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Gametocyte Carriage, Antimalarial Use, and Drug Resistance in Cambodia, 2008–2014

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Abstract. Gametocytes are the malaria parasite stages responsible for transmission from humans to mosquitoes. Gametocytemia often follows drug treatment, especially as therapies start to fail. We examined *Plasmodium falciparum* gametocyte carriage and drug resistance profiles among 824 persons with uncomplicated malaria in Cambodia to determine whether prevalent drug resistance and antimalarial use has led to a concentration of drug-resistant parasites among gametocyte carriers. Although report of prior antimalarial use increased from 2008 to 2014, the prevalence of study participants presenting with microscopic gametocyte carriage declined. Gametocytemia was more common in those reporting antimalarial use within the past year, and prior antimalarial use was correlated with higher IC_{50}s to piperaquine and mefloquine, as well as to increased *pfmdr1* copy number. However, there was no association between microscopic gametocyte carriage and parasite drug resistance. Thus, we found no evidence that the infectious reservoir, marked by those carrying gametocytes, is enriched with drug-resistant parasites.

Cambodia, long an epicenter of multidrug-resistant malaria, has been the focus of malaria containment efforts since artemisinin resistance was reported in 2008–2009.[1] Efforts to halt the spread of drug-resistant malaria with the use of artemisinin-based combination therapies (ACTs) combined with widespread private sector use of antimalarials has led to substantial in vivo drug pressure in the region.[2–4] Historically, drug treatment has been linked to carriage of *Plasmodium falciparum* gametocytes, the parasite stages responsible for human-to-mosquito transmission, especially as resistance emerges.[5] Patients with prolonged parasite clearance times in the Tracking Resistance to Artemisinin Collaboration study were more likely to carry gametocytes both pre- and post-treatment.[6] Recrudescent parasitemias are also associated with gametocyte carriage.[7,8] This raises the question of whether repeated treatment of drug-resistant parasites among a relatively small pool of at-risk persons in western Cambodia is leading to enhanced transmission of drug-resistant parasites.[9]

We sought to address this question by examining whether gametocyte carriers in Cambodia were more likely to harbor drug-resistant parasites. In pooled study data on 824 persons aged 13–65 years with uncomplicated smear-positive malaria, we evaluated the relationship of gametocyte carriage to prior antimalarial use and the association of both prior antimalarial use and gametocyte carriage with parasite drug resistance, as defined by phenotypic and molecular assays. Subjects were originally enrolled from north, west, and southern Cambodia from 2008 to 2014 as part of four clinical protocols: WR1396 (N = 143) in 2008–2009, WR1737 (N = 21) in 2010–2011, WR1877 (N = 119) in 2013–2014, and WR1576 (N = 541) in 2009–2014.[2] All subjects provided informed consent before participation, and all study protocols were approved by the Cambodian National Ethics Committee for Health Research and the Walter Reed Army Institute of Research Institutional Review Board.

At enrollment, participants were asked about duration of symptoms, history of malaria infection, and antimalarial use within the past year. Those with recent antimalarial use, within the previous 7–30 days (varying for each protocol), were excluded from participation. To facilitate recall of prior antimalarial use, the participants were shown samples of antimalarials available in Cambodia and local packages of commonly used antibiotics and cold medicines (Supplemental Figure 1). Pill sample packages were updated every year. Two microscopists examined Giemsa-stained blood smears to determine *P. falciparum* gametocytemia, based on the number of gametocytes per 200 white blood cells or 5,000 red blood cells. Molecular detection of gametocytes by reverse transcriptase polymerase chain reaction (RT-PCR) of Pfs25 was performed in two of the clinical studies (N = 256 from WR1396 and WR1877).[10,11] Parasite drug resistance profiles were measured before treatment by ex vivo drug susceptibility assays based on an histidine-rich protein-2 enzyme-linked immunosorbent assay.[2,12] For this analysis, we focused specifically on resistance to chloroquine (IC_{50} > 87 nM), mefloquine (MQ) (IC_{50} > 24 nM), and piperaquine (PPQ) (IC_{50} in top 25th percentile), as artesunate (AS) and MQ, and dihydroartemisinin (DHA) and PPQ are the most commonly used ACTs in the region. Measurement of piperaquine IC_{50}s began in 2010. In addition, genotyping of two molecular markers of resistance were included in the analysis: *P. falciparum* multidrug resistance gene (*Pfmdr1*) amplification associated with MQ resistance,[13] and *kelch*13 mutation associated with artemisinin resistance.[6,14]

We have previously reported high rates of drug resistance and prevalent antimalarial use in the region.[2] IC_{50}s to chloroquine and MQ in the cohort reflect high-grade resistance (median [interquartile range] 160 nm [94–237 nm] and 61 nm [33–106 nm], respectively), although rising IC_{50}s to PPQ in the latter years of the study (2013–2014) were accompanied by a reciprocal fall in MQ IC_{50}s. Kelch mutations have been present in the majority of tested isolates since

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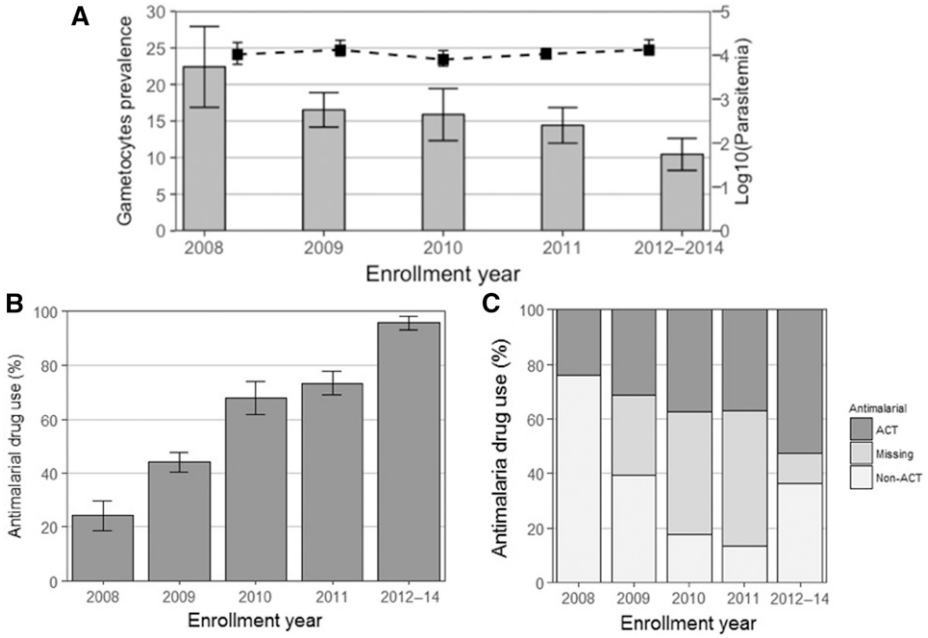

FIGURE 1. Gametocyte prevalence (**A**) and reported antimalarial use (**B** and **C**) in persons presenting with uncomplicated falciparum malaria in Cambodia from 2008 to 2014. Gametocyte prevalence (gray bars) refers to the proportion of study participants with gametocytemia detected by microscopy at enrollment. Antimalarial use denotes reported usage over the previous 12-month (recent antimalarial use in the preceding 7 days (WR1576) or 28–30 days (WR1877 and WR1396 were exclusion criteria). Artemisinin-based combination therapies (ACTs) reported included dihydroartemisinin–piperaquine or artesunate–mefloquine combinations, and non-ACT use was predominantly chloroquine for vivax malaria. Artesunate and artemether were included with ACTs, whereas antimalarial use reported as unknown is denoted as missing data.

2008 (431/493, 87%), predominantly C580Y (79%, $N = 340$) and R539T (21%, $N = 72$). More than half of participants reported antimalarial use within the previous 12 months (61%, 440/720), most commonly AS-MQ (34%, $N = 148$), DHA-PPQ (22%, $N = 95$), or chloroquine (13%, $N = 59$, typically given for *Plasmodium vivax* infection). Rates of prior antimalarial use rose over time, with widespread use of artemisinin-based therapies by 2012–2014 (Figure 1). In addition, a bioassay used to measure antimalarial activity in plasma found that roughly 18% of individuals in this cohort showed evidence of recent antimalarial use.[2]

Despite a high prevalence of multidrug-resistant parasites and increasing numbers of patients reporting multiple malaria treatment courses over 12 months, the proportion of gametocyte carriers at enrollment appeared to decline over time (Figure 1). Gametocyte prevalence in 2008 by microscopy was 22% (13/58) in 2008, falling to 11% (14/134) in 2012–2014. This apparent decline in gametocyte carriage was not associated with changes in the levels of parasitemia in patients seeking care (median parasite density 10,388 p/μL in 2008 and 9,621–12,325 p/μL in 2012–2014) or earlier access to care (median days of illness reported was 3 days in all years of the study). It may be associated with increasing use of artemisinin-based therapies (Figure 1) and/or a decline in transmission intensity as a result of malaria control efforts in the region.[15]

Among the 123 persons who were gametocyte positive by microscopy, risk factors for gametocyte carriage were similar to those seen in other surveys (Table 1).[5,7] Gametocytemic individuals were more likely to report prior malaria illness and antimalarial use within the past year. They also presented later in illness, had lower asexual parasite densities, and were less likely to be febrile, but more likely to be anemic. These risk factors are consistent with previous exposure to malaria and acquired immunity. Most of these associations were lost when expanding the definition of gametocyte carriage to include those with submicroscopic gametocytes detectable by RT-PCR (Table 1).

As might be expected, antimalarial use within the past year was associated with infection with drug-resistant parasites (Figure 2A, Supplemental Table 1). In those reporting prior antimalarial use, the mean number of illnesses believed or confirmed to be due to malaria over the past year was 2.8 episodes. Although most of the parasite isolates displayed chloroquine and mefloquine resistance, ex vivo PPQ resistance was more prevalent in persons reporting prior antimalarial use (prevalence ratio of 1.3 [95% confidence interval [CI] 0.9–2.0]), as was the increased *pfmdr1* copy number (prevalence ratio of 1.6 [95% CI 1.3–2.1]).

Although gametocytemia was more common in those reporting antimalarial use within the previous 12 months and antimalarial use was correlated with infection with drug-resistant parasites, we did not find that gametocyte carriers were more likely to harbor drug-resistant parasites (Figure 2B). In both crude and adjusted analyses, the prevalence of ex vivo drug resistance or molecular markers of drug resistance were not different in those with and without microscopic gametocytes (Supplemental Table 1). Accordingly, gametocytemia was not more common in those with mefloquine- or PPQ-resistant malaria (Supplemental Figure 2). To some extent, nearly all parasites in the region are drug resistant, in that only 13% (40/303) of persons in the cohort were both MQ and PPQ sensitive and 60% (24/40) of these still harbored a C580Y kelch mutation. However, taken together, our findings do not support the notion that increasing drug resistance and use of failing drugs are leading to preferential transmission of drug-resistant parasites.

There are several limitations of this study. This was not a population-based study, and the inclusion of consecutive

TABLE 1

Risk factors for gametocyte carriage, as detected by microscopy (top) or Pfs25 RT-PCR (bottom)

Potential risk factors	Smear positive for gametocytes *N* = 123	Smear negative for gametocytes *N* = 701	*P* value
Age (years), median (IQR)	27 (20–37)	27 (21–37)	0.36
Female gender (%)	23 (19)	97 (14)	0.16
Days of illness, median (IQR)	3 (2–6)	3 (2–3)	< 0.0001*
Fever (temperature ≥ 38°C) (%)	58 (47)	507 (72)	< 0.0001*
Number of illnesses believed to be due to malaria in the last 12 months, median (IQR)	2 (1–4)	1 (0–4)	< 0.0001*
At least one believed malaria infection in the last 12 months (%)	81 (84)	318 (56)	< 0.0001*
Antimalarial usage in last 28 days (%)	15 (13)	38 (6)	0.01
Antimalarial usage in last year (%)	95 (83)	345 (57)	< 0.0001*
Hematocrit, median (IQR)	35 (30–38)	40 (38–44)	< 0.0001*
Asexual parasite density (parasites/μL), median (IQR)	6,214 (2,726–19,596)	13,057 (4,768–48,450)	< 0.0001*
Mixed species infection (%)	8 (7)	45 (6)	0.97

Potential risk factors	Gametocytes detected by RT-PCR *N* = 79	Gametocytes not detected by RT-PCR *N* = 177	*P* value
Age, median (IQR)	25 (21–33)	25 (20–35)	0.52
Female gender (%)	6 (8)	27 (15)	0.09
Days of illness, median (IQR)	3 (2–4)	3 (2–3)	0.08
Fever (temperature ≥ 38°C) (%)	43 (54)	106 (60)	0.41
Number of illnesses believed to be due to malaria in the last 12 months, median (IQR)	0 (0–1)	0 (0–1)	0.50
At least one believed malaria infection in the last 12 months (%)	24 (49)	27 (40)	0.32
Antimalarial usage in last 28 days (%)	1 (2)	2 (2)	0.80
Antimalarial usage in last year (%)	37 (47)	54 (31)	0.18
Hematocrit, median (IQR)	40 (36–43)	41 (38–44)	0.01
Asexual parasite density, median (IQR)	19,548 (6,419–45,558)	14,499 (4,776–38,306)	0.40
Mixed infection (%)	5 (6)	7 (4)	0.41

IQR = interquartile range. Categorical variables are expressed as *N* (%) and continuous variables are expressed as median (IQR). Note that denominators may vary based on missing data and RT-PCR data were only available for studies WR1396 and WR1877.

* $P < 0.004$ with *P* values calculated by two-tailed Wilcoxon rank sum tests.

studies conducted in the same region may still draw from heterogeneous populations. However, when we confined the analysis to individual studies, no trends linking gametocyte carriage and drug resistance were found. Mefloquine- and PPQ-resistant parasites in the region display opposing resistance profiles,[16,17] which may render it difficult to detect associations with drug resistance. Because of limited data, results from the ring stage survival assay for artemisinin resistance and testing for amplification of *plasmepsin 2-3*, a recently described marker for PPQ resistance,[18,19] were not included. Finally, drug susceptibility assays are reliant on growth of parasites in short-term culture and tend to fail when antimalarials are present in plasma. This may lead to sampling bias, with the most resistant parasites potentially excluded from analysis because of missing IC_{50} data.[2]

Despite these limitations, our findings likely reflect that a complex interplay of host and parasite factors affect gametocytogenesis. Although persons reporting prior antimalarial use increased over time and these individuals were more likely to harbor gametocytes and drug-resistant parasites, the proportion of gametocyte carriers did not increase over time, and gametocyte carriers did not harbor more drug-resistant parasites compared with those without gametocytes. It is likely that host immunity plays an equally important role in determining who is gametocytemic and contributes to the infectious reservoir.[5] Although this study comprised a symptomatic cohort, it would be useful to evaluate the prevalence of drug-resistant parasites in asymptomatic gametocyte carriers.

In conclusion, increasing and prevalent drug resistance in Cambodia does not seem to have led to a rise in gametocyte carriage among malaria patients, perhaps because of widespread ACT use[20] and declining malaria transmission. We found no evidence that the infectious reservoir, marked by those carrying gametocytes, is enriched with drug-resistant parasites.

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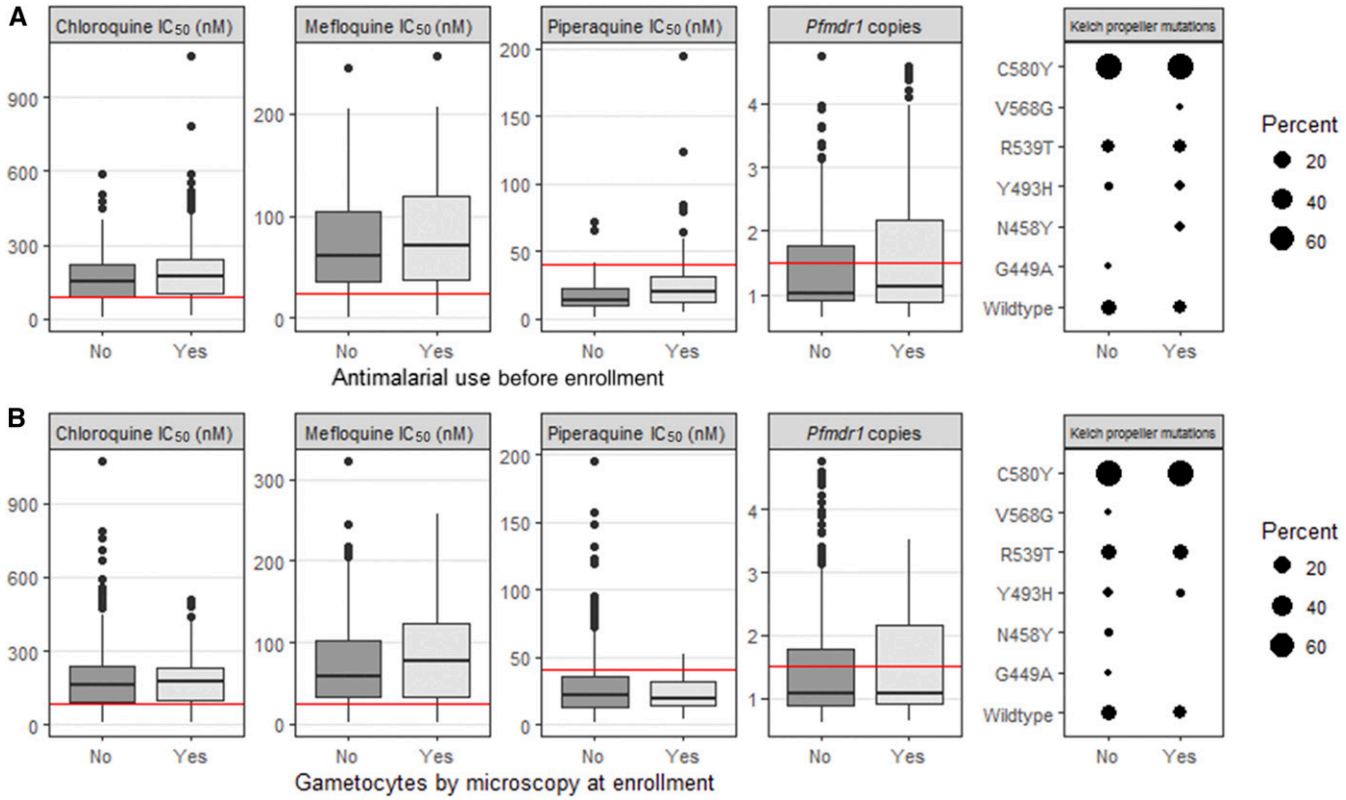

FIGURE 2. Relationship of antimalarial use within the past year (**A**) and gametocyte carriage (**B**) to drug resistance profiles based on ex vivo IC_{50}s and molecular markers of resistance. Red lines mark threshold for classifying parasites as resistant based on WHO-defined cutoffs (chloroquine and mefloquine) or the upper quartile for piperaquine resistance. This figure appears in color at www.ajtmh.org.

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REFERENCES

1. WHO, 2013. *Emergency Response to Artemisinin Resistance in the Greater Mekong Subregion*. Regional framework for action 2013–2015. World Health Organization Archived publication. Available at: http://www.who.int/malaria/publications/atoz/9789241505321/en/. Accessed February 4, 2018.
2. Chaorattanakawee S et al., 2015. Ex vivo drug susceptibility testing and molecular profiling of clinical *Plasmodium falciparum* isolates from Cambodia from 2008 to 2013 suggest emerging piperaquine resistance. *Antimicrob Agents Chemother 59:* 4631–4643.
3. Spring MD et al., 2015. Dihydroartemisinin-piperaquine failure associated with a triple mutant including kelch13 C580Y in Cambodia: an observational cohort study. *Lancet Infect Dis 15:* 683–691.
4. Lon CT, Tsuyuoka R, Phanouvong S, Nivanna N, Socheat D, Sokhan C, Blum N, Christophel EM, Smine A, 2006. Counterfeit and substandard antimalarial drugs in Cambodia. *Trans R Soc Trop Med Hyg 100:* 1019–1024.
5. Bousema T, Drakeley C, 2011. Epidemiology and infectivity of *Plasmodium falciparum* and *Plasmodium vivax* gametocytes in relation to malaria control and elimination. *Clin Microbiol Rev 24:* 377–410.
6. Ashley EA et al., 2014. Tracking resistance to artemisinin collaboration (TRAC). Spread of artemisinin resistance in *Plasmodium falciparum* malaria. *N Engl J Med 371:* 411–423.
7. Price R, Nosten F, Simpson JA, Luxemburger C, Phaipun L, ter Kuile F, van Vugt M, Chongsuphajaisiddhi T, White NJ, 1999. Risk factors for gametocyte carriage in uncomplicated falciparum malaria. *Am J Trop Med Hyg 60:* 1019–1023.
8. WWARN Gametocyte Study Group, 2016. Gametocyte carriage in uncomplicated *Plasmodium falciparum* malaria following treatment with artemisinin combination therapy: a systematic review and meta-analysis of individual patient data. *BMC Med 14:* 79.
9. Bell AS, Huijben S, Paaijmans KP, Sim DG, Chan BHK, Nelson WA, Read AF, 2012. Enhanced transmission of drug-resistant parasites to mosquitoes following drug treatment in rodent malaria. *PLoS One 7:* e37172.
10. Lin JT et al., 2011. *Plasmodium falciparum* gametocyte carriage is associated with subsequent *Plasmodium vivax* relapse after treatment. *PLoS One 6:* e18716.
11. Lin JT et al., 2016. Microscopic *Plasmodium falciparum* gametocytemia and infectivity to mosquitoes in Cambodia. *J Infect Dis 213:* 1491–1494.

12. Chaorattanakawee S et al., 2016. Ex vivo piperaquine resistance developed rapidly in *Plasmodium falciparum* isolates in northern Cambodia compared to Thailand. *Malar J 15:* 519.
13. Lim P et al., 2009. *Pfmdr1* copy number and arteminisin derivatives combination therapy failure in falciparum malaria in Cambodia. *Malar J 8:* 11.
14. Ariey F et al., 2014. A molecular marker of artemisinin-resistant *Plasmodium falciparum* malaria. *Nature 505:* 50–55.
15. Maude RJ et al., 2014. Spatial and temporal epidemiology of clinical malaria in Cambodia 2004–2013. *Malar J 13:* 385.
16. Parobek CM et al., 2017. Partner-drug resistance and population substructuring of artemisinin-resistant *Plasmodium falciparum* in Cambodia. *Genome Biol Evol 9:* 1673–1686.
17. Leang R et al., 2015. Evidence of *Plasmodium falciparum* malaria multidrug resistance to artemisinin and piperaquine in western Cambodia: dihydroartemisinin-piperaquine open-label multicenter clinical assessment. *Antimicrob Agents Chemother 59:* 4719–4726.
18. Witkowski B et al., 2017. A surrogate marker of piperaquine-resistant *Plasmodium falciparum* malaria: a phenotype-genotype association study. *Lancet Infect Dis 17:* 174–183.
19. Amato R et al., 2017. Genetic markers associated with dihydroartemisinin-piperaquine failure in *Plasmodium falciparum* malaria in Cambodia: a genotype-phenotype association study. *Lancet Infect Dis 17:* 164–173.
20. Price RN et al., 1996. Effects of artemisinin derivatives on malaria transmissibility. *Lancet 347:* 1654–1658.