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In Vitro Susceptibility of *Plasmodium vivax* to Antimalarials in Colombia

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The *in vitro* susceptibilities of 30 isolates of *Plasmodium vivax* to a number of antimalarials (chloroquine [CQ], mefloquine, amodiaquine, quinine, and artesunate [AS]) were evaluated. The isolates came from the region of Urabá in Colombia, in which malaria is endemic, and were evaluated by the schizont maturation test. The 50% inhibitory concentration (IC_{50}) was 0.6 nM (95% confidence interval [CI], 0.3 to 1.0 nM) for artesunate, 8.5 nM (95% CI, 5.6 to 13.0 nM) for amodiaquine, 23.3 nM (95% CI, 12.4 to 44.1 nM) for chloroquine, 55.6 nM (95% CI, 36.8 to 84.1 nM) for mefloquine, and 115.3 nM (95% CI, 57.7 to 230.5 nM) for quinine. The isolates were classified according to whether the initial parasites were mature or immature trophozoites (Tfz). It was found that the IC_{50}s for chloroquine and artesunate were significantly different in the two aforementioned groups ($P < 0.001$). The IC_{50}s of CQ and AS were higher in the isolates from mature Tfz (CQ, 39.3 nM versus 17 nM; AS, 1.4 nM versus 0.3 nM), and 10% of the isolates showed lower susceptibilities to one of the antimalarial drugs, 13.3% to two antimalarial drugs, and 3.3% to more than three antimalarial drugs. It should be highlighted that despite the extensive use of chloroquine in Colombia, *P. vivax* continues to be susceptible to antimalarials. This is the first report, to our knowledge, showing *in vitro* susceptibilities of *P. vivax* isolates to antimalarials in Colombia.

Plasmodium vivax is responsible for >50% of malaria cases worldwide and is prevalent in Southeast Asia, the Western Pacific, and Central and South America. *P. vivax* malaria is known for causing relapses (1). In recent years, complicated clinical conditions have been reported for this type of malaria that are similar to those found for *Plasmodium falciparum*. Indeed, the two species coexist in many parts of the world (2). In Colombia, 85% of the territory is suitable for the transmission of *Plasmodium* spp., and according to an epidemiological report from the National Health Institute of Colombia, 53,963 cases of malaria were reported in 2012, of which 40,314 (74.7%) corresponded to *P. vivax* malaria (3).

The therapeutic failure of chloroquine to treat *P. vivax* malaria has been reported in different regions around the world. The first case of therapeutic failure was reported in Papua New Guinea in 1989 (4, 5), and since then, cases have also been recorded in Asia, primarily in Indonesia, Malaysia, Myanmar, India, Philippines, Vietnam, South Korea, and Thailand. Cases have also been reported in Ethiopia in Africa (6–12) and in Guyana, Brazil, Venezuela, Peru, and Colombia in South America (13–17). In Colombia, whereas one study reported the therapeutic failure of chloroquine in 11% (3/27) of the patients evaluated (17), other studies reported 100% efficacy for this antimalarial (14, 18). Therefore, the *in vitro* susceptibility profile of *P. vivax* to antimalarials must be determined.

This work investigated the *in vitro* susceptibilities of fresh isolates of *P. vivax* from the region of Urabá (Antioquia, Colombia) to chloroquine, mefloquine, amodiaquine, quinine, and artesunate using the schizont maturation test.

MATERIALS AND METHODS

Predosing of plates with antimalarials. Seven serial dilutions of the antimalarials chloroquine (CQ; Sigma R-6628), mefloquine (MQ; Sigma R-2319), amodiaquine (AQ; Sigma R-2799), quinine (Qn; Sigma R-0132), and artesunate (AS; Sigma R-3731) were predosed. Each antimalarial plate was predosed in triplicate, in the ranges 1.646 nM to 1.200 nM, 12.5 nM to 800 nM, 2.34 nM to 150 nM, 3.7 nM to 2.700 nM, and 0.05 nM to 40 nM for CQ, MQ, AQ, Qn, and AS, respectively (4, 19–22). The predosed plates were stored at 4°C until they were used. The quality control of the predosing stage was carried out with the NF54 strain of *P. falciparum* (sensitive to all known antimalarials) (23), and parasitemia was recorded through the radioisotope method (24).

***P. vivax* isolates.** Between September 2010 and November 2012 at the malaria diagnosis stations in the municipalities of Turbo and Apartadó (Urabá, Antioquia, Colombia), where malaria was endemic, patients who met the following inclusion criteria were included: patients of any age and gender, women who were not pregnant, patients with single *P. vivax* infection (according to thick smear test and confirmed by the rapid diagnostic test SD Bio Line malaria Ag Pf/Pan; Standard Diagnostics, Inc., 05FK66), and patients with a parasitemia level of >2,000 parasites/μl of blood. Additional inclusion criteria were patients with a diagnosis of uncomplicated malaria according to the WHO criteria adapted for Colombia (25), patients who took no antimalarials in the month before the test, and patients with no chloroquine in the urine (according to the Saker-Solomons method). Each patient who fulfilled the criteria and was willing to take part in the study signed an informed consent form previously approved by the ethics committee of the Universidad de Antioquia through Act 012 of 18 June 2009. In the case of minors, the consent form was signed by a parent or guardian who was >18 years of age.

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TABLE 1 Parasitic stages, maturation times, and percentages of schizonts obtained by the isolate groups

Pre- and postculture isolate characteristic	Group 1[a] ($n = 15$)		Group 2[b] ($n = 15$)		*F* test *P* value
	Mean	95% CI	Mean	95% CI	
Immature Tfz (R + Ta)[c]	172.2	161.1–183.3	18.33	0.0–38.5	<0.0001
Mature Tfz[c]	23.3	13.8–32.9	174.7	151.4–198.1	<0.0001
Preschizonts[c]	3.0	0.7–5.3	1.4	0.2–2.6	0.19
Schizonts[c]	1.5	0.2–2.8	5.5	0.0–14.1	0.32
Maturation time (h)	34.9	31.0–38.8	22.7	20.1–25.2	<0.0001
% schizonts postmaturation	59.9	46.5–76.4	48	39.2–56.8	0.12

[a] Group 1 included isolates with predominance of immature trophozoites.
[b] Group 2 included isolates with predominance of mature trophozoites.
[c] Data are counts of parasites at the beginning of the assay carried out with 200 asexual parasites. R, rings; Ta, amoeboid trophozoites (27).

TABLE 2 IC_{50}s of the antimalarials evaluated in the *P. vivax* isolates from Urabá, Colombia

Antimalarial	No. (%) of valid assays[a]	IC_{50}[b] (95% CI) (nM)
Chloroquine	30 (100)	23.3 (12.4–44.1)
Mefloquine	28 (93.3)	55.6 (36.8–84.0)
Amodiaquine	26 (86.7)	8.5 (5.6–13.0)
Quinine	28 (93.3)	115.3 (57.7–230.5)
Artesunate	26 (86.7)	0.6 (0.3–1.0)

[a] Number of valid assays with IC_{50}s over total number of assays ($n = 30$).
[b] IC_{50}, geometric mean of the inhibitory concentration; 95% CI, 95% confidence interval.

Collecting and processing samples. In order to evaluate the *in vitro* susceptibility of *P. vivax* to antimalarials, 10 ml of venous blood was taken from each patient and put in a Vacutainer tube with heparin. The samples were processed within 4 h after being collected from the patients. Additionally, 200 μl of blood was poured onto filter paper (Whatman 3) to confirm *P. vivax* monoinfection in the analyzed samples using PCR (26).

In the thick smear test, 200 asexual parasites were counted in all of the different parasitic stages, including rings or immature trophozoites (Tfz imm), amoeboid trophozoites (Tfz amoeb), mature trophozoites (Tfz mat), preschizonts (pre-sch), and mature schizonts (Sch mat) (27). According to the initial parasitic stages, the isolates were divided into one of two groups. Group 1 consisted of isolates with >133 immature Tfz (rings plus amoeboid Tfz) out of 200 asexual parasites, and group 2 consisted of isolates that did not fulfill the previous criterion and had >133 mature Tfz out of 200 asexual parasites (21, 27).

Assay of *in vitro* susceptibility of *P. vivax* to antimalarials using the schizont maturation method. Before the susceptibility assay, the leukocytes in a 10-ml sample of heparinized blood were eliminated during two rounds of filtration using cellulose columns, in accordance with the method described by Russell et al. (22). The brand of cellulose used (S6288; Sigma-Aldrich) differed from that employed in the Russell et al. method, but it had similar characteristics.

The parasitized erythrocytes, recovered during the second filtration, were centrifuged at 1,500 rpm for 5 min. The supernatant liquid was then removed, and 800 μl of the parasitized erythrocytes was used to prepare a suspension at a hematocrit level of 2% in McCoy's 5A medium (M4892; Sigma-Aldrich), supplemented with 25% AB^+ of human serum. A total of 200 μl of this suspension was poured into each well of the predosed plates, which were incubated at 37°C in an atmosphere of 5% CO_2, 5% O_2, and 90% N_2 until 40% of the initial parasitic stages of each isolate matured into schizonts in the drug-free control wells. After incubation, the supernatant was removed from each well, and the thick smear test was performed (28). To obtain readings from the tests, 200 asexual parasites differentiated by their parasitic stages were counted as previously described (27).

Inhibition percentages and inhibitory concentrations. The inhibition percentage of schizont maturation in a treatment group was calculated by relating the number of schizonts present in the controls (>40%) with the number of schizonts in the treatment groups. Calculations were performed to determine the means or medians, standard deviations, and coefficients of variation of the schizont inhibition percentages obtained in three replicants of the antimalarial concentrations evaluated. The 50% inhibitory concentrations (IC_{50}s) were estimated using a dose-response regression model from the drug-free controls by the GraphPad Prism version 5.0 program. The IC_{50}s that were within the concentration range evaluated for each antimalarial and that had an r^2 value of >9.0 were classified as valid. The IC_{50} of each antimalarial was correlated with the antimalarial group to which it belonged (aminoquinolines and artemisinins) in order to find overlapping susceptibilities.

Statistical analysis. The statistical analysis was carried out using the GraphPad Prism version 5.0 software. The Kruskal-Wallis test was applied to determine the nonparametric comparisons, and the Fisher test was employed to calculate statistical significance. The nonparametric correlation analysis was carried out using the Spearman correlation (r_s) model.

RESULTS

Of the 68 *P. vivax* monoinfection isolates (confirmed by PCR) that fulfilled the inclusion criteria, 30 (44.1%) achieved a schizont maturation of >40%. The mean initial parasitemia level was 9,853.3 parasites/μl of blood (95% confidence interval [CI], 7,640.7 to 17,494.0 parasites/μl). The isolates took an average of 28.8 h (95% CI, 25.7 to 31.9 h) to reach schizont maturation, and each isolate reached an average maturation of 54% (95% CI, 46% to 100%). Of the evaluated isolates, 15 (50%) were classified as group 1, with >133 immature Tfz out of 200 asexual parasites and a mean initial parasitemia level of 12,605 parasites/μl of blood (95% CI, 8,985 to 16,226 parasites/μl). The other 15 (50%) were classified into group 2, with >133 mature Tfz out of 200 asexual parasites and a mean initial parasitemia level of 6,949 parasites/μl of blood (95% CI, 4,749 to 9,150 parasites/μl). The parasitemia levels were significantly different in the two groups ($P < 0.05$). In group 1, 66.7% (10/15) of the isolates achieved schizont maturation after 30 h, 20% (3/15) reached this stage after 40 h, and 13.3% (2/15) achieved maturation after 48 h; in group 2, 73.3% (11/15) achieved maturation after 30 h, and the rest reached this stage after 20 h. The majority of cultures in both groups had maturation times of 30 h. The maturation times were significantly higher in the isolates of group 1 than in those of group 2 ($P < 0.0001$) (Table 1).

***In vitro* susceptibility of *P. vivax* to antimalarials.** The number of valid tests and the IC_{50} found for each antimalarial can be seen in Table 2. Due to the fact that only 30 isolates matured successfully, the IC_{50} medians were analyzed based on the predominance of the initial parasitic stages, as shown in Table 3. The minimum and maximum values of the IC_{50}s were found for CQ (2.5 nM to 1,109.0 nM), MQ (10.6 nM to 522.3 nM), AQ (2.7 nM to 105.2 nM), Qn (4.0 nM to 1,398.0 nM), and AS (0.1 nM to 10.7 nM) (Fig. 1).

Correlation of the IC_{50}s of the different antimalarials for *P. vivax* from Colombia. The overlapping susceptibilities of the 30 isolates of *P. vivax* to all of the antimalarials evaluated are shown in Table 4. The susceptibilities of the quinoline group antimalarials were compared to that of AS (a derivative of artemisinin). Positive correlations to AS were found for CQ and MQ ($P < 0.001$) (Table 4).

TABLE 3 Comparison of median IC_{50} of each antimalarial based on initial parasitic stage in *P. vivax* isolates

Antimalarial	Group 1[a]		Group 2[a]		
	No. of isolates	IC_{50}[b] (median [IQR]) (nM)	No. of isolates	IC_{50}[b] (median [IQR]) (nM)	*F* test *P* value
Chloroquine	15	17.0 (3.2–59.8)	15	39.3 (11.5–73.7)	<0.0001
Mefloquine	13	24.1 (17.3–58.8)	15	72.2 (37.4–169.5)	0.644
Amodiaquine	12	3.7 (3.0–31.6)	14	7.2 (4.6–17.5)	0.199
Quinine	14	65.0 (24.8–946.2)	14	209.2 (53.5–378.3)	0.301
Artesunate	12	0.3 (0.1–1.0)	14	1.4 (0.2–2.0)	<0.0001

[a] Isolates with valid IC_{50} for each antimalarial.
[b] Analysis of the median of the concentration that inhibited 50% of the maturation of the schizonts. IQR, interquartile range (25% to 75%).

TABLE 4 Overlapping susceptibilities of *P. vivax* among different antimalarials

Antimalarial	r_s for indicated antimalarial[a]			
	Mefloquine	Amodiaquine	Quinine	Artesunate
Chloroquine	0.557**	0.489*	0.457*	0.689***
Mefloquine		0.456*	0.564**	0.639***
Amodiaquine			0.475*	0.414*
Quinine				0.483*

[a] Correlations are shown for the *in vitro* susceptibilities of the Colombian isolates of *P. vivax* to all of the antimalarials evaluated. Statistical significances are designated as follows: ***, $P < 0.00$; **, $P < 0.01$; *, $P < 0.05$. A positive correlation can be seen among the antimalarials evaluated.

DISCUSSION

This study analyzed the susceptibilities of 30 isolates of *P. vivax* to different antimalarials in Colombia. Chloroquine is a drug of special interest as it corresponds to a first-line treatment for *P. vivax* malaria in Colombia (25). Given the difficulty of maintaining *P. vivax* in continuous cultures (29–31), an *in vitro* short-term growth method was used which consisted of maintaining the parasites in culture for periods of <48 h until they reached schizont maturation (32). This method has helped to determine the *in vitro* susceptibility of *P. vivax* to antimalarials in different geographic regions of Africa and Asia (20–22, 33–35).

The IC_{50}s observed for the isolates from the region of Urabá, which is an area with low malaria endemicity for South America but high endemicity for Colombia, were low for all of the antimalarials. However, there were isolates that presented IC_{50}s for CQ that were 47.6 times higher than the mean IC_{50} (23.3 nM). Four (13.3%) out of 30 isolates presented IC_{50}s of >100 nM, which is similar to what has been found in Asian countries such as Thailand, Indonesia, and Myanmar and in Papua New Guinea and Brazil (36) (Table 5).

It is important to note that the schizont maturation method entails variables like the level of maturity of the parasitic stages at the beginning of the assay. These variables influence the maturation time of the schizonts and the reproducibility of the results. This is one of the possible reasons that the IC_{50}s of CQ and AS were significantly different ($P < 0.0001$) in the two groups established according to initial parasitic stages, as has been reported previously in the literature. Despite the differences found, the mean IC_{50}s for all of the antimalarials were observed to be within the ranges of susceptibility, suggesting that the Colombian isolates were susceptible even when they were cultured from mature trophozoites (21, 37).

Whereas the mature trophozoite in the case of *P. falciparum* is considered to be the target stage of CQ, this target is not recognized for *P. vivax*. It is believed that each species has characteristics

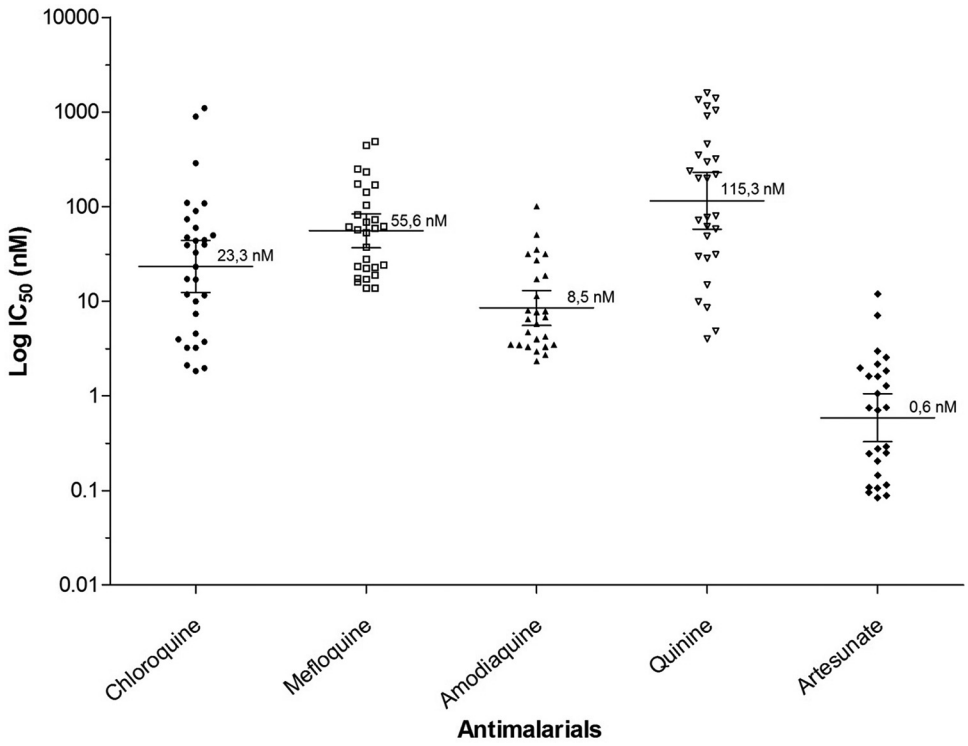

FIG 1 Geometric means of the IC_{50}s of antimalarials in *P. vivax* isolates in Urabá, Colombia.

TABLE 5 Susceptibility profiles of the *P. vivax* isolates to antimalarials in different geographical regions

Reference or source	Yr	Country	No. of successful assays	Geometric mean IC_{50} (nM) of antimalarials reported in the literature				
				CQ	MQ	AQ	AS	Qn
10	1995	Thailand	57	360.5[a]	—[d]	—	—	—
27	2002	Thailand	121	50.3[b]	—	—	—	—
22	2003	Thailand	34	96.5	—	—	—	—
19	2004	Thailand	20	96.9	335.9	30.1	1.3	393.4
42	2007	Thailand	81	46.8	—	—	—	—
		Indonesia	145	312[a]	—	—	—	—
20	2007	Myanmar	2	>100[a]	—	—	—	—
			15	<100	—	—	—	—
36	2008	Thailand	ND[c]	12[e]	—	—	—	—
			ND	415[f]	—	—	—	—
21	2008	Indonesia	216	295[a]	12.7	15.4	1.31	—
				55.2[e]	7.9[e]	16.6[e]	—	—
				2,812[f]	12.5[f]	24.2[f]	—	—
38	2008	Thailand	65	36.7	111	34	8.3	—
		Indonesia	85	114[a]	9.87	13.7	1.4	—
33	2009	Korea	50	75.6	103.2	—	1.5	75.4
		Tailandia	24	96.9	335.9	—	1.3	393.4
43	2011	Thailand	34	167.2[a]	139.4	—	32.6	—
37	2011	China	42	12.6	11.2	—	0.3	—
36	2014	Brazil	32	32	57	—	21	—
This study	2014	Colombia	30	23.3	55.6	8.5	0.6	115.3

[a] Susceptibility to chloroquine may be lost when the IC_{50} is >100 nM.
[b] Thirty-hour incubation.
[c] ND, no data.
[d] —, not analyzed in the study.
[e] Ring stage.
[f] Trophozoite mature stage.

that can explain the differences in susceptibility of each parasitic stage to chloroquine (21, 37). During studies that monitor the resistance of *P. vivax* to antimalarials, it is important to take into account the target parasite form. In this study, the cultures that began with a predominance of mature forms (group 2) presented significantly higher IC_{50}s than those that began with immature forms (group 1).

When determining the susceptibility of *P. vivax* to antimalarials, the monitoring of schizont maturation is fundamental in order to avoid taking premature readings, which would cause a smaller percentage of schizonts to be recorded than expected. However, excess maturation time would cause the schizonts to rupture, and their numbers would be reduced (21).

The positive correlation observed between the susceptibilities of the isolates to the quinolines CQ, MQ, AQ, Qn, and AS (a derivative of artemisinins) suggests a common mode of action, as proposed by other authors (21, 27). Artesunate is a first-line drug for the treatment of *P. falciparum* malaria in Colombia (25). This study shows that in Colombia, AS is also effective against *P. vivax*, as an IC_{50} of 0.6 nM (95% CI, 0.3 to 1.0 nM) was determined. Studies carried out between 2004 and 2009 in different regions of Asia, including Thailand and Indonesia, reported an IC_{50} of <10 nM for the effect of artesunate against *P. vivax*. Such results suggest that derivatives of artemisinins have been effective against this type of parasite. However, an IC_{50} of 32.6 nM was found for artesunate in Thailand in 2011 and an IC_{50} of 21.0 nM was found for this antimalarial in a Brazilian region in 2014, which suggests a susceptibility loss of *P. vivax* to this antimalarial in two different geographic regions (Table 5) (9, 19, 21, 22, 36, 38). In this study, when the isolates were classified according to the initial parasitic stages, IC_{50}s of 0.3 nM for AS in group 1 and 1.4 nM in group 2 were found (Table 3). Although these values are significantly different, they are below 10 nM, which indicates that this antimalarial is effective against *P. vivax* in Colombia. With respect to MQ, in this study, an IC_{50} of 55.6 nM (Table 2) was found, which differs from the values reported from 2008 to 2011 for Thailand, Indonesia, and China and from the values reported in 2004 and 2009 for Thailand and Korea (19, 33). In the case of AQ, an IC_{50} of 8.5 nM (Table 2) was recorded, which is similar to the values reported in different regions of Asia (IC_{50}, <30 nM) between 2004 and 2008 (Table 5) (19, 22, 39). This suggests that AQ is effective against *P. vivax* in the various geographical regions studied in Asia and South America. Finally, Qn in this study had an IC_{50} of 115.3 nM, which was almost three times below the values reported in the literature (393.4 nM) (Table 5). However, it should be noted that there are very few reports of quinine being evaluated *in vitro* against *P. vivax*. More monitoring of this antimalarial in different geographical regions would help to establish its effectiveness.

Given the difficulty in maintaining an *in vitro* culture of *P. vivax* (29–31), resistance studies have been complemented by use of genetic markers. One example of a genetic marker is the *pvmdr-1* gene that encodes the protein associated with multiple-drug resistance. The presence of mutations is associated with the loss of *in vitro* susceptibility of *P. vivax* to the antimalarials. Furthermore, the mutations in the *pvdhfr* and *pvdhpr* genes of *P. vivax* that encode the dihydrofolate reductase and dihydropteroate synthase proteins, respectively, have been shown to be associated with a loss of susceptibility to sulfadoxine-pyrimethamine (40).

In 2008, Suwanarusk et al. (39) evaluated how *in vitro* susceptibility is associated with the presence of mutations in the *pvmdr-1* gene. They found a significantly high IC_{50} (78.6 nM) for mefloquine and an increase in the number of copies of the *pvmdr-1* gene compared with isolates which had a low IC_{50} (38 nM) and a single copy of the gene. The presence of the Y976F mutation of the *pvmdr-1* gene has been associated with a greater loss of susceptibility to CQ (IC_{50}, 154 nM) compared to that of the isolates without mutation (IC_{50}, 34 nM) but with greater susceptibilities to artesunate (IC_{50}s, 1.8 nM [mutant] versus 9.5 nM [native]) and mefloquine (IC_{50}s, 14 nM [mutant] versus 121 nM [wild type]).

In Central America, polymorphisms have been found in the *pvmdr-1* and *pvdhfr* genes that may be related to the loss of susceptibility of *P. vivax* to antimalarials. However, there is no evidence from *in vitro* susceptibility studies to support this association (41). In South America, only isolated cases of the therapeutic failure to CQ for the treatment of *P. vivax* malaria have been documented, and no data on CQ drug levels to ensure therapeutic levels were included. Also, there are few reports of *in vitro* susceptibility of *P. vivax* to antimalarials. However, ongoing clinical efficacy monitoring would be important, in addition to integrated *in vitro* susceptibility and molecular marker studies. For example, in the Amazonian region of Brazil, in 2013, Chehuan et al. (42) reported that 12 (10.7%) out of 112 isolates with an IC_{50} of >100 nM were considered resistant to CQ, while 3 (6.4%) of 47 were considered resistant to MQ. The same study showed that Amazonian *P. vivax* strains with both CQ and MQ resistance may be common, and a nonsynonymous mutation at *pvdhps* codon 382 (S→C) was associated with *in vitro* susceptibility to CQ; thus,

further studies should be done to confirm this observation. On the other hand, in 2014, Aguiar et al. (36) found IC_{50}s similar to those reported for CQ and MQ in this region; however, the IC_{50}s for AS observed in this work for Colombia were 20 times lower than those reported in Brazil (Table 5).

In conclusion, this study showed that the Colombian isolates of *P. vivax* continue to be susceptible to all of the antimalarials evaluated. It is important to note that this is the first report that has investigated the effect of the IC_{50}s of antimalarials against *P. vivax* in Colombia. However, the presence of isolates with IC_{50}s of >100 nM for CQ, MQ, and Qn suggests the need for periodically monitoring the therapeutic responses to antimalarials and for the evaluation of antimalarial drug levels in blood. Importantly, it will be associated with the IC_{50s} *in vitro* of antimalarials in *P. vivax* and their tendency to increase toward an eventual therapeutic failure. It is also important to search for specific mutations in genetic markers as has been reported in Asia (10, 20, 21, 37, 39, 43–45).

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