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Determinants of In Vitro Drug Susceptibility Testing of *Plasmodium vivax*▽

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In Papua, Indonesia, the antimalarial susceptibility of *Plasmodium vivax* ($n = 216$) and *P. falciparum* ($n = 277$) was assessed using a modified schizont maturation assay for chloroquine, amodiaquine, artesunate, lumefantrine, mefloquine, and piperaquine. The most effective antimalarial against *P. vivax* and *P. falciparum* was artesunate, with geometric mean 50% inhibitory concentrations (IC_{50}s) (95% confidence intervals [CI]) of 1.31 nM (1.07 to 1.59) and 0.64 nM (0.53 to 0.79), respectively. In contrast, the geometric mean chloroquine IC_{50} for *P. vivax* was 295 nM (227 to 384) compared to only 47.4 nM (42.2 to 53.3) for *P. falciparum*. Two factors were found to significantly influence the in vitro drug response of *P. vivax*: the initial stage of the parasite and the duration of the assay. Isolates of *P. vivax* initially at the trophozoite stage had significantly higher chloroquine IC_{50}s (478 nM [95% CI, 316 to 722]) than those initially at the ring stage (84.7 nM [95% CI, 45.7 to 157]; $P < 0.001$). Synchronous isolates of *P. vivax* and *P. falciparum* which reached the target of 40% schizonts in the control wells within 30 h had significantly higher geometric mean chloroquine IC_{50}s (435 nM [95% CI, 169 to 1,118] and 55.9 nM [95% CI, 48 to 64.9], respectively) than isolates that took more than 30 h (39.9 nM [14.6 to 110.4] and 36.9 nM [31.2 to 43.7]; $P < 0.005$). The results demonstrate the marked stage-specific activity of chloroquine with *P. vivax* and suggest that susceptibility to chloroquine may be associated with variable growth rates. These findings have important implications for the phenotypic and downstream genetic characterization of *P. vivax*.

In vitro drug susceptibility assays assess antimicrobial activity in the absence of the confounding effects of the host. Although such assays have become useful for monitoring the antimalarial resistance of *Plasmodium falciparum*, the assay has been of limited use with *P. vivax.* This is in part a consequence of a perception of the importance of antimalarial drug resistance with *P. vivax*, compounded by difficulties in standardizing a field-based assay. Over the last decade, a number of clinical studies have demonstrated the emergence of high-grade chloroquine resistance in Papua, Indonesia, and Papua, New Guinea (1, 18, 21), and its spread to other regions of Asia (6) and South America (20). However, assessment of the clinical efficacy of antimalarial drugs against *P. vivax* infection is confounded by the occurrence of both reinfections and relapses, making the attributable fraction of recurrent infections due to intrinsic parasite resistance difficult to gauge (2, 3, 10). To confirm the emergence of the spread of antimalarial drug resistance of *P. vivax* and to investigate alternative antimalarial drugs, it is critical that a standardized in vitro assay be developed and validated. The aim of this study was to define the in vitro susceptibility profiles of a range of antimalarial drugs and to investigate the confounding factors that modulate the derived estimate of drug efficacy.

MATERIALS AND METHODS

Field location and sample collection. Between March 2004 and May 2007, *Plasmodium* isolates were collected from patients attending malaria clinics in Timika, located in the southern part of Papua province, Indonesia. Timika is a region of endemicity for multidrug-resistant strains of *P. vivax* and *P. falciparum*, with a risk of treatment failure of 65% within 28 days after chloroquine monotherapy for *P. vivax* malaria and 48% failure after multidrug therapy with chloroquine-sulfadoxine-pyrimethamine for *P. falciparum* malaria (16). In 2004, treatment guidelines were changed accordingly to recommend an artemisinin combination therapy for both *P. falciparum* and *P. vivax* infection, precluding further clinical studies of the use of chloroquine monotherapy in this region (15). Patients with symptomatic malaria who presented to an outpatient facility were recruited into the study if they were singly infected with *P. falciparum* or with *P. vivax*, with a parasitemia of between 2,000 μl^{-1} and 80,000 μl^{-1}. Patients treated with antimalarials in the previous 3 weeks were excluded from this study. Venous blood (5 ml) was collected by venipuncture and, after the host white blood cells were removed using a CF11 column, 2 ml of packed infected red blood cells were divided as follows: 1 ml was cryopreserved in Glycerolyte 57 solution (Baxter, Deerfield, IL), 200 μl was spotted onto a filter paper, and 800 μl was used for the in vitro drug susceptibility assay. Since most patients were not enrolled in clinical studies, the therapeutic response to treatment could not be determined.

In vitro drug susceptibility assay. The antimalarial susceptibility of *P. vivax* and *P. falciparum* isolates was measured using a protocol modified from the WHO microtest, as described previously (11, 19). Two hundred microliters of a 2% hematocrit blood medium mixture consisting of McCoy's 5A medium and 20% AB+ human serum was added to each well of predosed drug plates. The drug plates contained 11 serial concentrations of the antimalarials, with maxi-

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TABLE 1. Baseline characteristics of isolates with which in vitro assay was attempted

Isolate	Total no. of isolates assayed	Total no. of isolates harvested (% of isolates)	Median (range) delay from venipuncture to start of culture (h)	Geometric mean parasitemia (no. of parasites/μl [95% CI])	% of patients receiving antimalarial treatment in previous 2 mo (*n*/total)	Median initial % of parasites at ring stage (IQR)[a]	% of isolates with an RT ratio of ≥1 (*n*/total)
P. vivax	216	175 (81)	2.00 (0.33–11.0)	7,338 (6,665–8,079)	25 (39/158)	36.5 (8–66)	37 (73/196)
P. falciparum	277	226 (82)	2.96 (0.17–11.3)	10,968 (9,862–12,187)	14 (32/224)	100 (100–100)	98 (222/227)

[a] Not available for 20 isolates. IQR, interquartile range.

mum concentrations of 5,910 nM for chloroquine, 557 nM for amodiaquine, 93 nM for artesunate, 489 nM for lumefantrine, 338 nM for mefloquine, and 769 nM for piperaquine. A candle jar was used to mature the parasites at 37.5°C (22 to 42 h). Incubation was stopped when >40% of ring-stage parasites had matured to mature schizonts in the drug-free control. Preliminary studies demonstrated that once the 40% schizont threshold had been reached, further incubation did not increase the final count.

Thick blood films made from each well were stained with 5% Giemsa stain for 30 min and examined microscopically. Differential counts of 200 asexual parasites on the preincubation and test slides were divided into ring-stage parasites (ring-shaped trophozoites and early amoebids without pigment), mature trophozoites (in which single or double chromatin dot and hemozoin pigment were visible), and schizonts. Free merozoites and gametocytes were not included in the count. To ensure optimal maturity and ease of parasite identification and to reduce the parasite classification error between microscopists, only schizonts with at least five well-defined chromatin dots were classified as schizonts at harvest.

The number of schizonts per 200 asexual-stage parasites was determined for each drug concentration and normalized to the control well. The dose-response data were analyzed using nonlinear regression analysis (WinNonLin version 4.1; Pharsight), and the 50% inhibitory concentration (IC_{50}) value was derived using an inhibitory sigmoid Emax model. In vitro data were used only from predicted curves where the Emax and E_0 values were within 15% of 100 or 0, respectively.

Data analysis. Analysis was performed using SPSS software for Windows (version 15; SPSS, Inc., Chicago, IL). The Mann-Whitney U test or the Kruskal-Wallis method was used for nonparametric comparisons, and Student's *t* test (paired and unpaired) or one-way analysis of variance was used for parametric comparisons of log-transformed data. For categorical variables, percentages and corresponding 95% confidence intervals (CI) were calculated using Wilson's method. Proportions were examined using the χ^2 test with Yates' correction or by Fisher's exact test.

Ethical approval. Ethical approval for this study was obtained from the ethics committees of the National Institute of Health Research and Development, Ministry of Health, Indonesia, and Menzies School of Health Research, Darwin, Australia.

RESULTS

Between March 2004 and April 2007, venous blood was collected from 493 patients with single-species infections of either *Plasmodium vivax* ($n = 216$) or *P. falciparum* ($n = 277$) and assayed for drug susceptibility. Adequate growth for harvest was achieved with 81% of isolates (401/493). Baseline characteristics of the isolates processed are presented in Table 1. Whereas all *P. falciparum* isolates had ring-stage counts of more than 80% prior to culture, the *P. vivax* isolates were mostly asynchronous (median percentage of rings = 36.5% [interquartile range, 8 to 66%]) (Fig. 1). For those isolates that reached harvest, the median duration of the assay was 26 h (range, 24 to 48 h), with no differences between species. At harvest, the mean percentage of schizonts in the culture was 43.3% (95% CI, 41.7 to 44.9) and did not differ between the species of infection. The number of harvested isolates for which the susceptibility data were rejected differed between drug assays, varying from 0% (0/99) for mefloquine to 23% (51/221) for chloroquine. The overall geometric mean IC_{50}s are presented in Table 2.

The time taken for *P. vivax* isolates to reach 40% schizonts, and thus the duration of the assay, was correlated significantly with the ratio of rings to trophozoites (the RT ratio) prior to culture. The greater the initial proportion of trophozoites the shorter the duration of the assay (Spearman correlation coefficient, $r_s = 0.485$; $P < 0.001$) (Fig. 2). Both the RT ratio and the duration of the assay were negatively correlated with the delay between venipuncture and setting the isolate up in culture ($r_s = -0.171$ and $P = 0.025$; and $r_s = -0.209$ and $P = 0.006$, respectively). For the *P. vivax* isolates with a long delay before processing (greater than 4 h), 84% (27/32) of the isolates had a majority of parasites at the trophozoite stage (RT < 1) at the time of culture, compared to 57% (80/140) of those processed within 4 h ($P = 0.0077$).

Initial stage of parasite and in vitro susceptibility. For *P. vivax*, the RT ratio was correlated significantly with the IC_{50} values for chloroquine ($r_s = -0.349$; $P < 0.001$), for amodiaquine ($r_s = -0.251$; $P = 0.003$), for artesunate ($r_s = -0.162$; $p = -0.05$), and for mefloquine ($r_s = -0.258$; $P = 0.007$). Compared to isolates for which the assay was set up predominantly at the ring stage (RT > 4), isolates set up predominantly at the trophozoite stage (RT < 0.25) had significantly higher geometric mean IC_{50} results for chloroquine (478 nM versus 85.8 nM; $P < 0.001$), amodiaquine (22.1 nM versus 10.0 nM; $P = 0.007$), artesunate (1.61 nM versus 0.86 nM; $P = 0.024$), and mefloquine (17.0 nM versus 6.39 nM; $P = 0.001$) but not from lumefantrine or piperaquine.

To investigate the relationship between the stage of the isolate and in vitro susceptibility, isolates with greater than 90% rings were set up in culture in the presence of the drug directly and again after culture in the absence of the drug to achieve 90% trophozoites. *Plasmodium vivax* isolates added to

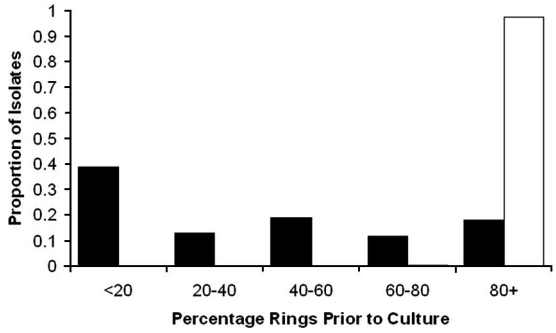

FIG. 1. Histogram of the initial percentage of rings prior to culture. Dark bars indicate *P. vivax*, and the light bar indicates *P. falciparum*.

TABLE 2. Overall in vitro sensitivity for each drug according to the species tested

Drug	*P. vivax*		*P. falciparum*	
	No. of acceptable assays/ total no. of assays harvested (%)[a]	Geometric mean IC_{50} value (nM) (95% CI)	No. of acceptable assays/ total no. of assays harvested (%)	Geometric mean IC_{50} value (nM) (95% CI)
Chloroquine	155/170 (91.2)	295 (227–384)	170/221 (77)	47.4 (42.2–53.3)
Amodiaquine	143/144 (99.3)	15.4 (12.7–18.7)	120/137 (88)	5.59 (4.96–6.29)
Artesunate	136/149 (91.3)	1.31 (1.07–1.59)	127/157 (81)	0.64 (0.53–0.79)
Lumefantrine	54/64 (83)	19.3 (13.2–28.0)	101/124 (82)	10.2 (7.6–13.9)
Mefloquine	108/108 (100)	12.7 (10.3–15.5)	146/166 (88)	8.5 (6.98–10.4)
Piperaquine	125/137 (91.2)	21.9 (18.5–25.8)	165/200 (83)	14.5 (13.2–16.1)

[a] Values shown are the total number of assays with acceptable IC_{50} values/number of assays harvested.

the assay at the ring stage had significantly lower chloroquine IC_{50}s than the same isolates added at the trophozoite stage (median IC_{50} of 55.2 nM versus 2,812 nM, respectively; $P = 0.008$). *P. falciparum* isolates exposed at the ring stage to chloroquine also resulted in lower IC_{50} values than those at the trophozoite stage, although the difference was more modest (median IC_{50} values of 36.1 versus 99.7 nM, respectively; $P = 0.008$ and $P = 0.04$, respectively). The effect of the initial stage of drug exposure for both *P. vivax* and *P. falciparum* was also significant for amodiaquine but did not reach significance for mefloquine (Table 3).

Duration of assay and in vitro susceptibility. The duration of the assay was negatively correlated with the in vitro drug susceptibility of chloroquine, amodiaquine, artesunate, and mefloquine for both *P. falciparum* and *P. vivax*; this was independent of the RT ratio and remained significant even after parasites with an RT ratio greater than 4 were selected (see Table 4). Of the 16 synchronous *P. vivax* isolates (RT $>$ 10) with valid chloroquine IC_{50} data, the median duration of the assay was 42 h (range, 24 to 48 h), with 31% (5/16) of the isolates reaching the harvest criteria within 30 h. The geometric mean chloroquine IC_{50} value for fast-growing synchronous parasites was 435 nM (95% CI, 169 to 1,118) compared to 39.9 nM (14.6 to 110.4) for isolates taking longer than 30 h ($P = 0.005$). Although the majority of *P. falciparum* isolates were synchronous at the start of the assay, the duration of the assay also ranged from 24 to 48 h, with 53% (83/155) of the isolates reaching harvest within 30 h. Faster growing *P. falciparum* parasites also had higher geometric mean chloroquine IC_{50} values (55.9 nM [95% CI, 48 to 64.9]) than the slower growing parasites (36.9 nM [31.2 to 43.7]; $P < 0.001$).

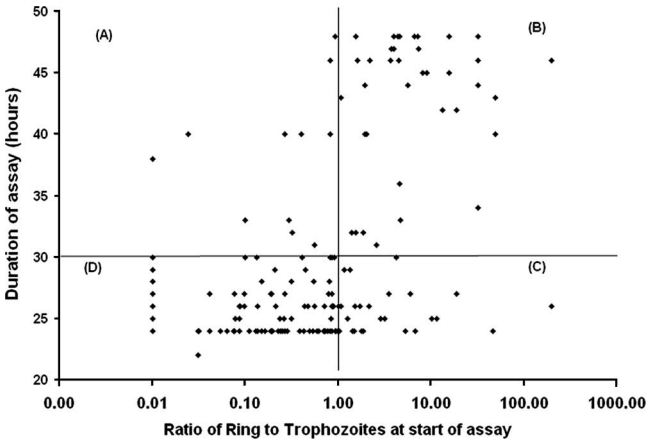

FIG. 2. Scatter plot of the ratio of rings to trophozoites stages present initially in *P. vivax* isolates relative to the time taken to reach 40% schizonts in the control wells.

Stratified results of in vitro susceptibility of *P. vivax*. Using criteria derived previously (23), *P. vivax* results were stratified into four groups according to whether the parasites were set up in culture with a majority of rings (RT $\geq$ 1) or a majority of trophozoites (RT $<$ 1) and whether the duration of the assay was below or above 30 h (Fig. 2). The stratified IC_{50} results are presented in Table 5. For isolates which were predominantly at the ring stage prior to culture (RT $\geq$ 1) (Fig. 2, quadrants B plus C), the geometric mean IC_{50} estimates for *P. vivax* were 142 nM (95% CI, 90.4 to 222) for chloroquine, 11.8 nM (95% CI, 8.6 to 16.2) for amodiaquine, 1.00 nM (95% CI, 0.70 to 1.42) for artesunate, 28.5 nM (95% CI, 15.6 to 51.8) for lumefantrine, 9.99 nM (95% CI, 6.98 to 14.3) for mefloquine, and 18.6 nM (95% CI, 14.0 to 24.6) for piperaquine. The correlation coefficients for *P. vivax* and *P. falciparum* isolates are presented in Table 6.

DISCUSSION

Unlike *Plasmodium falciparum*, *P. vivax* has a selective preference for invading young red cells (8), which has confounded the development of a standardized in vitro drug susceptibility assay. Susceptibility testing for *P. vivax* continues to rely on the modifications of the schizont maturation test (4, 7, 9, 14, 19, 23), whereas assays used for *P. falciparum* are increasingly incorporating red cell reinvasion and adopting alternative methods of growth quantification. In the present study, we compared the schizont maturation test for *P. falciparum* and *P. vivax* isolates in Papua, Indonesia, where clinical multidrug resistance of both species has emerged (16).

Whereas the isolates of *P. falciparum* were mostly synchronous, those of *P. vivax* were predominantly asynchronous, with all stages of the parasite present in the peripheral blood. The asynchronicity of *P. vivax* is well documented but, importantly, varies between areas of differing endemicity, a factor recently found to confound the in vitro susceptibility of chloroquine in both Thai and Indonesian isolates (22). In view of the high proportion of patients with asynchronous *P. vivax* infections in Papua, Indonesia, we adopted an inclusive approach for parasite testing based on the total parasitemia rather than the staging. As a conse-

TABLE 3. In vitro susceptibility for paired isolates tested at both the ring and the trophozoite stages[a]

Antimalarial	*P. falciparum*				*P. vivax*			
	No. of isolates	Median IC_{50} value (range) at the ring stage	Median IC_{50} value(range) at the trophozoite stage	*P* value	No. of isolates	Median IC_{50} value(range) at the ring stage	Median IC_{50} value(range) at the trophozoite stage	*P* value
Chloroquine	6	36.1 (11.9–55.0)	99.7 (19.6–157.8)	0.04	9	55.2 (18.3–171.8)	2812 (384–4611)	0.008
Amodiaquine	6	8.2 (2.6–16.2)	15.2 (5.9–22.4)	0.028	9	16.6 (6.7–37.9)	24.2 (12.5–59.9)	0.05
Mefloquine	6	4.6 (2.1–12.0)	6.4 (2.4–34.1)	0.075	9	7.9 (1.3–24.6)	12.5 (1.9–25.0)	0.123

[a] In vitro susceptibility for paired isolates was tested at both the ring (>90%) stage and after culture in the absence of the drug to the trophozoite stage (>90%).

quence, more than 60% of the isolates that were tested had a majority of trophozoites at the start of the assay. A major finding of our analysis is that in vitro susceptibility was correlated with the initial stage of the parasite, with isolates predominantly at the trophozoite stage having a twofold increase in IC_{50}s compared to those of parasites predominantly at the ring stage. To assess the stage specificity of drug activity further, synchronous isolates presenting with more than 90% ring stage were exposed to the drug immediately and after culturing to trophozoites in the absence of the drug. Exposure at the trophozoite stage resulted in higher IC_{50}s to amodiaquine and chloroquine, although the value did not reach statistical significance for mefloquine. Our findings confirmed the absence of chloroquine activity on *P. vivax* trophozoites, a developmental stage usually considered to be the specific target of this drug for *P. falciparum* (25), and concur with those made by Powell and Berglund some 30 years ago, using an in vitro MacroTest (13). Potentially, these observations have important biological implications, highlighting a fundamental difference between the activity of chloroquine with *P. vivax* and that with *P. falciparum*.

For convenience, most schizont maturation assays adopt a fixed duration of culture (usually 30 to 42 h) prior to the quantification of parasite growth (4, 12, 24). However, the time at which the assay is "harvested" is critical. Stopping the culture early reduces the proportion of schizonts present, thus decreasing the accuracy of quantification, whereas extending the culture beyond schizogony results in a significant reduction in the overall parasitemia as the mature schizonts start to rupture. In contrast to other methods, we report an assay in which in vitro culture was maintained until a threshold of 40% schizonts had been reached in the control wells. The time taken for isolates to reach this target varied considerably for both *P. vivax* and *P. falciparum*, from 22 to 48 h, even when synchronous ring-stage isolates were put into culture. The importance of this became apparent in the significant correlation of the in vitro susceptibility with the duration of the assay (Tables 4 and 5). Assays of shorter duration had significantly higher chloroquine, amodiaquine, artesunate, and mefloquine IC_{50}s than assays of longer duration. Predictably, the initial stage of the parasite was an important determinant of the time to harvest ($r_s = 0.489$; $P < 0.001$); isolates at the trophozoite stage had a shorter duration of assay (Fig. 2). However, the relation between the IC_{50}s and the duration of the assay remained after controlling for the initial stage of the parasite (Table 4). Moreover, after highly synchronous cultures (RT ratio > 10) were selected, 31% of *P. vivax* assays and 53% of *P. falciparum* assays were harvested within 30 h, and the isolates with a short assay duration had a 10-fold and a 1.5-fold increase in chloroquine IC_{50}, respectively, compared to isolates with longer assay durations.

Our findings raise the possibility that chloroquine resistant parasites grow faster than chloroquine susceptible parasites as has recently been reported in laboratory adapted strains of *P. falciparum* (17). Alternatively isolates rapidly reaching the threshold for harvest may represent parasites initially at a more mature ring stage. These findings have practical implications for interpreting in vitro assays and relevance for resent attempts to develop a nonmicroscopy assay. In assays that advocate culture for at least 36 h, faster growing isolates will rupture prior to quantification and thus will be recorded as unsuccessful. If these faster growing parasites are intrinsically more resistant then this will systematically bias the assessment of in vitro susceptibility toward a predominantly sensitive population. A recent comparison between Thai and Papuan isolates using the same assay highlighted the problem. In Thailand, where *P. vivax* is predominantly sensitive to chloroquine, the geometric mean IC_{50} was reported as 46.8 nM (22). In Papua, chemotherapeutic studies have confirmed high levels of clinical resis-

TABLE 4. Correlation of in vitro susceptibility (IC_{50}) with the duration of assay

Drug	*P. falciparum*			All *P. vivax*			*P. vivax* RT > 4		
	r_s value	No. of isolates	*P* value	r_s value	No. of isolates	*P* value	r_s value	No. of isolates	*P* value
Chloroquine	−0.245	168	0.001	−0.457	155	0.001	−0.616	30	<0.001
Amodiaquine	−0.220	118	0.017	−0.356	143	<0.001	−0.400	27	0.039
Artesunate	−0.290	125	0.001	−0.350	136	<0.001	−0.544	24	<0.001
Lumefantrine	−0.023	101	0.818	0.036	54	0.797	0.359	5	0.553
Mefloquine	−0.340	144	<0.001	−0.413	108	<0.001	−0.381	20	0.097
Piperaquine	−0.201	163	0.010	−0.147	125	0.101	−0.316	20	0.174

r_s values indicate the Spearman rank correlation.

TABLE 5. In vitro sensitivities of *P. vivax,* stratified by duration of assay and RT ratio prior to culture[a]

Drug	Quadrant A		Quadrant B		Quadrant C		Quadrant D		*P* value[d]
	No. of isolates	IC_{50} value(95% CI) for trophozoite majority and duration ≥30 h[b]	No. of isolates	IC_{50} value (95% CI) for ring majority and duration ≥30 h[c]	No. of isolates	IC_{50} value (95% CI) for ring majority and duration <30 h[c]	No. of isolates	IC_{50} value (95% CI) for trophozoite majority and duration <30 h[b]	
Chloroquine	18	298 (153–583)	35	59.8 (34.8–103)	25	475 (296–762)	75	539 (387–751)	<0.001*
Amodiaquine	15	16.5 (9.8–27.9)	35	7.85 (5.31–11.6)	21	23.4 (15.6–35.0)	71	18.9 (14.2–25.0)	0.001*
Artesunate	14	1.20 (0.71–2.02)	32	0.83 (0.56–1.23)	18	1.39 (0.69–2.80)	71	1.60 (1.23–2.09)	0.06
Lumefantrine	6	32.9 (5.59–193)	5	37.6 (22.4–63.0)	10	24.8 (9.8–62.6)	33	14.6 (8.9–24.1)	0.296
Mefloquine	12	7.27 (3.84–13.8)	30	7.26 (4.89–10.8)	12	22.2 (11.7–42.2)	53	17.4 (13.5–22.4)	<0.001**
Piperaquine	13	29.1 (22.3–38.1)	30	14.6 (10.3–20.7)	14	31.2 (20.9–46.5)	67	22.9 (17.9–29.3)	0.03***

[a] In vitro sensitivity of *P. vivax* for each drug (geometric mean IC_{50} [95% CI]) stratified by the duration of assay and the RT ratio prior to culture. Quadrants are the same subgroups as represented in Fig. 2.
[b] RT < 1; data on the initial parasite stage was unavailable for 20 isolates.
[c] RT ≥ 1; data on initial RT ratios were missing for two isolates.
[d] Overall between-group differences, by analysis of variance: *, group B versus A, C, or D, $P = 0.001$; **, group A or B versus C or D, $P = 0.006$; ***, group B versus A, C, or D, $P = 0.026$.

tance to chloroquine (16). If we had adopted a criterion based on the duration of the assay and initial parasite staging, then the derived estimate of IC_{50} would not have been significantly different (59.8 nM) (Table 5). Applying a criterion of selecting isolates with a majority of rings irrespective of the duration of the assay (i.e., quadrants B and C in Table 5) yielded a significantly higher population mean (142 nM).

These data have relevance for the development of nonmicroscopic methods for determining the in vitro susceptibility of *P. vivax*, such as enzyme-linked immunosorbent sensitivity or DNA detection (5). The absence of chloroquine sensitivity at the trophozoite stage combined with minimal reinvasion and parasitemia, generally less than 0.5%, will both contribute to a poor signal-to-background ratio, which are likely to undermine the sensitivity and reproducibility of such assays (11).

Although population estimates of drug susceptibility would best be based on isolates which are synchronous and are initially exposed at the tiny ring stage, in practice, such strict criteria are likely to exclude almost 90% of *P. vivax* isolates, which in itself may constitute an appreciable bias. Hence, to accommodate both the stage specificity of the drug action and the possible confounding effects of the speed of parasite growth, we recommend selecting parasites with ring stage predominance (RT > 1). Ensuring that isolates are set up in culture within 2 h of venipuncture will help to increase the proportion fulfilling this criterion. Although the trophozoite minority will still contribute to the schizont quantification, by 30 h of culture, most will have ruptured, leaving those present at harvest to represent parasites exposed to the drug at all stages. Cultures should be stopped upon reaching a certain threshold rather than used in a fixed-duration assay, which has the potential to bias toward sensitive isolates.

In conclusion, the primary objective of this study was to

TABLE 6. Correlation coefficients for the in vitro antimalarial susceptibilities of *P. falciparum* and *P vivax*[a]

Antimalarial	Combination	*P. Falciparum*			*P. Vivax*		
		Correlation (r_s)	*P* value	df	Correlation	*P* value	df
Chloroquine	Amodiaquine	0.648	<0.001	119	0.698	<0.001	51
	Artesunate	0.596	<0.001	111	0.285	0.05	46
	Lumefantrine	0.427	<0.001	65	−0.104	0.735	12
	Mefloquine	0.445	<0.001	136	0.618	<0.001	38
	Piperaquine	0.347	<0.001	139	0.620	<0.001	40
Amodiaquine	Artesunate	0.673	<0.001	119	0.743	<0.001	48
	Lumefantrine	0.571	0.006	21	−0.277	0.438	9
	Mefloquine	0.386	<0.001	112	0.703	<0.001	41
	Piperaquine	0.444	<0.001	115	0.766	<0.001	41
Artesunate	Lumefantrine	0.030	0.879	27	−0.112	0.793	7
	Mefloquine	0.340	<0.001	118	0.609	<0.001	39
	Piperaquine	0.422	<0.001	115	0.432	0.005	39
Lumefantrine	Mefloquine	0.016	0.914	50	0.702	0.504	2
	Piperaquine	0.152	0.215	67	−0.697	0.124	5
Mefloquine	piperaquine	0.225	0.011	125	0.644	<0.001	40

[a] Correlation coefficients (r_s) are shown for the in vitro antimalarial susceptibility of *P. falciparum* and *P vivax*. *P. vivax* isolates shown are restricted to those with a RT ≥ 1.

determine the major factors underlying *P. vivax* drug susceptibility, using a schizont maturation assay. Although we have demonstrated that this method can be used to discriminate between resistant and sensitive populations of *P. vivax* (22), the derived IC_{50} value needs to be interpreted in conjunction with the initial stage of the parasite as well as the duration of the assay.

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