Recurrent Parasitemias and Population Dynamics of Plasmodium vivax Polymorphisms in Rural Amazonia

Pamela Orjuela-Sánchez, Natal Santos da Silva, Mônica da Silva-Nunes, and Marcelo Urbano Ferreira*

Abstract. Clinical trials documented alarming post-treatment Plasmodium vivax recurrence rates caused by recrudescence of surviving asexual blood stages, relapse from hypnozoites, or new infections. Here we describe high rates of P. vivax recurrence (26–40% 180 days after treatment) in two cohorts of rural Amazonians exposed to low levels of malaria transmission after a vivax malaria episode treated with chloroquine-primaquine. Microsatellite analysis of 28 paired acute infection and recurrence parasites showed only two pairs of identical haplotypes (consistent with recrudescences or reactivation of homologous hypnozoites) and four pairs of related haplotypes (sharing alleles at 11–13 of 14 microsatellites analyzed). Local isolates of P. vivax were extraordinarily diverse and rarely shared the same haplotype, indicating that frequent recurrences did not favor the persistence or reappearance of clonal lineages of parasites in the population. This fast haplotype replacement rate may represent the typical population dynamics of neutral polymorphisms in parasites from low-endemicity areas.

INTRODUCTION

At a time when the Bill and Melinda Gates Foundation and the World Health Organization advocate eradication as the ultimate goal of malaria control strategies worldwide, it remains unclear why malaria proves so difficult to control in Brazil, a middle-income country with relatively low levels of transmission. Malaria burden in this country increased 10-fold between 1970 and the mid-1980s, after the massive settlement of non-immune subjects, involved in farming, timber extraction, and open-cast gold mining, on the fringes of the Amazonian rainforest. The Program for Malaria Control in the Amazon Basin, started in 1989, had a clear short-term impact; malaria morbidity decreased by 60% between 1989 and 1996, and an estimated 231,000 deaths were prevented. However, the gains were not sustained over the next years, and the overall malaria burden increased by 34% between 1998 and 1999. Further intensification of control efforts, through early diagnosis and treatment, once again resulted in a non-sustained decrease in malaria incidence between 2000 and 2002. The most recent incidence data available are for 2007, when 458,041 slide-confirmed malaria cases were reported country-wide, 99.9% of which were acquired across the Amazon Basin. These figures represent 57.4% of all clinical malaria cases recorded in the Americas and the Caribbean in 2007.

Recent data also show changes in the relative contribution of different malaria parasite species to the total malaria burden in Brazil. Whereas transmission of P. falciparum, which predominated between 1985 and 1990, decreased steadily, that of P. vivax maintained an upward trend throughout the 1990s. P. vivax now accounts for 80% of the malaria burden in this country. As in other parts of the world, the little success in reducing malaria incidence in Brazil over the past decade largely resulted from a failure to prevent P. vivax infections, providing a compelling stimulus for malaria vivax-oriented research in this country.

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Clinical trials of antimalarial drugs documented alarming P. vivax recurrence rates in Thailand and Papua New Guinea, which may reach 65% over 1–6 months of follow-up. Reappearance of parasitemia after drug treatment can result from either (1) recrudescence of surviving asexual blood-stage parasites, (2) relapse from dormant liver stages known as hypnozoites, or (3) new infections with unrelated parasites. Molecular genotyping of paired parasite samples usually makes a distinction between recrudescences (with the same genotype as the initial infection) and new infections (with a different genotype), but relapses may originate from reactivation of either the same parasite clone found in the primary bloodstream infection (homologous hypnozoites) or another, genetically different clone (heterologous hypnozoites). Recurrence patterns of vivax malaria after drug treatment have been predominantly studied in areas virtually free of malaria transmission in Brazil, where new infections are unlikely, but they remain little studied across malaria-endemic regions of this country. Here we evaluate the incidence and timing of parasite recurrences in two cohorts of rural Amazonians with laboratory-confirmed P. vivax infection treated with a standard regimen of chloroquine (CQ)-primaquine (PQ) therapy. We compare acute infection and recurrence parasite haplotypes and examine whether frequent recurrences affect the population dynamics of P. vivax polymorphisms.

MATERIALS AND METHODS

Study area. The state of Acre, in the Western Amazon Basin of Brazil, borders with Peru, Bolivia, and the Brazilian states of Amazonas and Rondônia. The study site, Granada (9°44′–9°49′ S, 67°05′–67°07′ W), was a sparsely peopled rubber tapper settlement in the eastern corner of Acre that became part of the Pedro Peixoto Agricultural Settlement Project in 1982. The area is characterized by a humid equatorial climate and receives the most rainfall (annual average, 2.198.5 mm) between December and March. The mean annual temperature is 24.5°C. The annual incidence rate of slide-confirmed vivax malaria in Granada ranged from 31.1 cases/100 person-years at risk in 2004 to 9.0 cases/100 person-years at risk in 2006 (N.S.d.S., M.d.S.-N., and M.U.F., unpublished observations).
Cohort A (2004–2005). Between March 2004 and May 2005, 509 inhabitants of Granada were maintained under clinical and laboratory surveillance for symptomatic malaria episodes through active case detection.\textsuperscript{22} A study clinician visited the area five times a week (Monday through Friday) and examined all study participants reporting current or recent fever, headache, or any other malaria symptom. Venous or finger-prick blood samples for malaria diagnosis by microscopy and polymerase chain reaction (PCR) were obtained from each symptomatic subject. The first laboratory-confirmed episode of \textit{P. vivax} malaria diagnosed in study participants after March 2004 was defined as the index infection and treated with CQ and PQ. Cohort A comprised 87 subjects <1–65 years of age (median, 14 years) who were followed up for 7–428 days (median, 336 days) after the index infection, until the end of the study (May 31, 2005) or the date when they left the study site, whatever came first. The timing of slide-positive malaria recurrences in cohort subjects was recorded, and parasite species were further confirmed by PCR using genomic DNA extracted from either venous or finger-prick blood samples.

Cohort B (2005–2007). The 77 participants in Cohort B, recruited between August 2005 and November 2006, were inhabitants of Granada 5–75 years of age (median, 19 years) with slide-positive vivax malaria confirmed by PCR at the time of the enrollment and treated with CQ and PQ. Women of childbearing age were tested for pregnancy and excluded if tested positive. The cohort was followed up for 181–647 days (median, 355 days), until the last follow-up visit (May 20, 2007) or the date the study subject moved away from Granada, whatever came first. The primary endpoint was the reappearance of \textit{P. vivax} in the bloodstream after drug treatment of the index infection. As in Cohort A, a field clinician visited the area five times a week and examined all cohort participants reporting malaria symptoms. Patients’ files kept at the three malaria diagnosis outposts located in Granada were examined daily to detect additional malaria episodes among study participants who reported directly to these facilities. All slide-positive recurrences were recorded, and parasite species were confirmed by PCR. Levels of CQ and its metabolite, desethylchloroquine (DCO), were measured in pre-treatment blood samples whenever the time interval between the current and the previous vivax malaria episode treated with standard CQ-PQ regimens was <2 months. Whole blood drug levels were measured in duplicate samples, under contract, by the Analytical Service of Gates Malaria Partnership at the London School of Hygiene and Tropical Medicine, London, UK, using high-performance liquid chromatography (HPLC) coupled with a photodiode array. The detection threshold for both compounds of interest was 50 ng/mL of blood.

Laboratory diagnosis of malaria. Giemsa-stained thick blood smears had at least 100 fields examined for malaria parasites under ×700 magnification by two experienced microscopists. Blood samples were further examined for malaria parasites by nested PCR-based amplification of a species-specific segment of the 18S rRNA gene of human malaria parasites. DNA templates for PCR amplification were isolated from 200 μL of whole venous blood, using Wizard genomic DNA purification kits (Promega, Madison, WI) or from finger-prick blood samples spotted onto FTA Micro Cards (Whatman, Clifton, NJ). The first round of PCR amplification (35 cycles) was carried out with 1 μL of parasite DNA solution with the genus-specific oligonucleotide primers P1-Up and P2, which amplify a 130-bp fragment of the 18S rRNA gene. The second round of PCR amplification (18 cycles) was carried out with 1 μL of the first PCR product (diluted 1:50 in distilled water), the forward primer P1, and one of the following species-specific reverse primers: F2, V1, or M1. The length of the second PCR product is ~100 bp. Primer sequences and assay protocols are described by Win and others.\textsuperscript{23}

Drug treatment of vivax malaria. \textit{Plasmodium vivax} malaria was treated according to the current therapy guidelines of the Ministry of Health of Brazil,\textsuperscript{24} with 25 mg/kg of CQ base over 3 days (maximum adult dose, 1.5 g over 3 days) and 0.5 mg/kg/day of PQ base for 7 days (maximum adult dose, 30 mg/day). Drug treatment of Cohort A subjects was provided by trained health workers, and only the first three doses were supervised; Cohort B subjects had their whole treatment supervised by a research clinician. Because the maximum adult doses of CQ and PQ were calculated according to the national therapy guidelines for subjects weighing 60 kg, patients who exceed this weight limit may have received subtherapeutic doses.\textsuperscript{25} All cohort subjects were screened for glucose-6-phosphate dehydrogenase (G6PD) deficiency, with the colorimetric method of Tantular and Kawamoto,\textsuperscript{26} before PQ administration. The prevalence of G6PD deficiency in Granada is ~3.9%.\textsuperscript{26}

Microsatellite typing. We typed 14 highly polymorphic single-copy microsatellites that map to 10 different chromosomes of \textit{P. vivax} (Table 1). Alleles were PCR-amplified with the oligonucleotide primers described by Karunaweera and others.\textsuperscript{27} For each PCR reaction, 3 μL of genomic DNA (prepared as described above) were used with 2 mmol/L MgCl\textsubscript{2}, 6 pmol/L of each forward and reverse primer, 0.1 mmol/L of each dNTP, 1 U of recombinant Taq polymerase, and 1.5 μL of 10× Taq polymerase buffer in a final volume of 15 μL. All reagents were purchased from Fermentas (Vilnius, Lithuania), except for primers (both labeled with fluorescent dyes and unlabeled), which were supplied by Applied Biosystems (Foster City, CA). The cycling parameters were set to 1 cycle at 94°C for 2 minutes, 40 cycles at 94°C for 30 seconds, 56°C ×

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* Forward primers were labeled with the fluorescent dyes 6-FAM (6-carboxyfluorescein; “blue” label), VIC (Applied Biosystems proprietary “green” fluorescent dye), or NED (Applied Biosystems proprietary “yellow” fluorescent dye). Primer sequences are given by Karunaweera and others.\textsuperscript{27}
† Chr, chromosome location in the Salvador-I strain according to the complete genome sequence assembly published by Carlton and others.\textsuperscript{28}
‡ Number of alleles (PCR-amplified fragments with different lengths) observed in 99 \textit{P. vivax} isolates from Granada, Acre, Brazil.
§ Virtual heterozygosity (average probability that a pair of alleles randomly obtained from the population is different) calculated for 99 \textit{P. vivax} isolates from Granada, Acre, Brazil.

Table 1: Characterization of the 14 polymorphic \textit{P. vivax} microsatellite loci used to type parasite samples from Granada, Acre, Brazil.
PLASMODIUM VIVAX RECURRENTS IN RURAL AMAZONIA

for 40 seconds, and 72°C for 50 seconds, and a final cycle at 72°C for 5 minutes. PCR products were analyzed on an automated DNA sequencer ABI310 (Applied Biosystems), and their lengths (in bp) and relative abundance (peak heights in electropherograms) were determined using the open-access STRand software (available at http://www.vgl.ucdavis.edu/informatics/strand.php). The minimal detectable peak height was set to 200 arbitrary fluorescence units. We scored two alleles at a locus when the minor peak was >33% of the height of the predominant peak. Infections were considered to contain multiple clones if at least one locus showed more than one allele; for each multiple-clone infection, we recorded the number of loci showing two or more alleles. Multilocus haplotypes were defined as unique combinations of alleles at each locus analyzed; in most analysis, only the most abundant alleles were considered for haplotype assignment in multiple-clone infections. However, given the risk of misassigning haplotypes because of biased amplification of co-existing alleles in multiple-clone infections, we considered all possible haplotypes when comparing acute infection and recrudescence parasites. For this purpose, we listed all combinations of alleles (considering major and minor peaks) for paired acute-phase and recrudescence samples and examined whether one of the possible haplotypes occurred in both samples of the pair.

Data analysis. A database was created with SPSS 16.0 software (SPSS, Chicago, IL). Continuous variables (age and time of residence in Granada, a proxy of cumulative exposure to malaria in migrants) were compared with the nonparametric U test of Mann-Whitney. Kaplan-Meier survival curves were used to estimate the proportion of subjects who remained free of P. vivax recurrence as a function of time of follow-up while allowing for censored data (when no recurrence was observed until the end of the subjects’ follow-up). Survival curves for Cohorts A and B were compared with the Cox proportional hazard model, with the endpoint defined as the first P. vivax recurrence diagnosed during the follow-up while controlling for subjects’ age and time of residence in Granada. We estimated the virtual heterozygosity (H_v) as a measure of overall genetic diversity in the parasite population. It is defined as \( H_v = \frac{\nu(n-1)}{n(1-\rho)^2} \), where \( n \) is the number of isolates analyzed and \( \rho \) is the frequency of the \( i \)th allele in the population. \( H_v \) gives the average probability that a pair of alleles randomly obtained from the population is different. In diploid organisms, this would give the average probability of having heterozygotes at that given locus. Virtual heterozygosity ranges between 0 and 1. The standardized index of association (\( I^2 \)) was used to test whether multilocus linkage disequilibrium occurred in local parasites. This test compares the variance (\( V_d \)) of the number of alleles shared between all pairs of haplotypes observed in the population (\( D \)) with the variance expected under random association of alleles (\( V_e \)) as follows: \( I^2 = (V_o/V_e - 1)(r - 1) \), where \( r \) is the number of loci analyzed. \( V_o \) is derived from 10,000 simulated data sets in which alleles were randomly reshuffled among haplotypes. Significant linkage disequilibrium is detected if \( V_o \) is greater than 95% of the values derived from the reshuffled data sets. Data were analyzed with LIAN 3.5 software (available at http://pubmlst.org/perl/mlstanalyse/mlstanalyse.pl?site=pubmlst&page=lian&referer=pubmlst.org). We used version 3 of eBURST software (available at http://eburst.pubmlst.net/) to identify clusters of closely related haplotypes, defined as groups of haplotypes that are identical to each other at 11 or more of the 14 loci analyzed. These clusters were used to examine patterns of genetic diversity of parasites over time. We further tested whether pairwise genetic relatedness (proportion of alleles shared between haplotypes) decreased with increasing distance between the dates of sample collection, by using the Mantel nonparametric matrix correlation test, implemented in Poptools 2.7.1 software (available at http://www.cse.csiro.au/poptools/). The significance of the correlation coefficient (\( r \)) was tested with 1,000 permutations. Statistical significance was defined at the 5% level in all analyses.

Ethical considerations. Approval of the study protocol was obtained from the Ethical Review Board of the Institute of Biomedical Sciences of the University of São Paulo, Brazil (538/2004 and 773/2007). Written informed consent was obtained from all study participants or their parents/guardians.

RESULTS

Plasmodium vivax recurrence rates. Recurrences of P. vivax parasitemia after CQ-PQ treatment were common in Granada. The proportions of subjects who remained free of recurrence at the end of the follow-up were 28.5% for Cohort A (maximum follow-up duration, 420 days) and 63.4% for Cohort B (maximum follow-up duration, 647 days), with the median time to the first P. vivax recurrence in Cohort A estimated as 212 days (Figure 1). After 180 days of post-treatment follow-up, 39.7% and 25.7% of subjects in Cohorts A and B, respectively, had experienced a recurrence. Overall, 72 recurrences (1–5 per subject) were diagnosed in Cohort A and 34 recurrences (1–3 per subject) in Cohort B, with a significantly lower risk of recurrences in Cohort B compared with Cohort A (risk ratio, 0.396; 95% confidence interval [CI], 0.239–0.657; \( \chi^2 = 13.80, 1 \text{ df}, P = 0.0002 \) by log-rank test). Because Cohort B subjects were older (median age, 19 versus 14 years; \( P = 0.016 \), Mann-Whitney U test) and had a longer median time of residence in the Granada area (13 versus

FIGURE 1. Kaplan-Meier estimates of the proportions of Cohort A and Cohort B participants who remained free of P. vivax recurrence after a drug-treated episode of infection with this species acquired in Granada, Acre, Brazil. Cohort A comprised 87 subjects <1–65 years of age who were followed up for 7–428 days between April 2005 and May 2005, after a laboratory-confirmed P. vivax infection treated with CQ and PQ. Cohort B comprised 77 subjects 5–75 years of age who were followed up for 181–647 days between August 2005 and May 2007, after a vivax malaria episode treated under supervision with CQ and PQ.
Timing of *P. vivax* recurrences. Figure 2 shows the distribution of the time intervals between consecutive *P. vivax* infections diagnosed in the same subjects during the follow-up of both cohorts. For subjects with multiple recurrences, we calculated the time distance between each laboratory-confirmed recurrence and the most recent previous drug-treated episode of *P. vivax* infection. The median time interval between laboratory-confirmed *P. vivax* infections was slightly shorter in Cohort A (86 days) than in Cohort B (100 days) (*P* = 0.162, Mann-Whitney *U* test), and most recurrences (79.2% of those observed in Cohort A and 76.5% in Cohort B) were diagnosed up to 180 days after drug treatment. Only four recurrences (three of them in Cohort A) were diagnosed up to 28 days after treatment. The only Cohort B subject (Subject 17) with an early reappearance of parasitemia after drug treatment had undetectable whole blood levels of CQ and DCQ at the time of the recurrence. The *P. vivax* isolates recovered from this early recurrence and the most recent previous infection (diagnosed and treated with CQ-PQ 26 days earlier) differed by a single microsatellite allele (Table 2).

Population dynamics of *P. vivax* polymorphisms. Frequent recrudescences and relapses can theoretically affect the temporal dynamics of polymorphisms by extending the persistence of clonal lineages of parasites in the population. To examine whether endemic haplotypes were maintained in the population by recrudescences and relapses, we analyzed the microsatellite diversity of *P. vivax* isolates over time. Because results for Cohort A have already been published,33 here we focus on 99 *P. vivax* isolates collected between August 2005 and December 2006 from 77 Cohort B subjects (more than one consecutive infection was analyzed for 16 subjects). *Plasmodium vivax* isolates collected during Cohort B follow-up were highly diverse, with virtual heterozygosity (*H*_E) estimates ranging between 0.4754 and 0.8677 for different markers (Table 1). Two or more genetically distinct clones were found in 42 (42.4%) isolates, as judged by the presence of more than one allele in at least one of the 14 single-copy microsatellite markers analyzed. The number of markers with more than one allele detected in multiple-clone infections ranged between one and nine (Figure 3). Consistent with previous reports,16,34 the proportion of multiple-clone infections decreased substantially when more strict criteria were applied to detect additional clones: only 18.2% of the 99 isolates analyzed had 2 or more of the 14 loci with more than one allele each and only 10.1% of them had more than one allele at 4 or more loci.

Despite the relatively high clonal diversity of blood stage infections, which would allow for frequent outcrossing during the sexual reproduction of parasites in the mosquito vector, we detected a highly significant multilocus linkage disequilibrium (*I*^S^ < 0.068, *P* < 0.0001), consistent with a predominantly clonal mode of reproduction of local isolates. We further analyzed single-clone infections (*N* = 57) separately to detect possible biases caused by misassignment of haplotypes in multiple-clone infections,28 but levels of linkage disequilibrium remained nearly unchanged (*I*^S^ = 0.083, *P* < 0.0001). We recovered 98 unique haplotypes from 99 *P. vivax* isolates analyzed (Supplementary Table 1 available online at www.ajtmh.org). A single pair of parasites (collected between July and August 2006, from two unrelated subjects, 27 days apart) shared the same multilocus haplotype (haplotype 5 in Supplementary Table 1 online). We found a weak, although statistically significant, negative correlation between pairwise genetic similarity and the time interval between dates of collection of isolates (r = −0.086, *P* < 0.001, Mantel correlation test). These findings are consistent with a high haplotype replacement rate as previously described for Cohort A subjects,33 despite the high frequency of *P. vivax* recurrences experienced by infected subjects.

The fact that identical haplotypes are rarely shared between isolates suggests that novel haplotypes are rapidly generated by mutation or recombination or introduced by migration. We explored the temporal dynamics of closely related haplotypes (which were identical to each other at 11–13 of 14 loci analyzed) during Cohort B follow-up (Figure 4). We found 11 clusters of related haplotypes, with 2–14 haplotypes each; 62 haplotypes were defined as singletons, because they shared <11 alleles with all other haplotypes found in the parasite population (Supplementary Table 1 online). The haplotypes that comprise the four largest clonal groups (Clusters 2, 3, 4, and 9, each comprising between 8 and 14 haplotypes) were recovered from parasites collected > 12 months apart (Figure 4), indicating that groups of related haplotypes may be endemic in the population.

Haplotypes in consecutive *P. vivax* infections. Because malaria is transmitted in Granada year-round, recrudescences, relapses,
and new infections may all originate *P. vivax* recurrences. We thus compared multilocus haplotypes in pairs of consecutive *P. vivax* infections diagnosed in Cohort A (7 pairs) and Cohort B (21 pairs). For subjects with multiple recurrences, we compared the parasite found in each recurrence with that found in most recent previous drug-treated episode of *P. vivax* infection in the same subject. For example, parasites recovered from the third *P. vivax* infection of subject 380 of Cohort A (diagnosed October 20, 2004) were compared with those recovered from the second infection (June 8, 2004) diagnosed in the same patient (Table 2). We classified pairs of parasites as identical (sharing identical alleles at all loci analyzed), related (sharing identical alleles at 11–13 loci), or different (sharing identical alleles at < 11 loci). We found only two pairs of identical haplotypes (consistent with recrudescences or relapses originating from the reactivation of homologous
of the recurrence in subjects with a recent (< 2 months) drug-treated *P. vivax* infection (Episode 4 of Subject 17 and Episode 2 of Subjects 46, 54, 63, and 81), suggesting that these recurrent parasites are not necessarily CQ resistant.

**Number of markers needed to describe the overall diversity of *P. vivax* infections.** With the growing use of microsatellite markers in epidemiologic studies and clinical trials,\(^{16,33–35}\) it is essential to define the minimal number of markers that would accurately estimate the overall genetic diversity and proportion of multiple-clone infections in *P. vivax* populations without incurring unnecessary cost. To examine how many markers are needed to score most or all haplotypes and multiple-clone infections found in Cohort B, we first ranked microsatellite loci according to their \(H_E\) values, shown in Table 1. The marker with the highest \(H_E\) value (MS5), when used alone, was able to discriminate 16 different alleles and detect eight multiple-clone infections. By combining MS5 and MS16 (which has the second largest \(H_E\)), we detected 50 different haplotypes and 16 multiple-clone infections. The remaining microsatellites were added one at time to the panel of markers, in order of decreasing \(H_E\). Figure 5 shows that all 42 multiple-clone infection and 95 (of 98) different haplotypes may be scored with a restricted panel of markers comprising the 10 microsatellite loci with the highest \(H_E\) values.

**DISCUSSION**

Here we report high rates of post-treatment *P. vivax* recurrence in a population exposed to relatively low levels of malaria transmission in rural Amazonia. The cumulative incidence of recurrent infections reached 26–40% after 180 days of CQ-PQ treatment, imposing a substantial burden to malaria control efforts. Similarly, a recurrence rate of 16% was observed after 60 days of follow-up of 61 *P. vivax* infections treated with CQ-PQ in the Porto Velho area of Rondônia, ~500 km east of Granada.\(^{21}\) Few early recurrences (up to 28 days after CQ-PQ treatment) were diagnosed in Granada (and none was observed in Porto Velho\(^{21}\)), suggesting that true recrudescences remain relatively rare in this region, despite...
the recent report of CQ resistance in *P. vivax* from Manaus, ~1,000 km north of Granada. 37 No instance of *P. vivax* recurrence despite therapeutic whole blood levels of CQ was found in Granada.

CQ-resistant *P. vivax* remains mostly confined to Indonesia, East Timor, and Papua New Guinea 38 and whether CQ-resistant *P. vivax* currently represents a major concern in Brazil remains to be determined. The recent report of synergism between PQ and CQ against blood stage parasites 36 suggests an explanation for the late emergence of CQ resistance in *P. vivax* from Brazil. Over half a century, PQ and CQ have been routinely associated for the radical cure of *P. vivax* infections in most countries in Latin America. The finding that PQ reverses CQ resistance in *P. falciparum* 39 suggests that a similar effect might occur in *P. vivax* isolates simultaneously exposed to both drugs. Accordingly, all 22 published reports of CQ-resistant *P. vivax* infections acquired in South America refer to patients to whom PQ was either given after CQ treatment, for several reasons, or not administered at all. 37,38 No patient given CQ and PQ simultaneously, as is usual in South America, has been documented thus far to harbor CQ-resistant *P. vivax*.

*Plasmodium vivax* populations circulating in the area were extraordinarily diverse and rarely shared the same multilocus haplotype. These haplotypes rapidly diversified to originate novel, genetically related variants that were detected in local parasites over the next months. Of 28 pairs of consecutive *P. vivax* infections, only 2 were genetically identical, as expected for recrudescences or relapses originating from the reactivation of homologous hypnozoites. In fact, most recurrent infections involved genetically distinct parasites, consistent with new infections or reactivation of heterologous hypnozoites.

Determining the relative contribution of relapses and new infections to parasitemia recurrences is of major public health significance for malaria-exposed populations. If relapses predominate, targeting the hypnozoite with improved drug treatment may be the most effective measure to reduce the vivax-associated malaria burden. 39 The frequent use of subtherapeutic PQ doses in adults is clearly a major risk factor for *P. vivax* relapses in Brazil that remains to be addressed. 39 The timing of most recurrences observed in Granada (76–79% of them diagnosed up to 6 months after drug therapy) is consistent with the known relapse patterns of *P. vivax* in Brazil: 95% of 330 relapses experienced by patients treated with CQ-PQ and followed in a malaria-free environment were diagnosed during the first 6 months of post-treatment follow-up. 39

The distinction between relapses and new infections is particularly challenging in the presence of multiple-clone infections. Until recently, relapses were thought to be caused by homologous hypnozoites, genetically identical to the bloodstage parasites found in primary infections. 4,13 but different parasite genotypes have been recently described in primary infections and relapses of Asian strains of *P. vivax*. 4,14 These findings suggest that relapses may originate from the reactivation of heterologous hypnozoites. 15,16 Alternatively, some haplotypes present in multiple-clone primary infections may have been missed or only partially characterized. Even worse, their alleles might have been combined to create artificial haplotypes during genotyping. 7 Relapsing clones could have been missed or incorrectly typed during the primary infection, leading to a false conclusion that different genotypes were present in paired samples. Drug pressure may further complicate data analysis. If the clonal mixture found in the primary infection comprises parasites with different levels of PQ resistance, less abundant PQ-tolerant clones (barely detectable by genotyping) may have been selected after drug treatment and may dominate the population of relapsing parasites. Further pairwise analyses of acute infection and relapse haplotypes are clearly required to determine the relative role of homologous versus heterologous hypnozoites in relapsing patterns of worldwide *P. vivax* isolates.

Despite the high *P. vivax* recurrence rate in Granada, we found no example of clonal lineages persisting (or reappearing) over several months in the parasite population. The strong linkage disequilibrium between alleles of markers on different chromosomes suggests a minor contribution of meiotic recombination to generate novel multilocus haplotypes. Although 10 of 14 microsatellite markers used to type *P. vivax* isolates map to coding sequences, none of them map to known surface antigens putatively under strong diversifying selection. We therefore suggest that the fast turnover of *P. vivax* microsatellite haplotypes found in Granada, which is little affected by recurrent infections, may represent the typical population dynamics of neutral polymorphisms in small populations. However, given the extraordinarily high mutation rates of microsatellite-type sequences, further analyses of additional, putatively neutral polymorphisms, such as single nucleotide polymorphisms, are essential to evaluate the relative contribution of stochastic (evolutively neutral) processes and natural selection to the population dynamics of antigenic variants and other phenotypes of interest in natural *P. vivax* populations.

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