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Decreasing *pfmdr1* Copy Number Suggests that *Plasmodium falciparum* in Western Cambodia Is Regaining *In Vitro* Susceptibility to Mefloquine

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Dihydroartemisinin-piperaquine is the current frontline artemisinin combination therapy (ACT) for *Plasmodium falciparum* malaria in Cambodia but is now failing in several western provinces. To investigate artesunate plus mefloquine (AS+MQ) as a replacement ACT, we measured the prevalence of multiple *pfmdr1* copies—a molecular marker for MQ resistance—in 844 *P. falciparum* clinical isolates collected in 2008 to 2013. The *pfmdr1* copy number is decreasing in Western Cambodia, suggesting that *P. falciparum* is regaining *in vitro* susceptibility to MQ.

Artemisinin-based combination therapy (ACT) is used worldwide to treat uncomplicated *Plasmodium falciparum* malaria. Artesunate plus mefloquine (AS+MQ) was adopted as Cambodia's first-line treatment in 2000. AS+MQ treatment failures were first observed in 10 to 20% of patients in Pailin and Battambang Provinces, Western Cambodia, in 2003 to 2004 (1, 2). The presence of multiple (i.e., ≥2) *pfmdr1* copies—a genetic marker of MQ resistance—was associated with AS+MQ failures and reduced parasite *in vitro* susceptibility to MQ not only in Pailin and Battambang but in neighboring Pursat and Kampot Provinces as well (3–5). This development, along with emerging evidence of reduced *in vitro* susceptibility to MQ in other provinces (6), prompted Cambodia's National Malaria Control Program to adopt dihydroartemisinin-piperaquine (DHA-PPQ) as the first-line ACT in Western Cambodia in 2008 and in the entire country in 2010. Unfortunately, recent clinical studies suggest that the efficacy of DHA-PPQ is rapidly declining in five Western Cambodian provinces: Pursat, Battambang, Pailin, Oddar Meancheay, and Preah Vihear. For example, DHA-PPQ treatment failures were observed in 25% and 11% of patients in Pailin and Pursat in 2010 (7) and in 36% of patients in Oddar Meancheay in 2013 (8). The rapidly increasing prevalence of DHA-PPQ failures in these provinces, likely due to entrenched artemisinin resistance (9, 10) and suspected PPQ resistance (7, 8), demands additional evaluations of newer antimalarial drugs (11, 12), as well as reevaluation of previously used ACTs.

Only a few ACTs, including AS+MQ and artemether-lumefantrine (AL), are presently available for widespread use in Cambodia to replace DHA-PPQ as it fails. Although AS+MQ and AL treatment failures have previously been reported from Battambang and Pursat (2, 13), the clinical efficacy of these regimens in the setting of DHA-PPQ resistance is not known. However, studies have suggested a lack of cross-resistance between PPQ and MQ *in vitro*; for example, *pfmdr1* amplification is associated with decreased susceptibility to MQ but increased susceptibility to PPQ (14). Given these and related findings (15), and the fact that earlier use of DHA-PPQ was associated with decreasing prevalence of multiple *pfmdr1* copies in Pailin (from 33% in 2005 to 5% in 2007) (16), we hypothesized that the recent substantially reduced use of AS+MQ in Pursat and Preah Vihear would select for parasites that have regained sensitivity to MQ. To explore this hypothesis, we used real-time PCR methods to quantify the *pfmdr1* copy number in 844 *P. falciparum* clinical isolates collected in 2008 to 2013 from Pursat and Preah Vihear, as well as Ratanakiri Province in Eastern Cambodia, where DHA-PPQ treatment failures have not been documented. *pfmdr1* copy numbers for 360 of these isolates were previously reported (17). Parasitized whole-blood samples were collected from patients enrolled in completed parasite clearance rate (10, 18) and ongoing DHA-PPQ efficacy (ClinicalTrials.gov Identifier, NCT01736319) studies. All patients or their parents provided written informed consent. The *pfmdr1* copy number in extracted DNA samples was quantified as previously described (17), and the *P. falciparum* lines 3D7 (copy number normalized to 1) and Dd2 (mean copy number ± SD, 2.05 ± 0.19; $n = 29$) were used as controls.

The distribution of 844 parasite isolates by province and year, in relation to Cambodia's recommended use of ACTs, is shown in Table 1. The prevalence of parasites with multiple *pfmdr1* copies decreased significantly in Pursat during the 2008-to-2013 period (Cochran-Armitage trend test, $P < 0.001$), decreased in Preah Vihear during the 2011-to-2013 period ($P = 0.065$), and did not change in Ratanakiri in the 2010-to-2013 period ($P = 0.869$). The latter finding may be explained by the fact that Cambodia's remaining stocks of AS+MQ were still being distributed to Ratanakiri until mid-2013, which may have maintained the low prevalence of multiple *pfmdr1* copies in this province. In 2013, the prevalence of parasites with multiple *pfmdr1* copies was estimated

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TABLE 1 *pfmdr1* copy number in 844 *P. falciparum* clinical isolates, stratified by Cambodian province and year of collection

Province	*pfmdr1* copy no.	No. (%) of isolates with indicated copy no. where the recommended ACT(s)[a] was as indicated in: 2008	2009	2010	2011	2012	2013	*P* value[b]
Pursat		AS+MQ, DHA-PPQ	DHA-PPQ	DHA-PPQ	DHA-PPQ	DHA-PPQ	DHA-PPQ	
	1	19 (50)	64 (70)	40 (67)	73 (66)	44 (90)	64 (90)	**<0.001**
	2	10 (26)	9 (10)	9 (15)	11 (10)	3 (6)	6 (9)	
	3	6 (16)	13 (14)	8 (13)	14 (13)	1 (2)	1 (1)	
	4	2 (5)	4 (5)	1 (2)	7 (6)	1 (2)	0 (0)	
	5	1 (3)	1 (1)	2 (3)	5 (5)	0 (0)	0 (0)	
Preah Vihear		AS+MQ	AS+MQ	AS+MQ	AS+MQ	AS+MQ, DHA-PPQ	AS+MQ, DHA-PPQ	
	1	NA[c]	NA	NA	63 (78)	67 (93)	28 (88)	0.065
	2	NA	NA	NA	10 (12)	2 (3)	2 (6)	
	3	NA	NA	NA	2 (3)	3 (4)	2 (6)	
	4	NA	NA	NA	1 (1)	0 (0)	0 (0)	
	5	NA	NA	NA	5 (6)	0 (0)	0 (0)	
Ratanakiri		AS+MQ	AS+MQ	AS+MQ	AS+MQ	AS+MQ, DHA-PPQ	AS+MQ, DHA-PPQ	
	1	NA	NA	54 (100)	91 (91)	52 (100)	33 (97)	0.869
	2	NA	NA	0 (0)	8 (8)	0 (0)	1 (3)	
	3	NA	NA	0 (0)	0 (0)	0 (0)	0 (0)	
	4	NA	NA	0 (0)	0 (0)	0 (0)	0 (0)	
	5	NA	NA	0 (0)	1 (1)	0 (0)	0 (0)	

[a] DHA-PPQ, combination therapy of dihydroartemisinin and piperaquine; AS+MQ, combination therapy of artesunate and mefloquine.
[b] *P* values were calculated using the Cochran-Armitage trend test (exact *P* values were estimated by Monte Carlo simulation using the coin R package; number of Monte Carlo replicates = 10^6). Boldface indicates a statistically significant difference.
[c] NA, not available.

TABLE 2 *In vitro* susceptibility of *P. falciparum* clinical isolates to eight antimalarial drugs in 2010 to 2013, stratified by Cambodian province and *pfmdr1* copy number

Province	Drug	Susceptibility of isolates with *pfmdr1* copy number of: One — No. of isolates	One — IC_{50} (nM) [GM[a] (range)]	Multiple — No. of isolates	Multiple — IC_{50} (nM) [GM[a] (range)]	*P* value[b]
Pursat	Chloroquine	133	474.4 (103.9–1,084.3)	52	294.1 (27.6–1,312.8)	**<0.0001**
	Mefloquine	172	14.2 (1.7–56.4)	53	28.2 (7.8–71.3)	**<0.0001**
	Quinine	174	273.2 (65.5–992.0)	56	319.4 (95.5–825.4)	**0.0311**
	Piperaquine	100	45.8 (5.6–185.2)	40	24.0 (2.5–125.7)	**0.0002**
	Atovaquone	64	0.5 (0.1–8.3)	6	0.8 (0.2–13.8)	0.7510
	Pyronaridine	64	5.4 (0.2–16.5)	6	3.3 (0.7–10.3)	0.1726
	Artesunate	148	2.9 (0.6–8.1)	40	4.0 (1.2–8.7)	**0.0003**
	DHA	176	2.7 (0.9–9.8)	56	3.0 (0.7–7.9)	0.1120
Preah Vihear	Chloroquine	105	318.4 (16.7–978.5)	17	339.4 (108.7–864.2)	0.5518
	Mefloquine	111	18.4 (3.6–46.6)	18	27.2 (4.3–69.7)	**0.0100**
	Quinine	113	200.8 (31.4–956.5)	19	297.3 (61.8–933.1)	**0.0231**
	Piperaquine	107	27.9 (4.9–130.1)	19	25.8 (7.9–66.2)	0.6976
	Atovaquone	27	0.6 (0.1–9.3)	3	0.4 (0.2–1.2)	0.3837
	Pyronaridine	27	4.5 (1.4–9.2)	3	6.6 (3.2–12.8)	0.4265
	Artesunate	111	2.4 (0.4–9.5)	19	3.8 (1.4–10.0)	**0.0132**
	DHA	111	2.1 (0.5–6.7)	18	2.8 (0.9–7.2)	0.0753
Ratanakiri	Chloroquine	155	175.8 (11.8–933.1)	5	149.6 (39.3–301.3)	0.6912
	Mefloquine	155	18.9 (4.2–64.7)	5	16.9 (9.7–32.5)	0.6554
	Quinine	160	131.8 (10.2–695.8)	4	184.4 (72.8–351.3)	0.3763
	Piperaquine	119	24.1 (5.5–67.1)	5	36.2 (13.6–72.3)	0.1567
	Atovaquone	28	0.4 (0.1–2.4)	0	NA[c]	NA
	Pyronaridine	29	3.6 (1.2–9.8)	0	NA	NA
	Artesunate	119	2.0 (0.9–5.6)	4	2.7 (2.3–4.7)	0.1619
	DHA	161	1.8 (0.5–5.8)	4	1.7 (1.0–2.8)	0.7787

[a] GM, geometric mean.
[b] *P* values were calculated using the Mann-Whitney test. Boldface indicates a statistically significant difference.
[c] NA, not available.

to be 10%, 12%, and 3% in Pursat, Preah Vihear, and Ratanakiri, respectively. Together, these data suggest that MQ resistance is presently decreasing in Pursat and Preah Vihear and remains consistently low in Ratanakiri, likely due to the relatively low prevalence of artemisinin resistance in this province (9, 10). To further investigate these possibilities, we measured the *in vitro* susceptibility of 514/844 (60.9%) parasite isolates to MQ using a SYBR green I-based growth inhibition assay as previously described (17). The 50% inhibitory concentrations (IC_{50}s) of at least four drugs were previously reported for 242 of these isolates (17). Parasites with multiple *pfmdr1* copies had higher geometric mean MQ IC_{50}s than those with one *pfmdr1* copy in Pursat (28.2 versus 14.2 nM, $P < 0.0001$, Mann-Whitney test) and Preah Vihear (27.2 versus 18.4 nM, $P = 0.0100$) (Table 2). Taken together, these genotype and phenotype data suggest that AS+MQ may now be effective for the treatment of malaria in Pursat, Preah Vihear, and Ratanakiri.

We also examined the association of *pfmdr1* copy number and the *in vitro* susceptibility of parasite isolates to chloroquine (CQ; $n = 467$), quinine (QN; $n = 526$), PPQ ($n = 390$), atovaquone (ATV; $n = 128$), pyronaridine (PYN; $n = 129$), AS ($n = 441$), and DHA ($n = 526$). As for MQ, parasites carrying one *pfmdr1* copy were significantly more susceptible to QN and AS in Pursat and Preah Vihear. As observed previously (14, 19–21), parasites harboring one *pfmdr1* copy were significantly less susceptible to CQ and PPQ in Pursat (Table 2), indicating that the mechanisms of resistance to MQ and QN differ from those mediating resistance to CQ and PPQ in this province. In Ratanakiri, *pfmdr1* copy number did not associate with IC_{50} for any of the antimalarial drugs tested, but the number of samples with multiple *pfmdr1* copies were very few (≤5) in this province. These data indicate that *pfmdr1* amplification is involved in decreasing parasite susceptibility to MQ and QN but not to CQ and PPQ in Cambodia. Whether the widespread decrease in the use of AS+MQ, increase in the use of DHA-PPQ, or both are driving the deamplification of *pfmdr1* requires further investigation. As expected, parasite isolates are highly susceptible to atovaquone and pyronaridine, which have not been extensively used as ACT partner drugs in Cambodia (12, 22).

In summary, our data indicate that *P. falciparum* is regaining *in vitro* susceptibility to MQ in Pursat and, possibly, in Preah Vihear as well and suggest that AS+MQ may be an effective first-line treatment for *P. falciparum* malaria in Cambodian provinces where DHA-PPQ treatment failures have been documented—a possibility we are presently investigating. In other areas of Southeast Asia where DHA-PPQ treatment failures are suspected, early quantification of the *pfmdr1* copy number and MQ IC_{50}s of contemporary isolates is a useful approach to rapidly investigate the suitability of AS+MQ as an alternative ACT.

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