

## Antimalarial Drug Sensitivity Profile of Western Kenya *Plasmodium falciparum* Field Isolates Determined by a SYBR Green I *in vitro* Assay and Molecular Analysis

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**Abstract.** *In vitro* drug sensitivity and molecular analyses of *Plasmodium falciparum* track drug resistance. DNA-binding fluorescent dyes like SYBR Green I may allow field laboratories, proximal to *P. falciparum* collection sites, to conduct drug assays. In 2007–2008, we assayed 121 *P. falciparum* field isolates from western Kenya for 50% inhibitory concentrations (IC<sub>50</sub>) against 6 antimalarial drugs using a SYBR Green I *in vitro* assay: 91 immediate *ex vivo* (IEV) and 30 culture-adapted, along with *P. falciparum* reference clones D6 (chloroquine [CQ] sensitive) and W2 (CQ resistant). We also assessed *P. falciparum* *mdr1* (*Pfmdr1*) copy number and single nucleotide polymorphisms (SNPs) at four codons. The IC<sub>50</sub>s for IEV and culture-adapted *P. falciparum* isolates were similar, and approximated historical IC<sub>50</sub>s. For *Pfmdr1*, mean copy number was 1, with SNPs common at codons 86 and 184. The SYBR Green I assay adapted well to our field-based laboratory, for both IEV and culture-adapted *P. falciparum*, warranting continued use.

### INTRODUCTION

Since the 1980s, measuring *in vitro* drug 50% inhibitory concentrations (IC<sub>50</sub>) against *Plasmodium falciparum* field isolates, coupled with molecular analysis, has been useful for tracking development of *in vivo* drug resistance, from Southeast Asia to sub-Saharan Africa.<sup>1–5</sup> However, one commonly used assay, <sup>3</sup>H-hypoxanthine uptake, is cumbersome, expensive, and often limits assay conduct to urban, well-resourced laboratories.<sup>4,5</sup> Moreover, blood samples containing *P. falciparum* parasites for testing may be stored at collection sites for days, transported long distances to the laboratory, and then culture adapted for assay, all potential sources of clonal selection.

Recently, non-radioisotope microtests using fluorescent DNA dyes such as SYBR Green I, which reliably depict *in vitro* *P. falciparum* parasite replication, have gained popularity by reducing some hurdles associated with *in vitro* *P. falciparum* drug sensitivity assays.<sup>6–9</sup> The SYBR Green I assays are considered convenient, relatively rapid, reproducible, and less costly than radioisotope assays.<sup>8,10</sup> These features suggest SYBR Green I drug sensitivity assays could be deployed to field laboratories, proximal to *P. falciparum* collection sites. The SYBR Green I is increasingly accepted as an alternate to <sup>3</sup>H-hypoxanthine uptake assays.<sup>11</sup>

In Kenya, malaria continues to cause significant morbidity and mortality, and is often a location where drug resistance emerges in Africa. For example, the development of *P. falciparum* resistance to chloroquine diphosphate (CQ), and later sulfadoxine-pyrimethamine, are well described.<sup>12–15</sup> This warrants continued *in vitro* drug IC<sub>50</sub> monitoring of *P. falciparum* field isolates, which is becoming more practical and safer, as more reliable assays like SYBR Green I become established, combined with simpler field isolate processing such as “immediate *ex vivo*” (IEV).

### SUBJECTS AND METHODS

**Protocol, sites, and subjects.** This study was approved by the Kenya Medical Research Institute (KEMRI) and Walter

Reed Army Institute of Research (WRAIR) institutional review boards (protocol numbers: KEMRI 1330, WRAIR 1384). Participating clinical centers, all Kenya Ministry of Health facilities located in West Kenya (Figure 1), including Kisumu, Kisii, and Kericho District Hospitals, and Chulaimbo Sub-District Hospital. Kisumu and Chulaimbo are lowland, malaria holoendemic areas, whereas Kisii and Kericho are highland, malaria hypoendemic areas.<sup>16</sup> At each participating facility, training of medical staff, capacity building, and facility upgrades were provided by the Global Emerging Infections Surveillance (GEIS) Program, U.S. Department of Defense.

Subjects attending outpatient clinics in 2007 and 2008, at least 6 months old and suspected of having non-complicated *P. falciparum* malaria were invited to participate. Written informed consent was obtained from adult subjects (≥ 18 years of age) or legal guardians for subjects < 18 years of age. Persons treated for malaria within the last 2 weeks were excluded.

**Sample collection and preparation.** Consented subjects with a positive *P. falciparum* rapid diagnostic test (RDT; Parascreen [Pan/Pf], Zephyr Biomedicals, Verna Goa, India) provided 2–3 mL of blood for transport to the laboratory. Parascreen detects *P. falciparum*-specific histidine protein-2 (Pf HRP-2) and pan-specific parasite lactate dehydrogenase (pLDH) in whole blood, the latter is useful for follow-up of antimalarial therapy. Three blood spots of about 100 µL each were placed on FTA filter paper (Whatman Inc., Bound Brook, NJ) for *P. falciparum* DNA extraction and molecular analysis, and two blood films on glass slides were made for Giemsa staining at the laboratory for microscopic examination, to confirm RDT results and determine parasitemia. For discrepancies between RDT and microscopy, microscopy determined the final result.

*Plasmodium falciparum* isolates from Kisumu District Hospital and Chulaimbo Health Center, 15-minute drive from the laboratory, were collected in acid citrate dextrose (ACD) vacutainer tubes (Becton-Dickinson, Inc., Franklin Lakes, NJ) and transported within 4 hours to begin IEV drug IC<sub>50</sub> testing (described below). *Plasmodium falciparum* isolates from Kericho and Kisii District Hospitals, 2-hour drive from the laboratory, were placed in storage-transport media, and refrigerated at 4°C until transported to the laboratory, generally within 72 hours, for culture adaptation and drug testing.<sup>17</sup>

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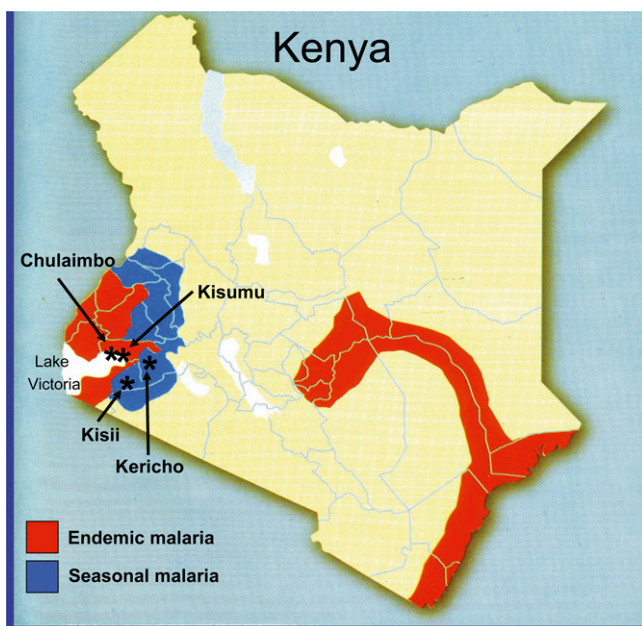


FIGURE 1. *Plasmodium falciparum* field isolates were collected from four western Kenya sites, denoted by (\*).

Subjects were treated with oral artemether-lumefantrine (AL; Coartem) administered over three consecutive days, a standard of care for *P. falciparum* malaria in Kenya. The first dose was observed by the study team and remaining doses were self-administered at home. Subjects living near study centers were asked to return on Day 7, for repeat malaria testing.

**In vitro drug sensitivity testing.** A SYBR Green I-based *in vitro* IC<sub>50</sub> drug sensitivity assay, described earlier,<sup>6,7,9</sup> was used to test each *P. falciparum* field isolate against a panel of six conventional antimalarials supplied as chloroquine diphosphate (CQ), mefloquine hydrochloride (MQ), quinine sulfate hydrate (QN), artemisinin (AR), amodiaquine hydrochloride (AQ), and doxycycline hyclate (DX). Because all test drugs, except AR (pure base), were provided as a salt and prepared by weight:volume convention, we used salt formulation weights (except AR) to calculate IC<sub>50</sub> values. Test drugs, except DX, were obtained from Walter Reed Army Institute of Research, (Silver Spring, MD). DX was obtained commercially (Sigma-Aldrich, Co., St. Louis, MO; catalog number D9891). Anti-folate drugs were omitted because *P. falciparum* resistance in Kenya is established.<sup>15,18,19</sup>

Reference *P. falciparum* clones assayed periodically for internal control against all six drugs included D6, considered CQ-sensitive and MQ-resistant (CQ-S; MQ-R) and W2, considered CQ-resistant and MQ-sensitive (CQ-R; MQ-S), as well as AR-sensitive.<sup>5</sup> Quinine sulfate hydrate generally parallels CQ for D6 and W2 IC<sub>50</sub> trends.<sup>7</sup> The D6 and W2 clones were obtained from frozen stocks and culture adapted for SYBR Green I IC<sub>50</sub> assays.

To prepare test drugs, stock drug solutions at 1 mg/mL were prepared in 5 mL 70% ethanol for MQ and QN or 100% dimethyl sulfoxide for AR, AQ, and DX. For CQ, 1.5 mL deionized water was combined with 3.5 mL of absolute ethanol. Further dilutions were in complete RPMI 1640 media to the desired starting concentration, followed by serial 2-fold dilutions to generate 10 concentrations for IC<sub>50</sub> testing.<sup>17</sup> The concentra-

tion ranges (ng/mL), from highest to lowest, were CQ (1,000 to 1.953), MQ (250 to 0.488), QN (2,000 to 3.906), AR (100 to 0.195), AQ (100 to 0.195), and DX (50,000 to 97.656). The drug solutions were prepared and used immediately, or stored at -80°C for not longer than 1 month before use. Basic and complete RPMI 1640 culture media, the latter with glucose and hypoxanthine enrichment, were prepared as described.<sup>7</sup>

*Plasmodium falciparum* field isolates refrigerated in transport media, and the two *P. falciparum* laboratory reference clones D6 and W2, were culture adapted before IC<sub>50</sub> SYBR Green I assay. The parasites were cultured at 6% hematocrit for 7–30 days, to reach 3–8% parasitemia.<sup>5</sup> For IC<sub>50</sub> drug assays, culture-adapted parasites were adjusted to 2% hematocrit and 1% parasitemia, and antimalarial drug aliquots in complete RPMI 1640 and added to the wells as described below.

*Plasmodium falciparum* isolates processed IEV were placed into assay within 6 hours of phlebotomy, without culture adaptation. Blood samples with > 1% parasitemia were adjusted to 1% parasitemia at 2% hematocrit, and those with ≤ 1% parasitemia were used unadjusted at 2% hematocrit.

For culture-adapted and IEV assays, transfer of parasite sample and antimalarial drug aliquots in complete RPMI 1640 drug aliquots onto 96-well microculture plates and addition of lysis buffer after 72 hours incubation was done as previously described.<sup>7</sup> The plates were then placed at room temperature in the dark, for 5–15 minutes. Parasite replication inhibition was quantified and the IC<sub>50</sub> for each drug calculated by an equation generating a sigmoidal concentration-response curve (variable slope), with log transformed drug concentrations on the X axis and relative fluorescent units (RFUs) on the Y axis (Graphpad Prism for Windows, version 4.0; Graphpad Software, Inc., San Diego, CA).<sup>6,7</sup>

**Assessing possible sources of SYBR Green I background signal.** In experiment set 1, to measure the intrinsic effect of each drug alone on SYBR Green I RFU, in comparison with complete assay wells, we plated each drug over the standard 10 dilutions in complete RPMI 1640 media alone (“drug only” wells), or in complete RPMI 1640 media containing 1% *P. falciparum* parasitemia and 32,000 peripheral blood mononuclear cells (PBMC)/well (“complete assay” wells), respectively. The PBMC were isolated by density gradient from the blood of a healthy donor. Drug only and 1 set of complete assay wells were processed without incubation, and a second set of complete assay wells were incubated for 72 hours at 37°C in a gas mixture, described earlier.<sup>7</sup> All plates were then processed for SYBR Green I RFU measurements by Genios Tecan. For complete assay wells, 32,000 PBMC/well approximated the number of white blood cells (WBC) in a typical IEV well, based on an estimate of 8,000 WBC/uL of whole blood in a subject. We also assessed a range of *P. falciparum* parasitemias, from 1% to 0.112% (2-fold dilutions), to determine the capability of SYBR Green I to discern IC<sub>50</sub>s in IEV samples with low parasitemias.

In experiment set 2, to assess the influence of PBMC concentration on SYBR Green I assay RFU, we plated a range of PBMC (61 to 250,000 cells/well) in complete RPMI 1640 media with 1% *P. falciparum* parasitemia, without incubation, and then measured RFUs within 15 minutes by Genios Tecan. All experiments were conducted in triplicate.

**Pfmdr1 copy number.** *Plasmodium falciparum* DNA for all molecular assays was extracted from FTA filter paper blots or

whole blood using QIAamp DNA Blood Mini Kit (Qiagen, Inc., Alameda, CA), according to manufacturer instructions.

The  $2^{-\Delta\Delta Ct}$  method of relative quantification was used to estimate *P. falciparum* multiple drug resistance gene 1 (*Pfmdr1*) copy numbers.<sup>20</sup> For this method, there should be at least 1 calibrator with a known number of copies of the gene under study, and a housekeeping gene with constant copy number to normalize the quantitative data.<sup>21</sup> For the calibrator, we used genomic DNA from *P. falciparum* reference clone 3D7, known to have 1 copy of *Pfmdr1* gene.<sup>21</sup> *Plasmodium falciparum* tubulin served as a housekeeping gene, and Dd2 *P. falciparum* clone DNA served as a *Pfmdr1* multiple copy control.

The relative quantification of the *Pfmdr1* gene was determined as previously described.<sup>20</sup> Briefly,  $\Delta\Delta Ct = (Ct, Pfmdr1 - Ct, P. falciparum-tubulin)x - (Ct, Pfmdr1 - Ct, P. falciparum-tubulin)y$ , where *x* is the field isolate and *y* is 3D7. The results were then calculated as *n*-fold changes in *P. falciparum* isolate *Pfmdr1* gene copies, normalized to *P. falciparum* tubulin, relative to the copy number of *Pfmdr1* in *P. falciparum* 3D7 using the equation  $2^{-\Delta\Delta Ct}$ , as described.<sup>20,21</sup> Each sample was run in triplicate and the Ct value of each well recorded at the end of the reaction. The average of the Ct values for each sample was used for the determination of *Pfmdr1* copy number.

***Pfmdr1* codon single nucleotide polymorphism (SNP) analysis.** SNP analysis was conducted for *Pfmdr1* codons 86 (N86Y), 184 (Y184F), 1034 (S1034C), and 1042 (N1042D) using real-time polymerase chain reaction (PCR).<sup>22,23</sup> Probes consisted of VIC for wild-type and 6-FAM (6-carboxyfluorescein) for the mutation.<sup>23</sup> *Plasmodium falciparum* reference clones 3D7 (MQ-S) and Dd2 (MQ-R), as well as D6 and W2, were also assayed.

## RESULTS

**Subject enrollment and follow up.** Three hundred fifty-two *P. falciparum* RDT (+) subjects provided blood samples. For Day 7 post-treatment appointments, 216 subjects tested by Parascreen remained PfHRP-2 (+), but were pLDH (-), the latter indicating absence of *P. falciparum*. All blood smears were (-) for *P. falciparum* asexual stages; five had gametocytes.

**SYBR Green I IC<sub>50</sub> drug assays.** We assayed 121 *P. falciparum* field isolates, 91 IEV, and 30 culture-adapted, against a panel of six drugs. A “successful” assay, defined as a concentration-response across the 10 drug concentrations for 1 or more drugs per each *P. falciparum* field isolate, occurred in 68% of all isolates. For IEV, the success rate for all assays was 70%, 78% with parasitemias > 0.5% and 61% for parasitemias ≤ 0.5%. For culture adaptation, 63% of the assays were successful.

Figure 2A–F shows individual and median IC<sub>50</sub>s for IEV and culture-adapted *P. falciparum* field isolates, against each of the six drugs, along with D6 (CQ-S, MQ-R) and W2 (CQ-R, MQ-S) *P. falciparum* reference clones. For CQ, QN, AQ, and DX median IC<sub>50</sub> values for IEV versus culture-adapted *P. falciparum* field isolates were similar ( $P \geq 0.05$ , Mann-Whitney rank sum test). For MQ and AR, median IC<sub>50</sub> values of culture-adapted *P. falciparum* isolates were higher than IEV isolates ( $P < 0.05$ , Mann-Whitney rank sum test), at 1.8- and 1.5-fold, respectively. The IC<sub>50</sub> ranges for all drugs, except AR, were generally larger for IEV than culture-adapted *P. falciparum* isolates in the upper half of the IC<sub>50</sub> range.

For all drugs, except AR, SYBR Green I median IC<sub>50</sub>s for IEV and culture-adapted *P. falciparum* isolates were simi-

lar to historical median IC<sub>50</sub>s from <sup>3</sup>H-hypoxanthine uptake assays on culture-adapted *P. falciparum* isolates collected in Kenya from 1997 to 2002 (Figure 2A–F).<sup>17</sup> The AR median IC<sub>50</sub> values for SYBR Green I IEV and culture-adapted *P. falciparum* isolates were about 2- to 4-fold higher than the earlier <sup>3</sup>H-hypoxanthine assays, respectively.

**SYBR Green I background RFU experiments.** Experiments are summarized in Figure 3A–C. In Figure 3A (Group 1), drug + media versus complete assay wells (drug + 1% parasitemia + 32,000 PBMC/well), both without incubation, showed a difference of about 1,000 RFU for each drug, with little overlap. Media alone values were similar to drug + media. These observations implied that none of the drugs, at any concentration, would obscure IC<sub>50</sub> readouts. Complete assay wells with no incubation (Group 2) versus with incubation for 72 hours at 37°C (Group 3; akin to IC<sub>50</sub> *P. falciparum* drug assays), the latter median RFU values were ~2-fold higher, indicative of parasite replication.

In Figure 3B, drug concentrations used in the IC<sub>50</sub> assays generated sigmoidal curves, indicating 50% parasite replication inhibition at about 4,000 RFU. Data from Figure 3A and other experiments (not shown) indicated background RFU signal (drug + medium + 32 K PBMC) subtracted from the RFU value of each IC<sub>50</sub> replicate was unnecessary, consistent with earlier work.<sup>6</sup> Finally, SYBR Green I assay discerned IC<sub>50</sub>s for complete assay wells with *P. falciparum* parasitemias ranging from 1% to 0.112%, against all six test drugs (data not shown). These observations, and Figure 3B, are relevant to IEV processing.

In Figure 3C, to determine the RFU signal of PBMC plated across a wide concentration range in complete assays wells (+1% *P. falciparum* parasitemia), RFU values for wells with ≤ 30,000 PBMC were about 2,500 RFU, a value that would not be expected to obscure the identification of drug IC<sub>50</sub> values, at about 4,000 RFU.

**Molecular assays of *P. falciparum* samples.** *Plasmodium falciparum* field isolates ( $N = 308$ ) had a mean *Pfmdr1* gene copy number of 1, quantified relative to the *P. falciparum* reference clones 3D7, with a mean of 1 copy, and multiple drug-resistant Dd2 clone, with a mean of 3.5 copies.

The mutation (86Y) rates in *Pfmdr1* codons were highest at codon 86 (66%), with fewer at codons 184 (38%) and 1034 (12%), and none at codon 1042, regardless of the collection site. The SNP rates at the four codons were similar among the four locations where *P. falciparum* isolates were collected, including lowland versus highland sites. As in earlier work, mutant and mixed (mutant + wild) genotypes were combined to depict total mutant genotype populations.<sup>17</sup>

Associations between the *Pfmdr1* codon 86 SNPs and CQ, MQ, and AR IC<sub>50</sub> values are shown in Figure 4A–C. *Plasmodium falciparum* field isolates with *Pfmdr1* codon 86 mutation (86Y) (66% of isolates) had a higher median CQ IC<sub>50</sub> (lower CQ sensitivity), in comparison with *Pfmdr1* codon 86 wild (N86) samples. For MQ and AR, *P. falciparum* isolates with *Pfmdr1* codon 86Y had a lower median IC<sub>50</sub>, in comparison with *Pfmdr1* codon 86 wild, both statistically significant ( $P < 0.05$ ).

## DISCUSSION

This is the first report from Kenya describing a SYBR Green I assay for measuring *in vitro* drug IC<sub>50</sub>s against *P. falciparum* field isolates. Notably, SYBR Green I assay was conducted at

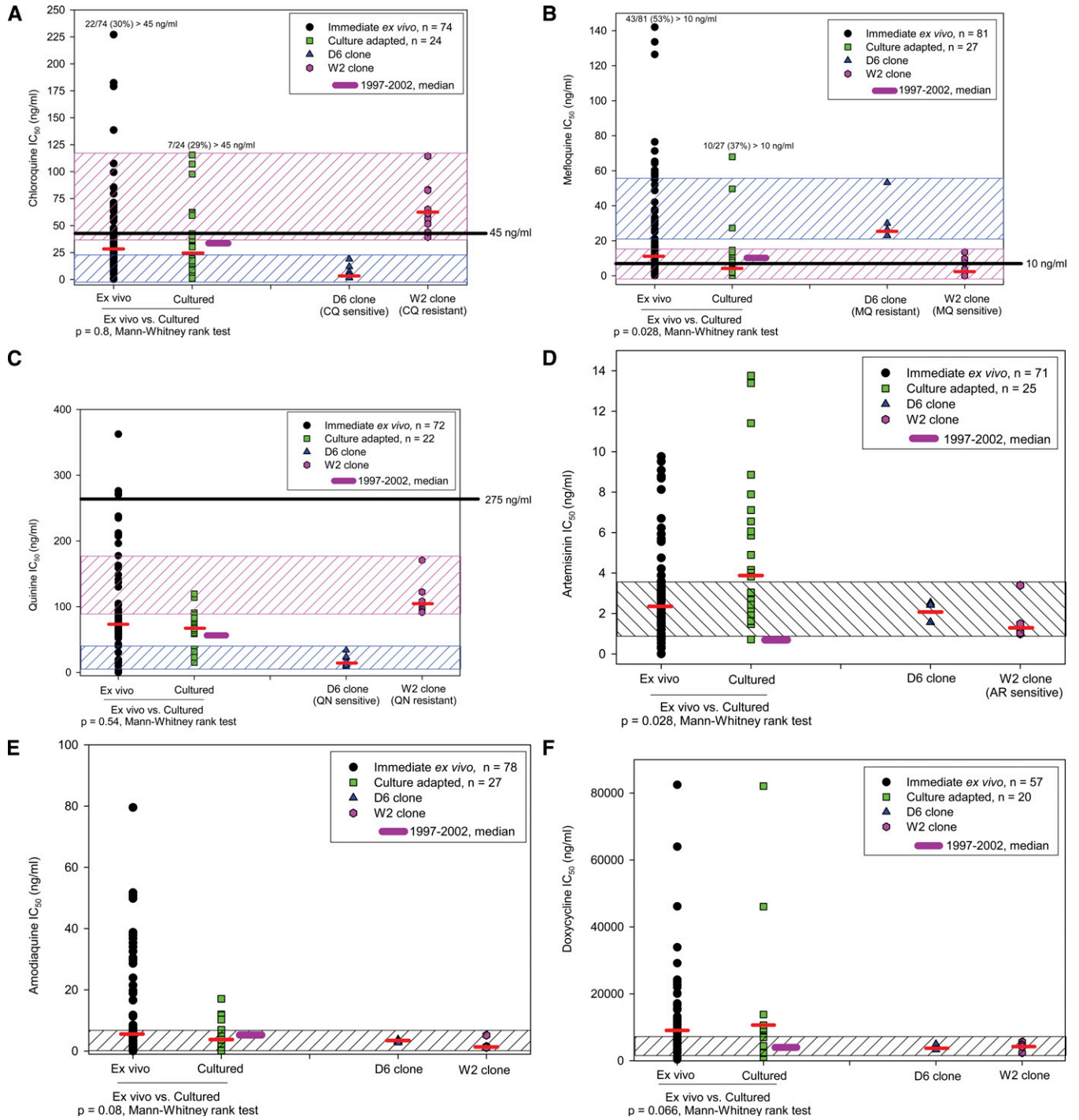


FIGURE 2. (A–F) SYBR Green I drug IC<sub>50</sub>s for *Plasmodium falciparum* field isolates, shown as scatter plot and median (red bar). IC<sub>50</sub> ranges of *P. falciparum* reference clones D6 and W2 for chloroquine diphosphate (CQ), mefloquine hydrochloride (MQ), and quinine sulfate hydrate (QN) shown in two hatched boxes; for artemisinin (AR), amodiaquine hydrochloride (AQ), and doxycycline hyclate (DX), in single hatched box. Purple bars: median IC<sub>50</sub> in *P. falciparum* field isolates obtained 1997–2002 (culture-adapted, radioisotope uptake assay).<sup>17</sup> Black lines on CQ, MQ, and QN graphs: IC<sub>50</sub> threshold values considered discriminative for “resistant” *P. falciparum* isolates (culture-adapted, radioisotope uptake assay).

our field-based laboratory, proximal to *P. falciparum* collection sites, using six drugs. Several earlier reports describe SYBR Green I assays in developed countries, assessing *P. falciparum* clones against a few drugs.<sup>7,9</sup> Second, we assayed *P. falciparum* field isolates IEV and by culture adaptation, obtaining IC<sub>50</sub> values in 70% and 63% of assays, respectively. These success

rates, higher than historical radioisotope uptake assays in our laboratory, may reflect simpler processing. The overall expedience of SYBR Green I supports continued use.

Median IC<sub>50</sub>s in SYBR Green I assays for each of six drugs, among IEV and culture-adapted samples, generally paralleled our earlier data using <sup>3</sup>H-hypoxanthine uptake in

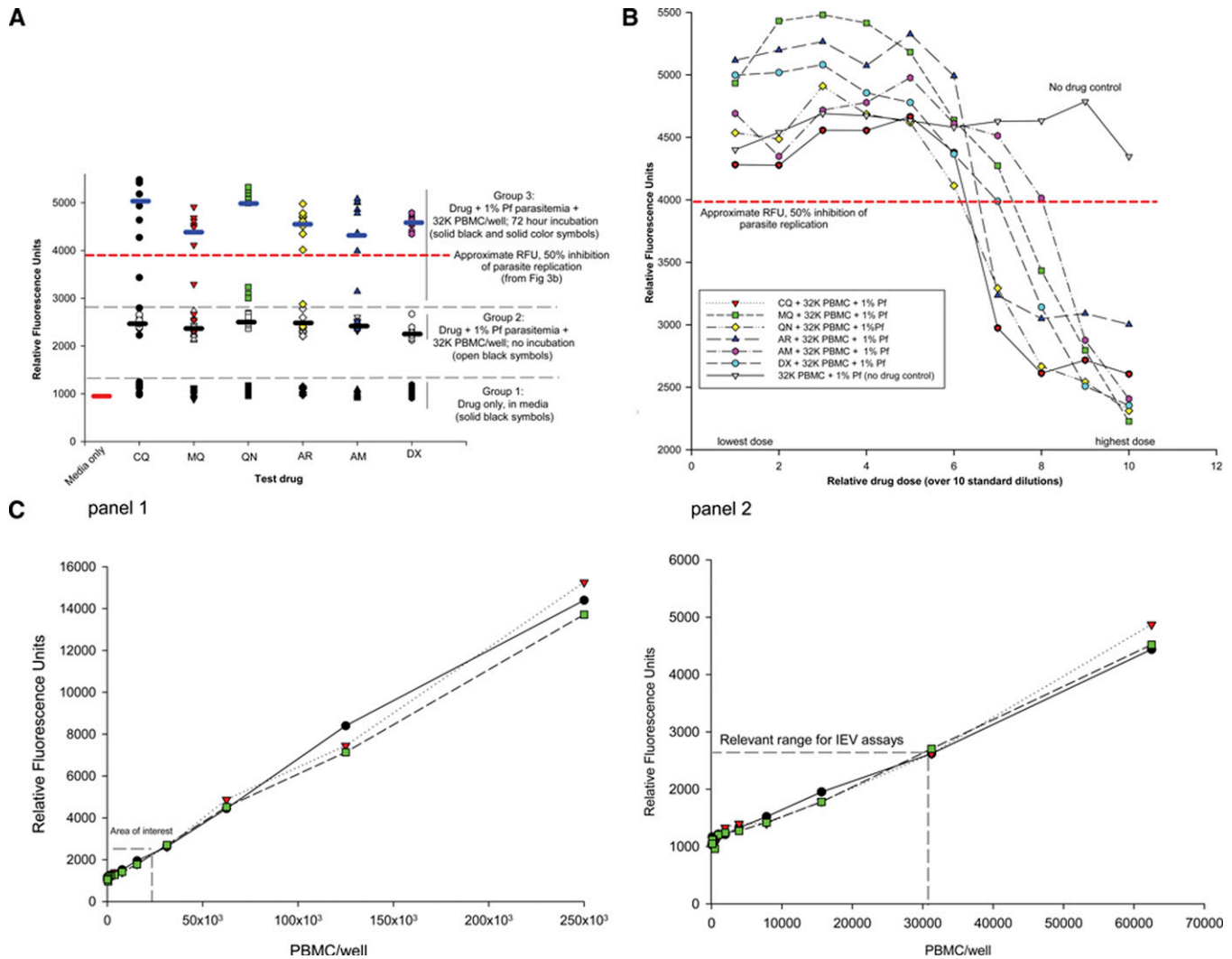


FIGURE 3. Experiments assessing SYBR Green I relative fluorescent unit (RFU) background signal of each drug, and peripheral blood mononuclear cells (PBMCs). (A) SYBR Green I individual and median RFUs among three groups: Group 1: each drug in RPMI 1640 media only. Group 2: Each drug in RPMI 1640 media + 32K PBMC + 1% parasitemia/well; no incubation. Group 3: Same as Group 2, after 72 hours incubation (37°C). Symbols represent RFU value for each drug, across 10 concentrations used in the SYBR Green I assay. Medians for Groups 2 and 3, shown as black and blue bars, respectively; differences in medians and minimal overlap implies determinable  $IC_{50}$ s after 72 hours incubation. Dotted red line at 4,000 RFU approximates 50% inhibition of parasite replication, obtained from Figure 3B. (B) SYBR Green I RFUs in complete assay wells, over 10 drug concentrations, with 32,000 PBMC and 1% parasitemia/well, incubated 72 hours (37°C). Dotted red line approximates 50% inhibition of parasite replication. (C) Panels 1 and 2 show SYBR Green I RFUs for PBMC concentrations ranging from 61 to 250 K cells/well, plated in complete RPMI 1640 media. Each colored symbol is 1 PBMC spiked replicate, diluted over the range shown. Panel 2: “Area of interest” denotes range for number of white blood cells in a typical immediate *ex vivo* blood sample, relevant to IEV SYBR Green I  $IC_{50}$  assays. Linear relationships occur over entire range.

culture-adapted *P. falciparum* isolates.<sup>17</sup> For example, about 30% of CQ  $IC_{50}$ s and 50% of MQ  $IC_{50}$ s in SYBR Green I assays exceeded previously established *in vitro* discriminative “resistance” values of 45 ng/mL and 10 ng/mL, respectively.<sup>17</sup> The  $IC_{50}$  ranges for two *P. falciparum* reference clones, D6 and W2, paralleled their expected CQ and MQ sensitivity profiles. These observations underscored adaptability of the SYBR Green I  $IC_{50}$  assay to a field-based laboratory.

We processed *P. falciparum* field isolates IEV or culture adapted, depending on proximity to the laboratory. For *P. falciparum* isolates stored for several days at 4°C, we waited until parasitemias reached 3–8% before drug  $IC_{50}$  testing. Here, IEV was feasible because of laboratory proximity to two collection sites, with *P. falciparum* isolates placed into assay soon

after collection. Encouragingly, against each of four drugs (CQ, QN, AQ, DX), median  $IC_{50}$ s of IEV and culture-adapted isolates were similar, differing only for MQ and AR, at 1.5- and 1.8-fold, respectively, albeit relatively low magnitudes for  $IC_{50}$  testing. The IEV was simpler than culture adaptation, as others observed.<sup>6</sup>

To partly address background concerns for SYBR Green I assay, we confirmed each drug alone did not affect RFU values. Second, complete RPMI 1640 media with 1% *P. falciparum* and 32,000 PBMC/well, approximating WBC in an IEV assay well, appeared unlikely to elevate RFUs to a level obscuring  $IC_{50}$  interpretations. Finally, relevant to IEV, we found that SYBR Green I assay discerns drug  $IC_{50}$ s with *P. falciparum* parasitemias as low as 0.112%, an issue in debate.<sup>7,24</sup>

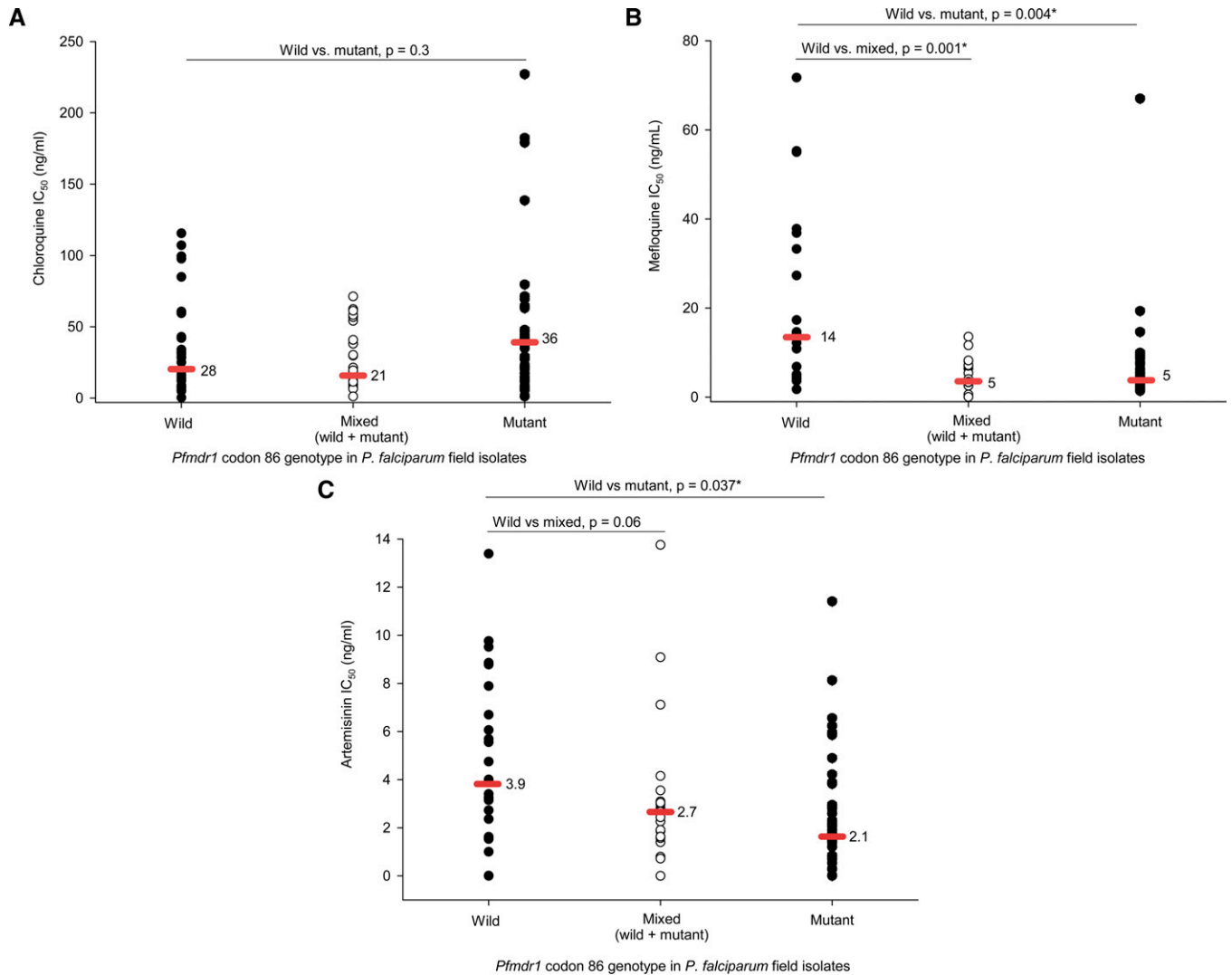


FIGURE 4. Association between *Pfmdr1* codon 86 genotype and chloroquine diphosphate (CQ), mefloquine hydrochloride (MQ), and artemisinin (AR) IC<sub>50</sub> values, respectively. *P* values determined by Mann-Whitney rank test (\* denotes  $P \leq 0.05$ ). (A) *Pfmdr1* codon 86 mutation (86Y), modestly associated with higher CQ IC<sub>50</sub>. (B) *Pfmdr1* codon 86 mutation (86Y), associated with lower MQ IC<sub>50</sub>. (C) *Pfmdr1* codon 86 mutation (86Y), associated with lower AR IC<sub>50</sub>.

Among IEV assays, the upper half of IC<sub>50</sub> ranges was notably higher for five drugs, in comparison with culture adaptation. This might reflect greater biological variability in IEV *P. falciparum* isolates, including better preserved sub-populations of drug-resistant and sensitive parasites.<sup>11,25,26</sup> It may be useful to determine multiplicity of infection of each *P. falciparum* isolate, processed IEV, and culture adapted, along with comparative IEV and culture-adapted SYBR Green I assays.

AR was the lone drug without larger IEV IC<sub>50</sub> ranges, in comparison with culture-adapted isolates, perhaps because there are few, if any, AR-resistant clones in Kenya. Alternatively, this may reflect the activity of AR against most *P. falciparum* blood stages.<sup>27</sup> Median IC<sub>50</sub> values for AR, among IEV and culture-adapted assays, were 2- to 3-fold higher in comparison with earlier radioisotope assays, and a recent report of SYBR Green I assessing freeze-thawed, culture-adapted *P. falciparum* samples.<sup>7,17</sup> Higher AR IC<sub>50</sub>s are unlikely to represent resistance, but perhaps methodological variability.

The SYBR Green I IC<sub>50</sub> values for six drugs, establishing a modest baseline for western Kenya, support continued work

to better define IC<sub>50</sub>s. For radioisotope assays using culture-adapted *P. falciparum* isolates, IC<sub>50</sub> values discriminative for *in vivo* “resistance” exist (CQ, MQ, QN, and five others).<sup>17</sup> If SYBR Green I is widely implemented for *in vitro* *P. falciparum* drug testing, reference IC<sub>50</sub> values discriminative for resistance, ideally linked with *in vivo* responses, could enhance usefulness.

In Kenya, with artemether + lumefantrine (Coartem) implemented as the artemisinin combination therapy (ACT) standard in 2003, and with no clinical evidence of resistance, there is an opportunity with SYBR Green I assay to track A+L IC<sub>50</sub> values. Indeed, in East Africa, Kenya is often a sentinel site for drug resistance.<sup>15,28</sup> As such, we have developed SYBR Green I assays for artemether, lumefantrine, artemisinin combinations, as well as atovaquone, halofantrine, primaquine, and tafenoquine.<sup>11</sup>

*Pfmdr1* is a constitutive gene in *P. falciparum* that confers multiple drug resistance when more than 1 copy is present.<sup>2</sup> Parasites with multiple *Pfmdr1* copies are most common in Southeast Asia, where multiple drug-resistant *P. falciparum*

malaria, including MQ, exists.<sup>2,29,30</sup> As expected, our *P. falciparum* field isolates from western Kenya contained an average of 1 copy number of *Pfmdr1*,<sup>31</sup> consistent with a wild-type genotype not necessarily associated with multidrug resistance.<sup>32</sup> MQ has not been widely used in Kenya, precluding comment on *in vitro* MQ IC<sub>50</sub>s and *in vivo* responsiveness.

SNPs in certain *Pfmdr1* codons may confer drug resistance.<sup>32–34</sup> Among *P. falciparum* isolates we collected earlier (1997–2002), *Pfmdr1* codon 86 mutation (86Y) rates in two western sites (Kisumu, Kericho) were 35% and 68%, respectively (mean, 51%), whereas two eastern sites (Entosopia, Magadi) had rates > 85%.<sup>17</sup> This reflected CQ resistance in Kenya.<sup>17,35</sup> Here, in *P. falciparum* isolates from western Kenya, mutation rates in codon 86 were 66%, far less at 184 and 1,034. The mutation rates in codons 86, 184, and 1034 were similar for lowland and highland sites, suggesting *Pfmdr1* SNPs are not necessarily associated with severe malaria presentation, often anemia and cerebral malaria, respectively.<sup>36</sup>

We interpret the similarity in *Pfmdr1* codon 86 mutation (86Y) rates for 1997–2002 (51%) and this study (66%), along with similar CQ IC<sub>50</sub>s, as sustained CQ resistance in Kenya.<sup>14,17,37</sup> Indeed, most *P. falciparum* isolates contained codon 86 mutation (86Y), modestly associated with higher CQ IC<sub>50</sub>s. Mutation in *pfcr*, well established in Kenya, also confers CQ resistance. In contrast, *Pfmdr1* codon 86 mutations (86Y) were associated with lower median IC<sub>50</sub>s for MQ, and AR. This parallels earlier reports describing *Pfmdr1* codon 86Y and drug IC<sub>50</sub> relationships, typically direct with CQ, and inverse with MQ.<sup>38</sup> For AR, a lower median IC<sub>50</sub> in *P. falciparum* field isolates expressing *Pfmdr1* codon 86 mutation (86Y) supports the notion this mutation is unlikely associated with emerging AR resistance.<sup>39,40</sup>

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