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Prospective Study of *Plasmodium vivax* Malaria Recurrence after Radical Treatment with a Chloroquine-Primaquine Standard Regimen in Turbo, Colombia

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***Plasmodium vivax* recurrences help maintain malaria transmission. They are caused by recrudescence, reinfection, or relapse, which are not easily differentiated. A longitudinal observational study took place in Turbo municipality, Colombia. Participants with uncomplicated *P. vivax* infection received supervised treatment concomitantly with 25 mg/kg chloroquine and 0.25 mg/kg/day primaquine for 14 days. Incidence of recurrence was assessed over 180 days. Samples were genotyped, and origins of recurrences were established. A total of 134 participants were enrolled between February 2012 and July 2013, and 87 were followed for 180 days, during which 29 recurrences were detected. The cumulative incidence of first recurrence was 24.1% (21/87) (95% confidence interval [CI], 14.6 to 33.7%), and 86% (18/21) of these events occurred between days 51 and 110. High genetic diversity of *P. vivax* strains was found, and 12.5% (16/128) of the infections were polyclonal. Among detected recurrences, 93.1% (27/29) of strains were genotyped as genetically identical to the strain from the previous infection episode, and 65.5% (19/29) of infections were classified as relapses. Our results indicate that there is a high incidence of *P. vivax* malaria recurrence after treatment in Turbo municipality, Colombia, and that a large majority of these episodes are likely relapses from the previous infection. We attribute this to the primaquine regimen currently used in Colombia, which may be insufficient to eliminate hypnozoites.**

Plasmodium vivax has the widest global distribution of the human malaria parasites (1, 2). In the Americas, more than 60% of the annual cases of malaria are caused by *P. vivax* (2). This parasite has a dormant stage known as hypnozoite, in which it remains in the liver for an indeterminate length of time (3). Hypnozoites might be activated months after a previous episode that has been treated and cured, causing relapses (3). Relapses after treatment can cause a new clinical episode with risk of complications for the patient. Moreover, they may contribute to continue *P. vivax* transmission (4, 5).

In areas of endemicity, recurrent *P. vivax* infections have three origins: (i) recrudescence of parasites from blood, which could be caused by resistance to treatment, inadequate dosage, or suboptimal drug absorption; (ii) reinfection by inoculation of new parasites from mosquito bites; or (iii) relapse by reactivation of hypnozoites from the liver (6). Diagnostic methods alone cannot distinguish among these three types of recurrence. However, a comprehensive study, including supervised treatment, individual follow-up for therapeutic efficacy, microscopic and molecular diagnosis, quantification of drugs in blood, and genetic characterization of the parasites, may reveal the source of the recurrence (7, 8).

Per current recommendations of the World Health Organization, the treatment for *P. vivax* infection consists of two drugs: chloroquine (CQ), a blood schizonticide against circulating parasites, and primaquine (PQ), a tissue schizonticide that clears the liver of schizonts (9). CQ remains widely used against *P. vivax* although treatment failures have been reported (10). In Colombia, to this date, there have been no reports of CQ therapeutic failure for *P. vivax* infections (11). PQ is the only antimalarial commercially available to treat liver stages (12).

Previous studies have compared the efficacy of different PQ regimens in preventing *P. vivax* recurrence (13). Although the standard PQ regimen (0.25 mg/kg/day for 14 days) has a significant incidence of recurrence, it is the most commonly treatment used worldwide (2). It was estimated that 16.2% of patients in Colombia treated with this regimen have at least one recurrence within 6 months (14). Nevertheless, the standard PQ regimen yields a lower incidence of recurrences than regimens employing an equal total dose administered for a shorter time (13, 14).

We conducted a prospective study in an area of endemicity in Colombia to determine the incidence of *P. vivax* recurrences in a 6-month period posttreatment in patients who underwent a su-

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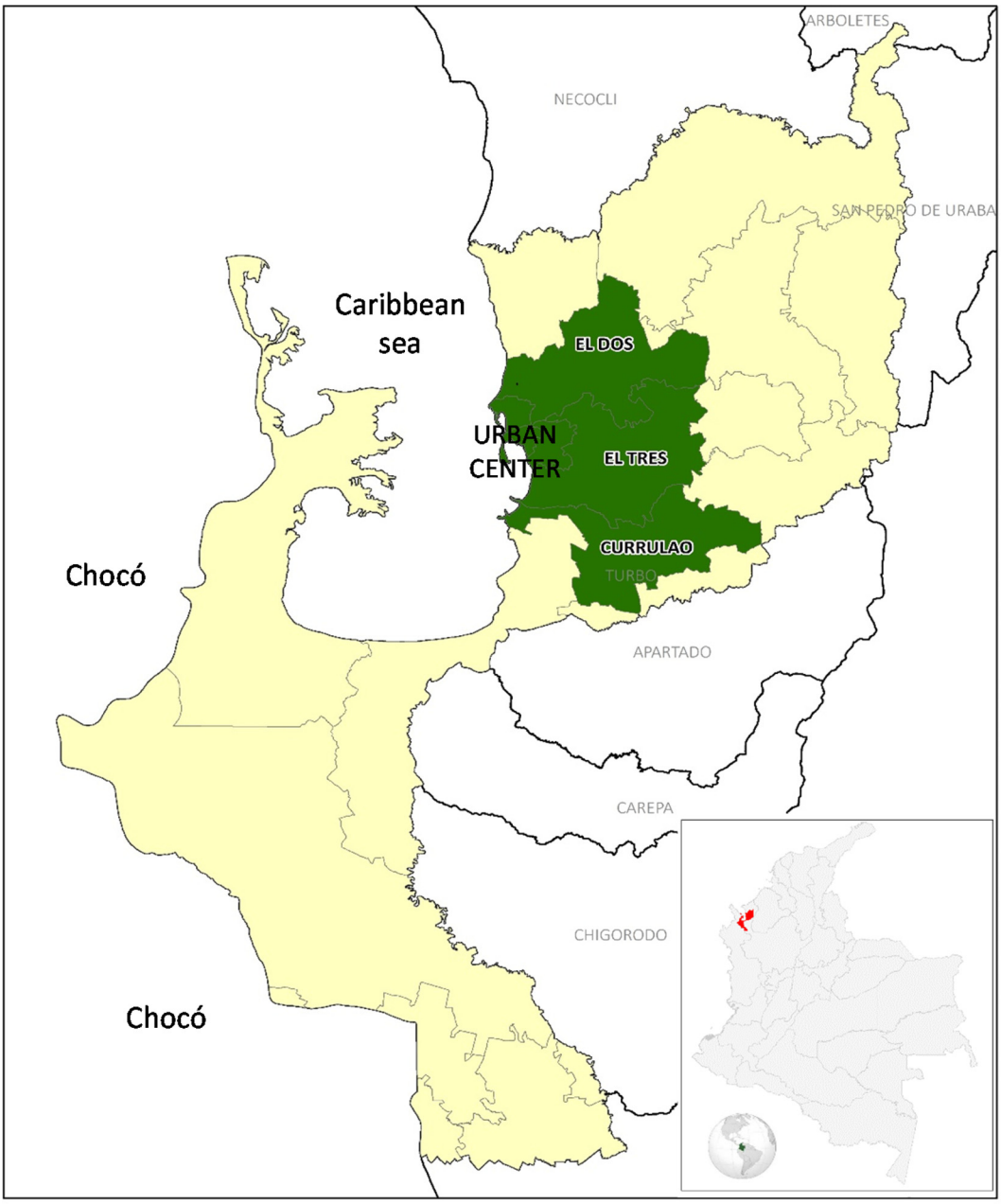

FIG 1 Map of study area. Participants were recruited in the hospital of Turbo, located in an urban center, and in three health centers in the periphery (El Dos, Tres, and Currulao).

pervised CQ-PQ regimen. Associations between risk factors and recurrence were explored, and *P. vivax* parasites from all episodes were genotyped in order to distinguish between relapses and reinfections.

MATERIALS AND METHODS

Study site. This study was conducted in Turbo municipality, Antioquia department, located in northwest Colombia (8°5′42″N, 76°44′123″W) (Fig. 1). Turbo has an area of 3,055 km^2 and approximately 135,967 inhabitants, 61% of whom live in a rural area. The main economic activity is banana and plantain cultivation. Malaria is endemic in Turbo, with peaks of highest transmission between February and June; according to recent statistics, 80% of malaria cases in the area are *P. vivax* monoinfections (15). Over the past 7 years, the number of cases has been reduced significantly from an annual parasite incidence (API) of 65 cases per 1,000 people in 2007 to 3 cases per 1,000 people in 2014 (15).

Study design. A longitudinal observational study was conducted. Participants were selected from four malaria diagnostic centers (the municipal hospital and three nearby health centers) between February 2012 and July 2013 (Fig. 1). These centers account for about 20% of all reported *P. vivax* cases in the Turbo municipality.

All individuals diagnosed with *P. vivax* malaria in the diagnostic centers during the study period were invited to participate. Eligibility criteria were the following: monoinfection with *P. vivax*, asexual parasite count greater than 250 parasites/μl, age over 4 years, absence of general danger

signs or signs of severe malaria according to the criteria adopted by the World Health Organization (7, 9), negative pregnancy test, not breastfeeding, no reported intake of antimalarials in the preceding 4 weeks, residence in the study area, and the ability and willingness to comply with the protocol for the duration of the study.

Participants received supervised simultaneous treatment with CQ (25 mg/kg for 3 days) and PQ (0.25 mg/kg/day for 14 days) (16). Patients weighing 60 kg or over received the maximum dose of CQ (1.5 g), while the PQ dose was adjusted for body weight. Information about demographic characteristics, current disease, history of malaria, travel to other regions of endemicity, and use of preventive measures for malaria was collected. Participants were monitored by thick blood smears and surveillance for symptoms on days 1, 2, 3, 7, 13, 21, and 28 postdiagnosis to evaluate therapeutic responses during the current episode (7). The evaluation for recurrences was carried out by monthly thick blood smears on days 60, 90, 120, 150, and 180 days and at any time the patient had symptoms consistent with malaria.

Recurrence was defined as a positive thick blood smear for *P. vivax* between days 29 and 180, with or without clinical symptoms. In all recurrences, participants were treated again with the same CQ-PQ regimen, and they continued the follow-up as scheduled until day 180.

For patients who did not meet one or more inclusion criteria, a sample of capillary blood was taken before treatment, but no study treatment or follow-up was provided. These samples were used for malaria diagnosis and parasite genotyping; results provided information for baseline parasite genetic diversity in the study area.

All participants enrolled signed an informed consent. This study was approved by the ethics committee from the School of Medicine of the University of Antioquia, Colombia. The investigators from the Centers for Disease Control and Prevention (CDC) did not engage in field study or have access to participants' personal identifying information. Therefore, their participation in this study was determined to be nonengaged after human subject review at the CDC.

Malaria diagnosis and genetic characterization. Malaria diagnosis on the day of inclusion (considered day zero) and on each scheduled visit was carried out by microscopy (17). Diagnosis was confirmed by PCR on days 0 and 28 and on the day of recurrence. DNA samples were extracted from filter paper with blood spots by the saponin-Chelex method (18), and they were analyzed using the nested PCR protocol described by Snounou et al. (19).

All samples determined to be *P. vivax* monoinfection by PCR were genotyped at seven neutral microsatellite (MS) loci (20, 21). Microsatellites MS2 (chromosome 6), MS6 (chromosome 11), and MS20 (chromosome 10), previously described by Karunaweera et al., were amplified by simple PCR (21), while microsatellites 2.21 (chromosome 2), 3.502 (chromosome 3), 11.162 (chromosome 11), and 12.335 (chromosome 12), described by Imwong et al., were amplified by seminested PCR (20). For each MS amplification, a forward primer labeled with a fluorophore (6-carboxyfluorescein [FAM] or hexachlorofluorescein [HEX]) was used. PCR products were analyzed by capillary electrophoresis on an ABI Prism 3130xl sequencer (Applied Biosystems) using ROX 350 (Applied Biosystems) as an internal standard. Fragment size and allele determination were obtained using Genemapper, version 4.1 (7 (Applied Biosystems, Foster City, CA). Samples in which some loci did not amplify were reamplified twice. If two or more alleles were amplified in a single locus of a given sample and if the signal from the minor allele was greater than 33% of that of the predominant signal allele, the sample was defined as being polyclonally infected (22).

Data analysis. (i) Incidence of recurrences and risk factors. The information was reviewed in the field, validated for quality control, and then entered into a database. Cumulative incidence of first recurrence was calculated using Kaplan-Meier survival analysis. Association of potential risk factors with recurrence was analyzed using a Cox proportional hazards model with the extension proposed by Andersen and Gill, which allows multiple events per participant (23). Crude and adjusted hazard ratios (HR) and their 95% confidence intervals (95% CI) were calculated. Selection of variables for calculating adjusted HR was made by biological plausibility and according to a P value of <0.25 in bivariate analysis (24). For quantitative variables, linear assumption of risk was evaluated, and if it was not met, the variables were dichotomized at the median or previously reported thresholds (25, 26). All analyses were carried out using Stata, version 11.2, software (StataCorp, College Station, TX).

(ii) Genetic diversity and genetic structure. For samples with monoclonal infection, the total number of alleles per locus and expected heterozygosity (H_e) were estimated. The H_e represents the probability of finding two different alleles for a given locus on a pair of randomly selected samples from the study population (27). Additionally, the number of multilocus haplotypes (MLH), defined as unique combinations of alleles for the seven MSs analyzed, was estimated. These analyses were conducted with GenAlEx (28) and Microsatellite Toolkit applications from Microsoft Excel (29). The percentage of polyclonal infection per locus and average number of alleles per locus were calculated for all samples on day zero and day of recurrence.

Given that unknown population substructures could bias our observations, we explored whether the sampled parasites could be considered one population undergoing random mating. In particular, the parasite population structure was evaluated in Structure, version 2.3.4, which uses a Bayesian clustering approach to assign samples to one of K genetic groups according to allele frequency per locus; the data were evaluated using values of K from 2 to 10. For each value of K, 10 independent runs were performed, with a burn-in period of 10,000 iterations followed by 100,000 iterations (30). The Structure Harvester, version 0.6.94, program was used to visualize the Structure output results (31). The most likely number of genetic groups within the sample was found according to the method of Evanno et al. (32). These analyses were complemented by inferring the MLH genealogies using the global optimal eBURST algorithm (33), as implemented in PHYLOViZ (34). Using an extension of the goeBURST rules up to the n locus variant (nLV, where n equals the number of loci in our data set, i.e., seven) level, a minimum spanning tree-like structure was drawn to cluster the MLH into a clonal complex based on their multilocus genotypes.

(iii) Classification of recurrences. The multilocus genotypes of the samples from day zero and the recurrence day in participants with recurrence were compared. If both samples had exactly the same allele at each locus for all seven MSs analyzed, i.e., having identical MLH, it was considered an identical recurrence. In case of a polyclonal infection on day zero, the recurrence was considered to be from the same MLH when all alleles for the seven MSs analyzed in the recurrence day were present on day zero.

For participants with identical haplotypes at day zero and on recurrence day, the probability that a second infection with same haplotype occurred by chance, P_{match}, was estimated by considering the relative frequency of each MLH in the day zero population (35–37). The P_{match} value was estimated by multiplying the relative frequency of the MLH within each of the two genetic clusters (i.e., subpopulations) obtained by Bayesian analysis in the day zero sample set by their relative frequencies in the recurrence population. Polyclonal infections were excluded for this analysis, except for samples from participants with a recurrence in which the haplotype frequency from the recurrence day was considered. When P_{match} was <0.05, the recurrence was classified as a relapse; otherwise it was considered a reinfection by the same haplotype.

RESULTS

Incidence of malaria recurrences caused by *P. vivax* and risk factors. Of 134 participants screened, 87 met the inclusion criteria (Fig. 2). Participants included and excluded were similar in demographic characteristics and history of malaria (see Table S1 in the supplemental material). All participants included in the recurrence surveillance had a negative malaria PCR result on day 28 post-initial treatment. During the 6-month follow-up, 29 recurrences in 21 participants were detected. Seven participants had two recurrences while one had three recurrences. The remaining

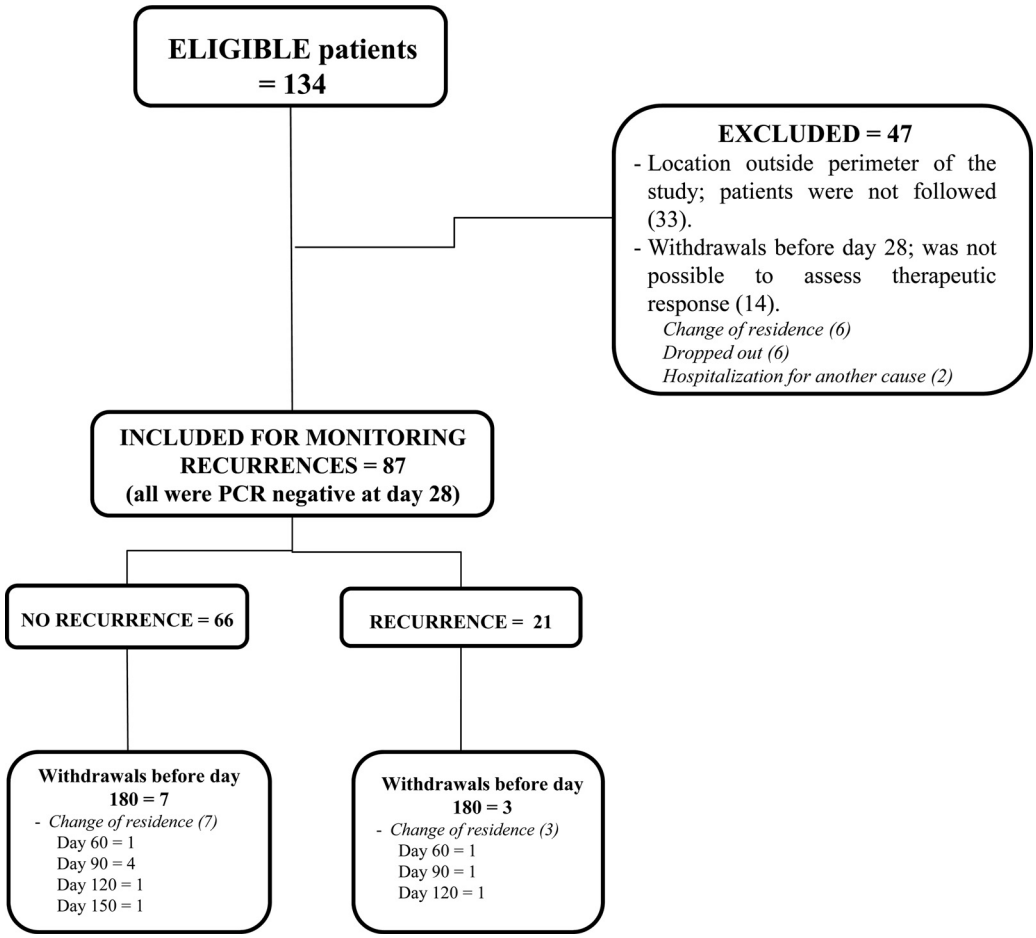

FIG 2 Study flowchart of enrolled participants under supervised treatment with chloroquine and primaquine.

66 participants (75.9%) did not develop recurrent malaria during the study period (censored cases) although 7 of them were lost to follow-up within the 6-month period.

The average follow-up time was 170.34 days, with a standard deviation (SD) of 3.06 days; cumulative incidence to first recurrence was 24.1% at 180 days posttreatment (95% CI, 14.6 to 33.7%); 86% (18/21) of the participants had their first recurrence between 51 and 110 days after enrolling in the study (Fig. 3). Seven participants had a second recurrence event between days 118 and 177, and one presented a third recurrence on day 179.

Characteristics of participants with and without recurrence are presented in Table 1, while the crude and adjusted HR are shown on Table 2. After adjusting for history of malaria in the preceding year, time of residence in an area of endemicity for longer than 5 years was the only factor associated with malaria recurrence (HR of 2.5; 95% CI, 1.04 to 4.87).

Genetic diversity of *P. vivax* samples. A total of 157 samples were genotyped: 128 day zero samples (participants included and excluded from monitoring of recurrences) and 29 recurrences. A total of 87.5% of samples from day zero ($n = 112$) and 100% of the recurrences were monoclonal infections. All polyclonal infections had two clones. Two samples collected on day zero (1.4%) did not amplify an allele at one MS locus (MS20 and MS12.335) while all seven loci were successfully amplified in all recurrence samples.

The average H_e was 0.721 ± 0.036 in samples from day zero. The average number of alleles per locus was 8.71 ± 3.2, and the average number of alleles/locus/per sample was 1.04. MS20 was the most polymorphic locus with an H_e of 0.860, and it discriminated 43.7% of polyclonal infections. (Table 3). A total of 52 MLH

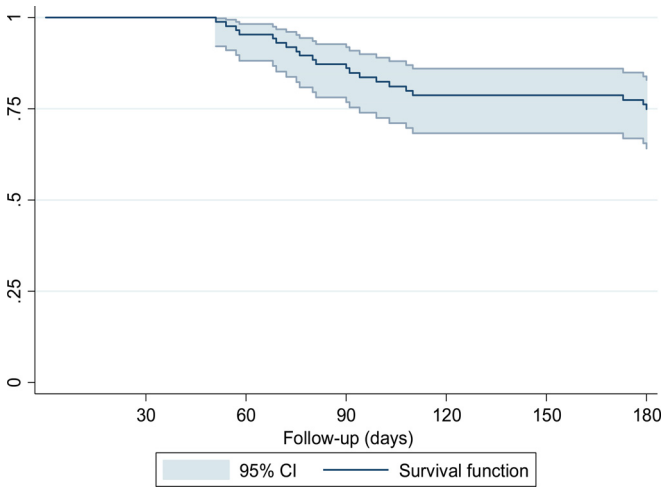

FIG 3 Kaplan-Meier survival curve for participants included in the monitoring of malaria recurrences caused by *Plasmodium vivax*.

TABLE 1 Characteristics of participants enrolled for monitoring malaria recurrences caused by *Plasmodium vivax*

Parameter[a]	Value for the group		*P* value[b]
	Patients with recurrence ($n = 21$)	Patients without recurrence ($n = 66$)	
Male sex (no. [%])	14 (66.7)	37 (56.1)	0.209
Median age (IQR [yr])	25 (17.0–35.5)	29 (15.7–45.2)	0.258
Median body mass index (IQR [kg/m^2])	23.0 (20.2–26.2)	22.7 (20.0–24.8)	0.659
Occupation (no. of patients [%])			
Farmer	8 (38.1)	19 (29.7)	0.616
Housewife	5 (23.8)	16 (25.0)	
Student	5 (23.8)	18 (28.1)	
Other job	3 (14.3)	11 (17.2)	
Likely infection source within Turbo (no. of patients [%])	17 (80.9)	60 (90.9)	0.182
Rural residence (no. of patients [%])	14 (66.7)	41 (62.1)	0.486
Median parasitemia on day zero (IQR [parasites/μl])	4,480 (2,280–7,080)	4,940 (2,100–9,590)	0.797
Median no. of days with symptoms before diagnosis (IQR)	5 (4–6.5)	5 (3–7)	0.210
Median time of residence in a region of endemicity (IQR [yr])	12 (2.5–20)	4 (1–12)	0.043
Symptomatic malaria in the last year (no. of patients [%])	8 (38.1)	14 (21.1)	0.390
Median no. of malaria episodes in the last year (IQR)	1 (1–1.7)	1 (1–2)	0.390
Last episode of symptomatic malaria (no. of patients [%])			
1–2 mo	5 (25.0)	3 (4.8)	0.882
3–6 mo	2 (10.0)	10 (15.9)	
7–12 mo	1 (5.0)	1 (1.6)	
>12 mo	5 (25.0)	25 (39.7)	
No history of malaria	7 (35.0)	24 (38.1)	
Median CQ dosage (IQR [mg/kg])	24.2 (22.3–26.1)	23.9 (22.1–26.4)	0.387
Median PQ dosage (IQR [mg/kg])	3.6 (3.4–4.1)	3.7 (3.5–4.1)	0.500
Travel to other region(s) of endemicity during follow-up (no. of patients [%])	7 (35.0)	19 (37.2)	0.954
Use of bednet during follow-up (no. of patients [%])			
Always	12 (60.5)	33 (61.9)	0.776
Never	5 (25.0)	9 (16.7)	
Some months	3 (15.0)	12 (22.2)	
Use of insecticides to spray house during follow-up (no. of patients ([%])	14 (70.0)	25 (48.1)	0.286

[a] IQR, interquartile range.
[b] Cox regression, Andersen-Gill extension.

were detected in 112 monoclonal samples from day zero, of which 34 (65.4%) were present only in a single sample (Fig. 4). H33 and H3 MLH were the most frequently detected from the day zero group, in 21.4% (24/112) and 9.8% (11/112) of the samples, respectively.

Average H_e was 0.664 ± 0.050 in the 29 recurrence samples. Among them, 13 MLH were identified, of which H33 had a frequency of 24.1%, and H16 had a frequency of 20.7%; H54 and H55 were new haplotypes not identified in samples from day zero, and H53 was identified only in a polyclonal sample from day zero.

In Fig. 4, the monthly distribution of haplotypes detected during the study period is presented. Two genetic clusters were inferred using the Structure program, including the data from monoclonal infections (see Fig. S1 in the supplemental material), and all samples had a greater than 75% probability of belonging to one of the two possible genetic clusters. A total of 87 isolates (60.4%) with 46 haplotypes belonged to cluster 1 while 57 isolates (39.6%) with 9 haplotypes belonged to cluster 2. The samples were classified into these groups to estimate haplotype frequencies on day zero and P_{match} values. Both clusters of parasites circulated simultaneously in Turbo during the study period, but their geographic distributions were slightly different (see Fig. S2). These clusters, however, could be genetically interrelated, as indicated by the haplotype network (see Fig. S3), with the putative primary founders in the less diverse cluster 2. Importantly, all MLH lineages were found in all localities, so this source of bias did not affect our results.

Classification of recurrences caused by *P. vivax*. MLH from participants with recurrences are presented in Table 4. Of a total of 29 recurrences, 27 had haplotypes identical to those present on day zero (Table 4; see also Table S2 in the supplemental material), and the results from the P_{match} analysis for classification of recurrences indicated that 65.5% (19/29) were relapses from initial infection.

Cluster 1 contained 12 participants with recurrences and a total of 18 recurrence events (Table 4). The recurrences in two participants from this group were classified as reinfections (i.e., new infections) because the MLH from day zero and day of recurrence

TABLE 2 Potential risk factors for *Plasmodium vivax* malaria recurrence during 6-month follow-up

Parameter	Crude HR (95% CI)[a]	Adjusted HR (95% CI)[b]
Sex		
Female	1	
Male	1.66 (0.75–3.69)	1.59 (0.71–3.56)
Age		
<18 yr	1	1
≥18 yr	1.69 (0.73–3.94)	1.52 (0.66–3.51)
Time of residence in region of endemicity		
≤5 years	1	1
>5 years	2.19 (1.00–4.77)	2.25 (1.04–4.87)
No. of days with symptoms before diagnosis		
<5 days	1	1
≥5 days	0.79 (0.38–1.66	0.85 (0.40–1.80)
Parasitemia day zero		
<4,920 parasites/μl	1	1
≥4,920 parasites/μl	1.11 (0.50–2.44)	1.19 (0.54–2.61)
Symptomatic malaria in last year		
No	1	1
Yes	1.41 (0.65–3.07)	1.50 (0.70–3.25)
Place of residence		
Urban	1	1
Rural	1.33 (0.60–2.93)	1.39 (0.63–3.09)
Travel to other region(s) of endemicity during follow-up		
No	1	1
Yes	0.98 (0.45–2.11)	1.02 (0.48–2.14)
Use of bednet during follow-up		
Always	1	1
Never	1.32 (0.52–3.36)	1.11 (0.41–3.00)
Some months	0.75 (0.26–2.17)	0.79 (0.29–2.16)
Use of insecticides to spray house during follow-up		
Yes	1	1
No	0.64 (0.29–1.45)	0.83 (0.34–2.03)

[a] Cox regression, Andersen-Gill extension. HR, hazard ratio; CI, confidence interval.
[b] Cox regression, Andersen-Gill extension. Values are adjusted for symptomatic malaria in the last year and time of residence of >5 years in an area of endemicity.

TABLE 3 Genetic diversity of *Plasmodium vivax* strains per locus from day zero samples

Locus	Total no. of alleles	Allele size range (bp)	Expected heterozygosity (H_e)[a]	No. of polyclonal samples (%)	Avg no. of alleles/locus/sample
MS2	11	181–251	0.791	5.47	1.05
MS6	6	211–249	0.746	3.13	1.03
MS20	15	206–263	0.860	5.51	1.06
2.21	7	83–115	0.644	3.91	1.04
3.502	7	133–199	0.768	7.03	1.07
11.162	8	181–243	0.643	2.34	1.02
12.335	7	160–179	0.598	3.94	1.04

[a] Only from monoclonal samples ($n = 112$). The H_e represents the probability of finding two different alleles for a given locus on a pair of isolated samples randomly selected from the study population ($H_e = n/(n-1)(1 - \Sigma p_i^2)$, where p is ith frequency allele and n is sample size.

were not identical. Nevertheless, the recurrence haplotypes were highly genetically related to the infection episode at the time of inclusion in the study, with differences at only two alleles (see Table S2 in the supplemental material).

A group of nine participants were assigned to cluster 2 with a total of 11 recurrence events, all of which were caused by haplotypes that were identical to those of the previous episode(s) (Table 4). There were six recurrences by the predominant haplotype (H33), which had a very high P_{match}, thus making it highly likely that participants were reinfected with the same parasite strain that was detected on day zero.

DISCUSSION

In this study, we found that 24.1% (21/87) of *P. vivax*-infected participants had at least one recurrence within 180 days of treatment with a standard CQ-PQ regimen, despite receiving full treatment and being monitored. Additionally, 8% (7/87) of participants had three or more episodes within 6 months. Moreover, it is highly unlikely that these recurrences were recrudescence by therapeutic failure to CQ since all participants had a negative PCR for malaria on day 28 posttreatment. Results from similar studies in other parts of the world reported a cumulative incidence of recurrence between 0% and 13.5% after treatment with 0.25 mg/kg/day PQ for 14 days and a follow-up time between 180 and 210 days, which is much lower than what was found in this study (38–43).

A similar study carried out in two regions of endemicity of Colombia (2003 to 2004) reported a cumulative incidence of 16.2% for a first recurrence of *P. vivax* during 6 months after treatment with the same regimen as in the present study, and only 1.5% of the participants had more than one recurrence event (14). Although the difference in cumulative incidences of recurrence is seemingly little between these two studies, recurrences in the previous study are more likely to have been cases of reinfection, rather than relapses, since the API in that study was much higher than that in the present one (30 versus 3) (15, 44). In addition, a maximum PQ dose of 210 mg was used in the previous study, so participants weighing more than 60 kg received a lower dose than recommended; in the present study, all participants were dosed by body weight to avoid relapse due to underdosing, as previously reported in other studies (26, 45, 46).

P. vivax recurrences may have an impact on patient well-being via clinical symptoms and the risk of complicated malaria. In this study, participants presented clinical symptoms in all episodes of malaria although recurrent episodes were not always febrile. Moreover, *P. vivax* gametocytes were present at recurrence, which may have contributed to ongoing transmission (4).

Potential risk factors for *P. vivax* recurrence were explored. Duration of residence of more than 5 years in this area of endemicity was associated with an increased risk of recurrence, even after adjusting for malaria history in the last year. Although this result seems to contradict previous reports (25), our interpretation is that a longer time of exposure to malaria, and therefore a greater probability of having had previous episodes, may increase

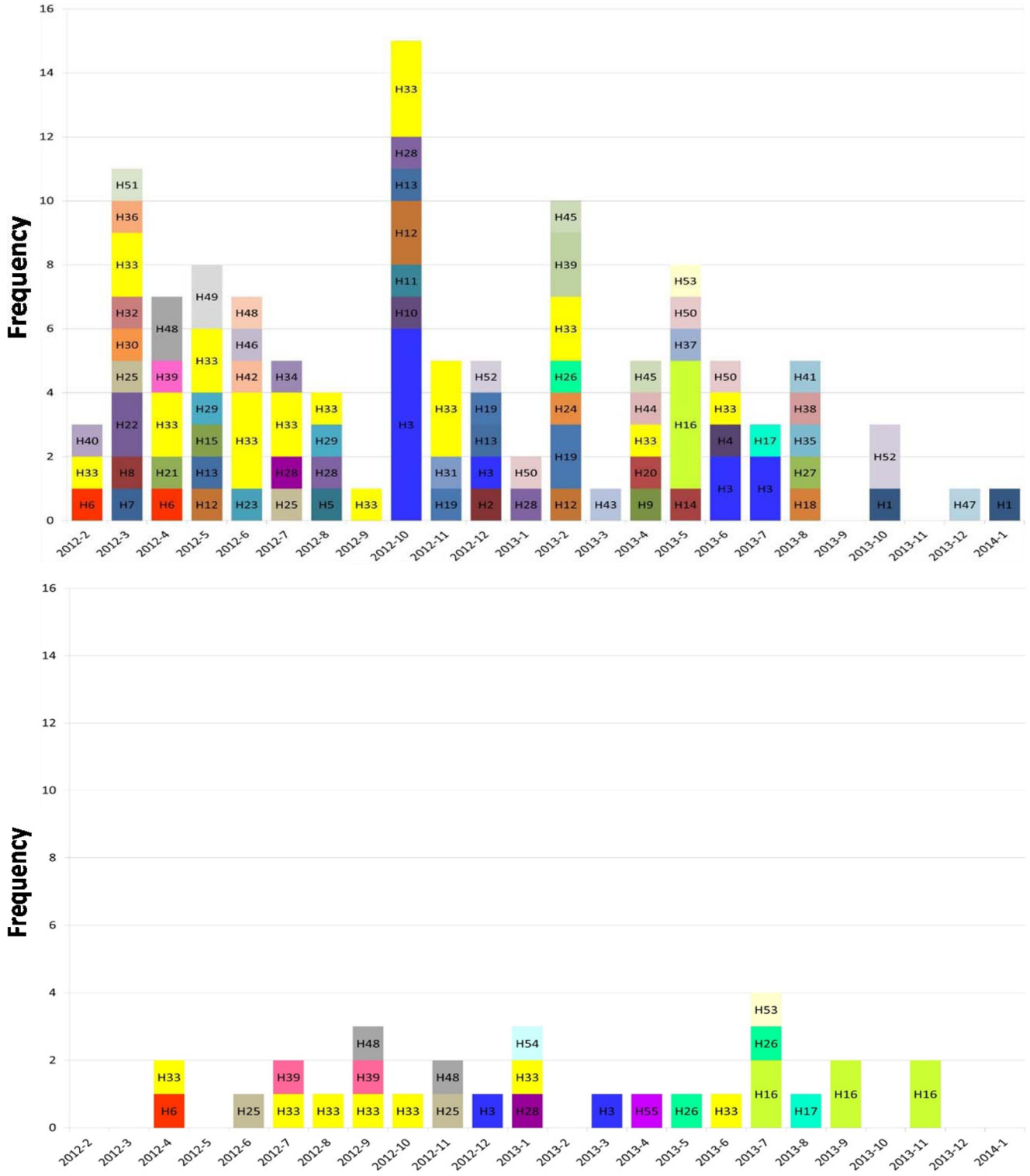

FIG 4 Monthly distribution of multilocus haplotypes of *Plasmodium vivax* in Turbo, Colombia, identified during the study period. Vertical axes are numbers of cases, and horizontal axes are dates (year-month). Data show monthly frequency of haplotypes in monoclonal samples from day zero ($n = 115$) (top) and from the recurrence day ($n = 29$) (bottom). Each color represents a different haplotype.

the likelihood of hosting dormant hypnozoites. Importantly, in this study a sample size was not considered to assess risk factors associated with recurrence, which limited precision in estimates of risk factors for recurrence.

This study included 74% of the reported *P. vivax* malaria cases in four diagnostic centers from Turbo municipality during the study period, so the parasite sample genotyped ($n = 157$) can be considered a reliable representation of the *P. vivax* strains respon-

TABLE 4 Multilocus haplotypes in 21 participants with recurrences caused by *Plasmodium vivax* during 6 months

Sample identification no.	Time to first recurrence (days)[f]	Time to second recurrence (days)[f]	Day zero haplotype vs recurrence day haplotype	Haplotype code(s)[e]	Cluster no.	P_{match}[a]	Classification[b]	Residence zone	Last episode of malaria before study (no. of mos.)	Travel to other region(s) of endemicity during follow-up	Family member with malaria at time of recurrence
1014	54		Identical	6	2	0.0019	Relapse	Rural	>12	No	No
1015	68	154	Identical	33[c]	2	0.5146	Reinfection	Rural	Never	No	No
1018	173		Identical	33	2	0.5146	Reinfection	Rural	7–12	No	No
1022	94		Identical	25	1	0.0008	Relapse	Rural	3–6	No	No
1034	103	170	Identical	39[c]	1	0.0019	Relapse	Urban	Never	Yes	No
1050	180		Identical	33	2	0.5146	Reinfection	Rural	1–2	Yes	No
1051	90		Identical	33	2	0.5146	Reinfection	Rural	Never	No	No
1052	81	154	Identical	48[c]	1	0.0019	Relapse	Rural	>12	Yes	No
1055	110		Identical	25	1	0.0008	Relapse	Urban	>12	Yes	No
1057	91		Identical	33	2	0.5146	Reinfection	Rural	Never	No	No
1063	108		Different	13 (day zero), 54 (R1)	1		Reinfection	Rural	>12	No	No
1065	72	142	Identical	3[c]	2	0.0572	Reinfection	Rural	>12	No	No
1066	80		Identical	28	2	0.0075	Relapse	Urban	Never	Yes	No
1075	179		Different	10 (day zero), 55 (R1)	1		Reinfection	Urban	1–2	Yes	No
1089	75	145	Identical	26[c]	1	0.0002	Relapse	Urban	1–2	No	No
1094	69		Identical	33	2	0.5146	Reinfection	Rural	3–6	No	No
1098	76		Identical	53	1	0.0002	Relapse	Rural	1–2	No	No
1101[d]	58	118	Identical	16[c]	1	0.0034	Relapse	Rural	>12	No	Yes
1102	99		Identical	16	1	0.0034	Relapse	Rural	Never	No	No
1103	57	177	Identical	16[c]	1	0.0034	Relapse	Rural	Never	No	Yes
1111	51		Identical	17	1	0.0002	Relapse	Urban	1–2	No	No

[a] Probability of finding by chance an identical haplotype in different episodes for a participant.
[b] Relapse when P_{match} is less than 0.05 and reinfection when P_{match} is greater than or equal to 0.05.
[c] Second recurrence with haplotype identical to that of the previous two episodes in the study.
[d] Third recurrence on day 179 with haplotype 16, identical to that of previous three episodes in the study.
[e] R, recurrence number.
[f] Time to recurrence is measure from day zero.

sible for symptomatic infections in the study area. The detection of 52 haplotypes in 112 day zero samples and the high percentage of unique haplotypes (65.4%) show high genetic diversity, consistent with other reports from South America using *P. vivax* microsatellites (37, 47–50). Overall, the percentage of samples with polyclonal infection was 12.5%, which is similar to reports from other populations in Colombia, Venezuela, and Peru (47, 50, 51). The genetic diversity of the selected markers indicates that they have enough resolution to detect differences among samples of *P. vivax* in Turbo.

About 93.1% of recurrences analyzed were caused by MLH that were identical to those of the first episode (day zero sample). This percentage is higher than that reported in other studies that used the same PQ supervised regimen and similar length of follow-up and genotyping approach for recurrences (microsatellite loci) (37, 40). Additionally, 62.1% of recurrences were classified as relapses according to established criteria based on estimation of the P_{match} within each genetic cluster, which could indicate a problem with efficacy of the PQ regimen used. The criteria used in this study for classification of recurrences were strict, and additional information collected during follow-up, such as travel to other areas of endemicity, family members with malaria in the same household, and use of preventive measures for malaria, supported this interpretation.

Several aspects of this study support the finding that at least 6 out of 10 *P. vivax* recurrences in Turbo, Colombia, are caused by relapses. A PQ regimen with low incidence of recurrences reported worldwide was used. CQ-PQ treatment was supervised to completion. PQ daily dose was adjusted for body weight, even in patients weighing more than 60 kg. No recrudescence was confirmed by negative *P. vivax* PCR on day 28. Remarkably, most recurrences were caused by parasites genetically identical to those of previous episodes. Given the high genetic diversity of *P. vivax* strains in the study area, the probability of reinfection with an identical MLH in this area is extremely low. Most recurrences occurred between 51 and 110 days of a previous episode, which agrees with previously reported timing for *P. vivax* relapses from South America, which are characterized by a short latency time (3). API was low during the study period, and therefore the probability of reinfection was also low. Most participants with recurrences had not visited another area of endemicity during follow-up, and they had no family members infected with malaria. There were recurrences in participants who resided in urban areas where currently there is no malaria transmission, and therefore it was not possible for the participants to acquire a new infection.

The high incidence of relapses found in this study was possibly caused by the current standard dose of PQ used in Colombia being insufficient to eliminate hypnozoites. It was recently reported that PQ metabolism by the cytochrome P450 (CYP) 2D family of enzymes is required for antimalarial activity in humans, and lower CYP 2D6 enzyme activity, as in the poor-metabolizer phenotype, could compromise its radical curative efficacy (52). It is not clear if any such variation in the genetic background of this population could account for this observation. In addition, it has been shown

that when higher doses of PQ, such as 0.5 mg/kg for 14 days, are used as recommended by the CDC (53), lower incidences of recurrence are reported (between 1.9% and 6.6% during 180 to 365 days) (43, 54, 55). However, no direct comparison had been made between a PQ regimen of 0.5 mg/kg for 14 days and the standard regimen in countries where malaria is endemic (9). Further studies addressing this subject and evaluating the optimal dose and type of treatment are required, as well as the establishment of a minimum effective concentration of PQ in order to define therapeutic failure.

For elimination of malaria by *P. vivax*, surveillance of recurrences is necessary, including genotyping and monitoring of the different factors associated with them. This type of approach not only allows the classification of recurrence as reinfection or relapse but also opens the prospect of identifying PQ tolerance markers, evaluating the efficacy and safety of therapeutic PQ regimens with optimal doses for different epidemiological contexts, and advancing the search for new agents against liver hypnozoites.

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