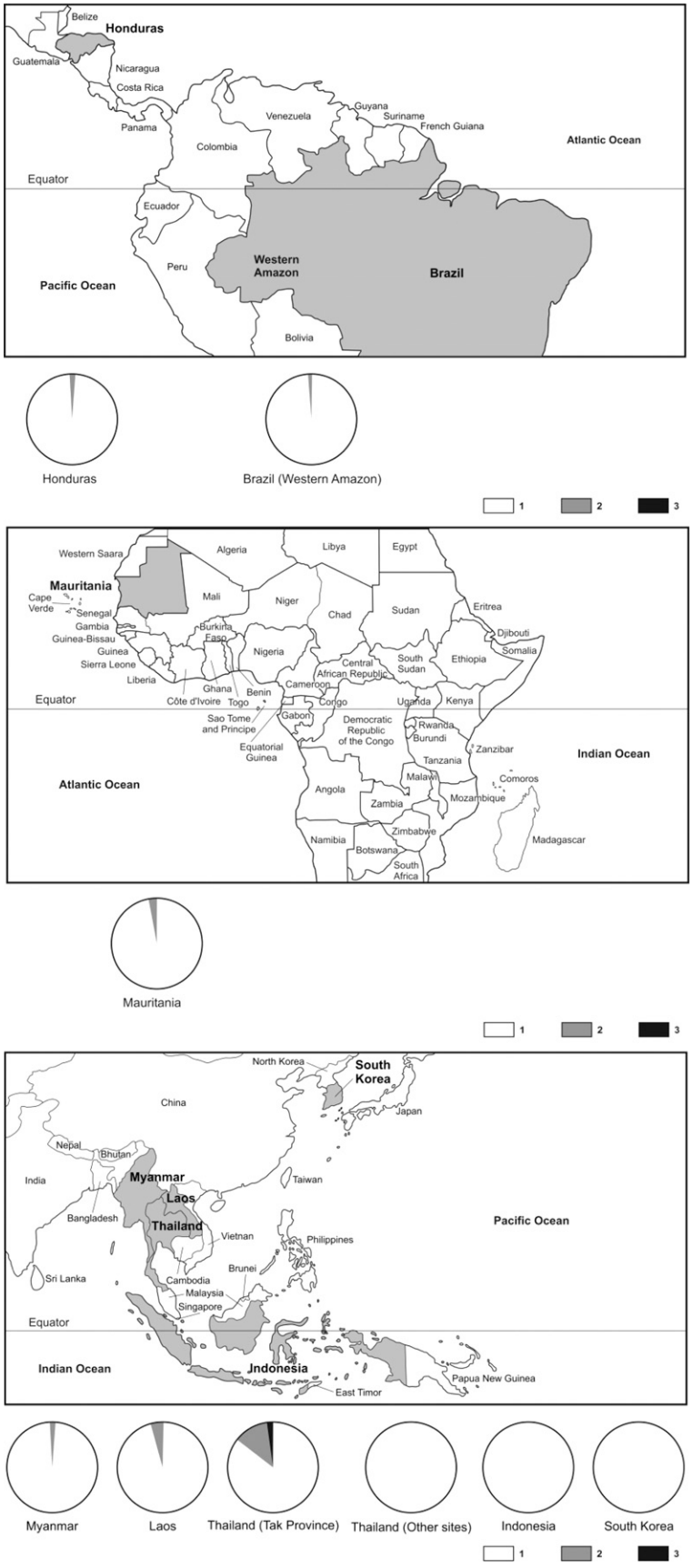

FIGURE 3. Distribution of the number of *Plasmodium vivax* multidrug resistance 1 (*pvmdr-1*) gene copies in worldwide isolates. Upper panel, Latin America; central panel, Africa; lower panel, Southeast Asia. Sources of data, number of isolates analyzed in each country, and genotyping methods used are 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,[3] 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] but 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.

More than half of the 883 isolates examined worldwide carry the F1076L mutation, but using this polymorphism for predicting early CQ resistance, before the Y976F change becomes widespread, may be misleading in regions where parasites carrying the single-mutant FF haplotype are commonly found. We conclude that the Y976F change is not necessarily preceded by the F1076L mutation.

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.[16] 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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