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Clinical Infectious Diseases

MAJOR ARTICLE

Clinical and In Vitro Resistance of *Plasmodium falciparum* to Artesunate-Amodiaquine in Cambodia

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(See the Editorial Commentary by Petersen and Picot on pages 414–5.)

Background. Artesunate-amodiaquine is a potential therapy for uncomplicated malaria in Cambodia.

Methods. Between September 2016 and January 2017, artesunate-amodiaquine efficacy and safety were evaluated in a prospective, open-label, single-arm observational study at health centers in Mondulkiri, Pursat, and Siem Reap Provinces, Cambodia. Adults and children with microscopically confirmed *Plasmodium falciparum* malaria received oral artesunate-amodiaquine once daily for 3 days plus single-dose primaquine, with follow-up on days 7, 14, 21, and 28. The primary outcome was day-28 polymerase chain reaction (PCR)-adjusted adequate clinical and parasitological response (ACPR). An amodiaquine parasite survival assay (AQSA) was developed and applied to whole genome sequencing results to evaluate potential amodiaquine resistance molecular markers.

Results. In 63 patients, day-28 PCR-adjusted ACPR was 81.0% (95% confidence interval [CI], 68.9–88.7). Day 3 parasite positivity rate was 44.4% (28/63; 95% CI, 31.9–57.5). All 63 isolates had the *K13*(C580Y) marker for artemisinin resistance; 79.4% (50/63) had *Pfpm2* amplification. The AQSA resistance phenotype (≥45% parasite survival) was expressed in 36.5% (23/63) of isolates and was significantly associated with treatment failure ($P = .0020$). *Pfmdr1* mutant haplotypes were N86/184F/D1246, and *Pfcrt* was CVIET or CVIDT at positions 72–76. Additional *Pfcrt* mutations were not associated with amodiaquine resistance, but the G353V mutant allele was associated with ACPR compared to *Pfmdr1* haplotypes harboring F1068L or S784L/R945P mutations ($P = .030$ and $P = .0004$, respectively).

Conclusions. For uncomplicated falciparum malaria in Cambodia, artesunate-amodiaquine had inadequate efficacy owing to amodiaquine-resistant *P. falciparum*. Amodiaquine resistance was not associated with previously identified molecular markers.

Keywords. artesunate-amodiaquine; artemisinin; *Plasmodium falciparum*; Cambodia; drug resistance.

Artemisinin-based combination therapy (ACT) includes a rapid-acting artemisinin with a longer-acting partner drug. ACTs support effective malaria treatment globally, contributing to recent declines in mortality [1]. In 2006, artemisinin-resistant *Plasmodium falciparum* was confirmed in Cambodia's western provinces [2] and subsequently verified in multiple studies [3]. Artemisinin resistance delays parasite killing, but resistance to the partner drug is required before treatment failure rates increase [4, 5]. Unfortunately, *P. falciparum* resistant to artemisinins and partner drugs (piperaquine, mefloquine) circulate in Cambodia and the Greater Mekong subregion, undermining clinical efficacy and limiting treatment options [1, 6–8].

Artesunate-amodiaquine was not deployed systematically in Cambodia and requires evaluation as a potential replacement for failing ACTs. In Africa, artesunate-amodiaquine is used extensively with 98.5% clinical efficacy [1]. *P. falciparum* with *multidrug resistance 1* (*Pfmdr1*) alleles N86Y/Y184/D1246Y (YYY haplotype) is associated with amodiaquine treatment failures in Africa [9] but has not been detected in Cambodia [10]. The most prevalent chloroquine resistance transporter gene (*Pfcrt*) haplotype in Cambodia is CVIET at positions 72–76 (wild-type CVMNT) [10], which is also prevalent in Africa [11, 12], and insufficient to confer amodiaquine resistance in vivo [13, 14]. In Viet Nam, 2 artesunate-amodiaquine clinical trials showed encouraging results with 98% efficacy [15, 16]. However, there are no recent data from Southeast Asia on artesunate-amodiaquine efficacy.

This study investigated artesunate-amodiaquine clinical efficacy for uncomplicated falciparum malaria in Cambodia. In the event of clinical failures, molecular markers associated with amodiaquine resistance were to be investigated.

Received 21 December 2019; editorial decision 13 April 2020; published online 27 May 2020.

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Clinical Infectious Diseases® 2021;73(3):406–13

DOI: 10.1093/cid/ciaa628

METHODS

Study Design

This prospective, single-arm, open-label therapeutic efficacy trial of artesunate-amodiaquine plus single-dose primaquine was conducted between September 2016 and January 2017 at 3 health centers in Cambodia: Koh Gnek (Koh Gnek district, Mondulkiri province), Promoy (Veal Veng district, Pursat province), and Khvav (Chi Kraeng district, Siem Reap province) (Supplementary material Figure S1).

The study confirmed to Good Clinical Practice and the Declaration of Helsinki (2000). The protocol followed the standard World Health Organization protocol for the surveillance of antimalarial treatment efficacy [17] and was approved by the National Cambodian Ethical Board and the World Health Organization Regional Office, Western Pacific Region. Owing to an administrative error, this study was registered retrospectively at https://www.anzctr.org.au (identifier ACTRN12619001628134). All patients or their guardians provided written informed consent. Additionally, assent was obtained from children aged ≥12 years.

Patients

Eligible patients were aged 5–60 years with microscopically confirmed *P. falciparum* monoinfection (1000–250 000 μL^{-1} blood), fever or history of fever during the past 24 h, who could swallow oral medication. Unmarried girls and women aged 12–18 years were excluded because a pregnancy test would be culturally unacceptable; pregnant and lactating women were also excluded. All other women of child-bearing potential were given a pregnancy test. Exclusion criteria were severe falciparum malaria, severe malnutrition, febrile conditions other than malaria or other underlying chronic illness, medication that might interfere with antimalarial pharmacokinetics, or a history of hypersensitivity to artemisinin or amodiaquine.

Treatment

Artesunate-amodiaquine (ASAQ Winthrop®, Sanofi, Paris, France) was administered under supervision once-daily for 3 days. Doses were determined by bodyweight to achieve 4 mg/kg/day (range 2–10 mg/kg) artesunate and 10 mg/kg/day (range 7.5–15 mg/kg) amodiaquine. Primaquine was given as a single 15-mg dose (0.25 mg base/kg). All patients were treated as in-patients with out-patient follow-up visits on days 7, 14, 21, and 28. Any recurrence during follow-up was treated with artesunate-mefloquine.

Procedures

At enrollment, a clinical examination was performed and a full medical history taken. Adverse events were recorded at all study visits. Parasitemia and *Plasmodium* species identification was assessed using Giemsa stained thick and thin blood films obtained at screening, every day following the first treatment dose until samples were parasite negative, at each weekly follow-up visit, and if clinically indicated. Parasite counts were recorded as the average from 2 microscopists using standard methods [17]. Treatment failures were verified as recrudescence using polymerase chain reaction (PCR) genotyping by comparing *P. falciparum* genes *msp1*, *msp2,* and *glurp* in pretreatment blood samples versus those obtained at recurrence [18].

Molecular Surveillance

Using samples collected on day 0, the *Kelch13* (*K13*) gene was sequenced to identify mutations associated with artemisinin resistance [19], and gene copy numbers for *P. falciparum plasmepsin 2/3* (*Pfpm2*) and *Pfmdr1* were determined, as per published methods [20]. The threshold for gene amplification was defined as > 1.5 copies.

Amodiaquine Susceptibility in Vitro

Pretreatment blood samples were collected into acid-citrate-dextrose tubes (Becton-Dickinson, Franklin Lakes, NJ, USA) and processed within 48 h at Institut Pasteur, Cambodia. Clinical isolates were culture adapted using standard methods [4]. *P. falciparum* reference strains 3D7 (amodiaquine susceptible, from MR4) and 7G8 (amodiaquine resistant, from the European Malaria Reagent Repository) were similarly maintained and used as controls. Molecular markers obtained from day 0 samples were confirmed as identical to those obtained from the corresponding culture-adapted parasites via whole genome sequencing except for 1 isolate that had *Pfpm2* amplification at day 0, which reverted to a single gene copy under culture.

In vitro susceptibility to mono-desethyl-amodiaquine (from the WorldWide Antimalarial Resistance Network) was assessed using the [^{3}H]-hypoxanthine assay, according to published methods [4]. Half-maximal inhibitory concentration values (IC_{50}) were determined using ICEstimator software (http://www.antimalarial-icestimator.net).

The amodiaquine survival assay (AQSA) was based on a similar assay for piperaquine [21]. Tightly synchronized ring-stage parasites (0–3 h postinvasion) were exposed to 200 nM mono-desethyl-amodiaquine for 48 h and maintained for a further 24 h in drug-free medium. Live parasites were then enumerated microscopically from Giemsa-stained thin blood films on examination of ≥ 10 000 erythrocytes. Parasite survival following exposure to mono-desethyl-amodiaquine was determined as a percentage relative to untreated controls.

Amodiaquine Resistance and Association With Molecular Markers

Investigation of potential molecular markers associated with amodiaquine resistance used an expanded data set including culture-adapted *P. falciparum* isolates from this study plus 34 culture-adapted clinical isolates collected from sentinel sites between February 2017 and February 2018 (n = 10 Kampong Speu, n = 13 Mondulkiri, n = 3 Pursat, n = 8 Ratanakiri).

Pfcrt and *Pfmdr1* were sequenced using whole genome sequencing with Illumina paired-reads sequencing, according to published protocols [19, 20]. After processing, data were integrated into the Whole-genome Data Manager (version 2.0) [19, 20]. Single-nucleotide polymorphisms (SNPs) were investigated using Phen2gen software [20].

Outcomes

The primary efficacy outcome was day-28 adequate clinical and parasitological response (ACPR) adjusted for reinfection using PCR genotyping. Day-3 parasite positivity rate was the secondary efficacy outcome. Safety outcomes were the frequency of adverse events, serious and severe adverse events.

Statistical Analysis

Data were analyzed with Excel StatX and Graphpad Prism (version 8.3.0). ACPR was evaluated using Kaplan-Meier survival curves and associated 95% confidence intervals (95% CI) and compared using the log-rank test (Mantel-Cox). IC_{50} and AQSA values versus clinical outcome were compared using the Mann-Whitney test. Resistance thresholds for IC_{50} and AQSA parasite survival were determined with receiver operating characteristic (ROC) analysis. Kruskal-Wallis tests were used to identify significant differences in *Pfcrt–Pfmdr1* haplotype AQSA parasite survival results. Significant *P* values were <.05.

RESULTS

Patients

Most patients were adult males (87.3%, 55/63) (Table 1). There were no withdrawals or patients lost to follow-up; all 63 patients were included in the analysis. Thirty-one patients were from Mondulkiri, 29 from Pursat, and 3 from Siem Reap.

Therapeutic Efficacy and Molecular Surveillance

Day-28 ACPR was 81.0% (51/63). All recurrences were late clinical failures (days 21–28) and PCR-confirmed as recrudescence—9 in adults (18–52 years) and 3 in children (8–15 years). The Kaplan-Meier day-28 ACPR estimate was 81.0% (95% CI, 68.9–88.7) (Figure 1); 77.4% (95% CI, 58.4–88.5) for Mondulkiri, 86.2% (95% CI, 67.3–94.6) for Pursat, and 66.7% (95% CI, 5.4–94.5) for Siem Reap. No severe or serious adverse events were reported during the study.

Day-3 parasite positivity rate was 44.4% (28/63; 95% CI, 31.9–57.5); 22.6% (7/31; 95% CI, 9.6–41.1) for Mondulkiri, 69.0% (20/29; 95% CI, 49.2–84.7) for Pursat, and 33.3 (1/3, 95% CI, .8–90.6) for Siem Reap. All 63 isolates had the *K13*(C580Y) marker for artemisinin resistance. None had increased *Pfmdr1* copy number, but 85.7% (54/63) had *Pfpm2* amplification.

Table 1. Baseline Characteristics of the Safety and Efficacy Population

Characteristic	Study Population (N = 63)
Males/females, n	61/2
Adults aged > 15 years, n	56
Children aged 5–15 years, n	7
Mean age (SD) [range], years	28.2 (11.9) [5–56]
Mean weight (SD) [range], kg	53.7 (10.5) [23–78]
Geometric mean parasitemia (range) μL^{-1} blood	22 695 (1756–248 000)

Abbreviation: SD, standard deviation.

In Vitro Amodiaquine Resistance

In the [^{3}H]-hypoxanthine assay, the mono-desethyl-amodiaquine median IC_{50} for the 63 clinical isolates was 174.5 nM (interquartile range [IQR], 90.7–213.1). Isolates from patients with recrudescence (n = 12) had a median IC_{50} of 193.8 nM (IQR, 156.6–240.3) versus 165.0 nM (IQR, 88.3–212.0) for patients with day-28 ACPR (n = 51) (*P* = .084, Mann-Whitney) (Figure 2A). ROC analysis indicated an IC_{50} threshold value most strongly correlated with day-28 ACPR of <181 nM (Supplementary material Figure S2); sensitivity was 61% (95% CI, 47–73) at a specificity of 75% (95% CI, 47–91). Area under the curve (AUC) was 0.66 (95% CI, .49–.83; *P* = .083). Thus, IC_{50} had inadequate discriminatory value for predicting clinical outcome.

Amodiaquine Survival Assay (AQSA)

In the 63 clinical isolates, AQSA median parasite survival was 23.5% (IQR, 1.6–65.5). Quality control values were 72.8% parasite survival for the amodiaquine-resistant 7G8 strain and 0% for the susceptible 3D7 strain. There was a strong positive correlation between IC_{50} and AQSA parasite survival (Pearson *r* value 0.75 [95% CI, .62–.84] *P* < .0001) (Figure 3A). Recrudescent isolates had a significantly higher median survival (64.0% [IQR, 29.1–77.0]) versus those from patients with day-28 ACPR (13.3% [IQR, 0.9–48.1]) (*P* = .0054, Mann-Whitney) (Figure 3B). ROC analysis indicated an AQSA threshold predictive of day-28 ACPR of <45% survival, with 75% sensitivity (95% CI, 47–91) and 73% specificity (95% CI, 59–83). AUC was 0.75 (95% CI, .60–.91; *P* = .0063) (Supplementary material Figure S3). ACPR occurred with 92.5% (37/40) of isolates with <45% AQSA parasite survival but decreased significantly to 60.9% (14/23) for those with ≥45% survival (*P* = .0020, log-rank Mantel-Cox) (Figure 3C). Therefore, ≥45% parasite survival in the AQSA was a clinically relevant resistance phenotype.

Molecular Signature Associated With Amodiaquine Resistance

In the expanded data set of 97 clinical isolates, median AQSA parasite survival was 8.0% (IQR, 1.0–50.2), and 28.9% (28/97) had ≥45% parasite survival. Ninety-six isolates were *K13*(C580Y) and 1 was *K13*(Y493H). One isolate had *Pfmdr1* amplification, whereas 78.4% (76/97) had *Pfpm2* amplification. There was no significant difference in the median AQSA value of strains with single-copy

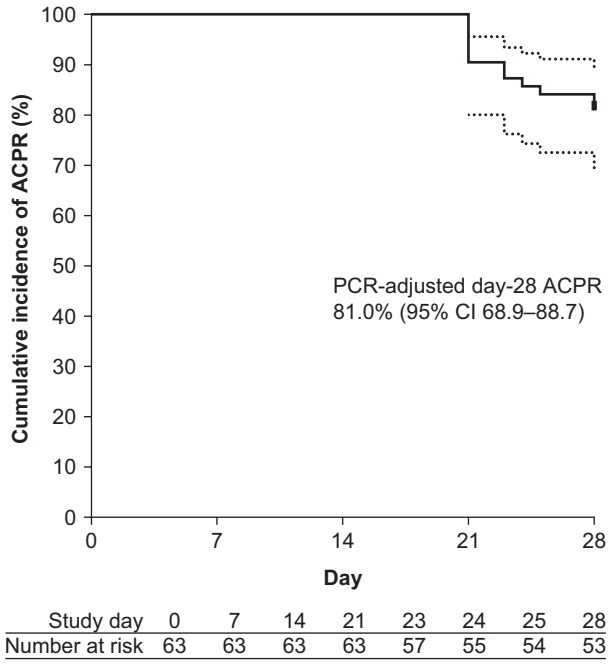


Figure 1. Kaplan-Meier estimates of ACPR with artesunate-amodiaquine for uncomplicated malaria. There were no reinfections on PCR-adjustment and no patients were censored. Abbreviations: ACPR, adequate clinical and parasitological response; CI, confidence interval; PCR, polymerase chain reaction.

Pfpm2 (6.3% [IQR, 1.1–56.4]) versus those with multiple copies (12.3% [IQR, 0.91–47.6]) ($P = .74$, Mann-Whitney).

The frequency of SNPs for the key *P. falciparum* resistance genes *Pfcrt* and *Pfmdr1* are shown in Supplementary material Figure S4. For isolates with complete *Pfcrt* haplotypes (N = 94), 12 different haplotypes were identified, of which 97.9% (92/94) were either Dd2 or on the Dd2 background (Table 2). For *Pfmdr1*, 6 different haplotypes were found, with 51.1% (48/94) having Y148F plus at least 1 other mutation (Table 2).

AQSA results for isolates with complete *Pfcrt–Pfmdr1* sequences (N = 92) indicated significant differences in survival between 4 haplotypes: Dd2–Y184F/S784L/R945P versus Dd2 F145I–Y184F and versus Dd2 G353V–Y184F ($P = .0075$ and $P = .0003$, respectively; Kruskal-Wallis); and between Dd2 T93S–Y184F/F1068L versus Dd2 F145I–Y184F and versus Dd2 G353V–Y184F ($P = .0003$ and $P < .0001$, respectively; Kruskal-Wallis) (Figure 4A). When examining clinical outcomes, although the data set was smaller (N = 60), there was still a significant difference between Dd2 G353V–Y184F versus Dd2–Y184F/S784L/R945P or Dd2 T93S–Y184F/F1068L ($P = .0004$ and $P = .0304$, respectively; log-rank Mantel-Cox) (Figure 4B).

DISCUSSION

Antimalarial drug resistance curtails ACT efficacy for uncomplicated malaria in Cambodia, with the emergence of triple mutants (artemisinin, piperaquine, and mefloquine resistant) underlining the need for new therapeutic options [22–24]. High artesunate-amodiaquine efficacy in Africa and Viet Nam, and the absence of known amodiaquine resistance markers in Cambodia, suggested that this ACT would be efficacious. Surprisingly, 19.0% of patients had recrudescence, sufficient to exclude artesunate-amodiaquine as an uncomplicated malaria treatment in Cambodia.

The *K13*(C580Y) artemisinin resistance marker was ubiquitous in this study and is the predominant *K13* mutant in Cambodia [25]. Consistent with this, the day-3 parasite positivity rate was 44.4%. However, artemisinin resistance increases recrudescence probability only if there is also partner drug resistance [4, 5]. Thus, the high treatment failure rate is most likely explained by amodiaquine resistance.

Previous studies in Africa indicated a mono-desethyl-amodiaquine IC_{50} resistance threshold of >60 nM with amodiaquine monotherapy [26], compared with >180 nM for artesunate-amodiaquine in the current study. This could be because artemisinin resistance is partial, and a higher degree of amodiaquine resistance is necessary to support parasite recrudescence following artesunate-amodiaquine versus amodiaquine monotherapy.

In the current study, IC_{50} lacked sufficient discriminatory power to differentiate between ACPR and recrudescence. This lack of correlation between IC_{50} and clinical outcome was observed previously for artemisinin and piperaquine, promoting the development of parasite survival assays measuring

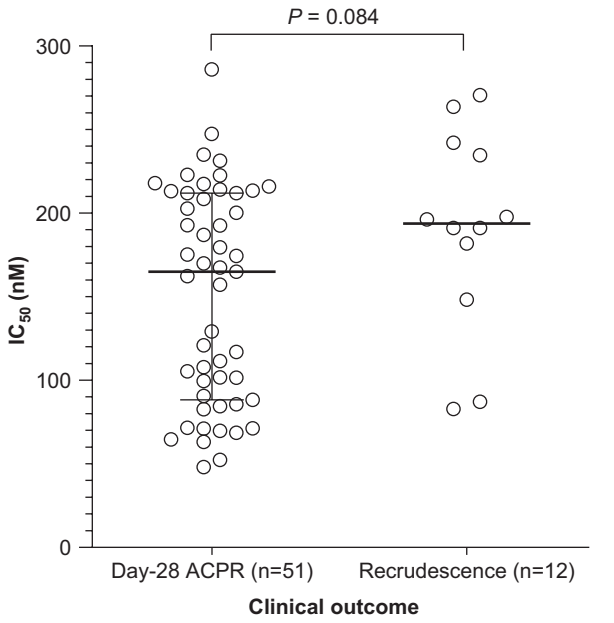


Figure 2. Relationship between mono-desethyl-amodiaquine IC_{50} determined in the [^{3}H]-hypoxanthine uptake inhibition assay and clinical outcome at day 28 following treatment with artesunate-amodiaquine for 63 *P. falciparum* clinical isolates. Open circles represent *P. falciparum* isolates and black horizontal bars and I bars indicate the median and interquartile range, statistical comparison used the Mann-Whitney test. Abbreviations: ACPR, adequate clinical and parasitological response; IC_{50}, half-maximal inhibitory concentration.

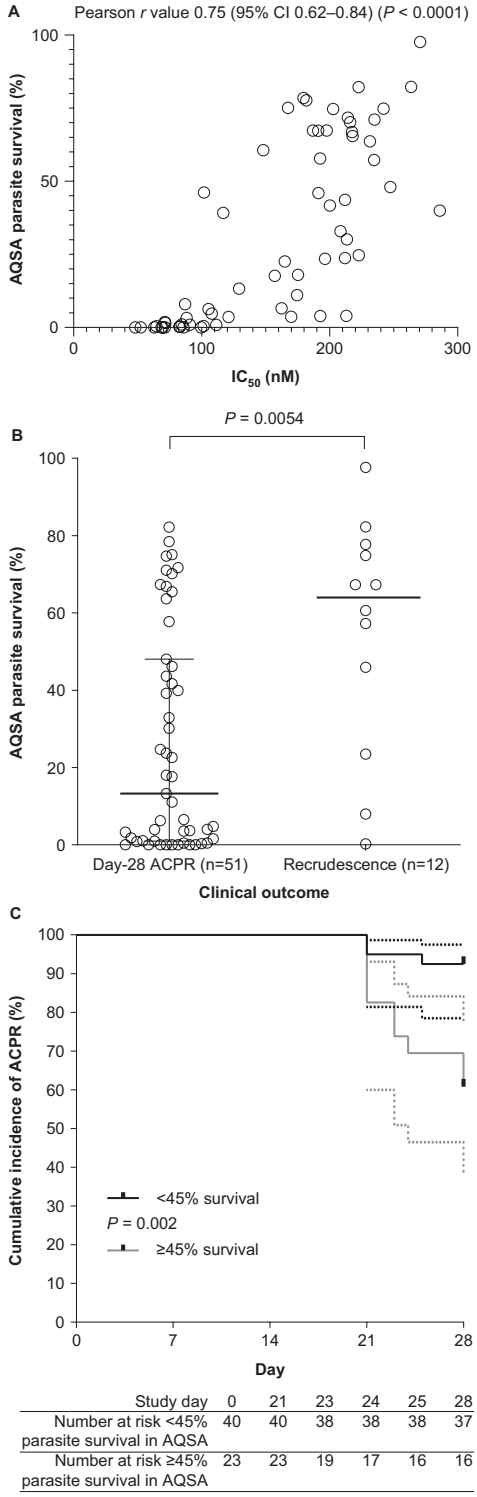


Figure 3. Parasite survival in the amodiaquine survival assay (AQSA). *A*, Correlation between AQSA parasite survival and IC_{50} determined in the [^{3}H]-hypoxanthine uptake inhibition assay. *B*, Relationship between AQSA parasite survival and clinical outcome at day 28 following treatment with artesunate-amodiaquine. Open circles represent *P. falciparum* isolates and black horizontal bars and I bars indicate the median and interquartile range, statistical comparison used the Mann-Whitney *U* test. *C*, Kaplan-Meier estimates of ACPR for parasites with the AQSA resistance phenotype (≥45% survival) versus those with the susceptible phenotype (<45% survival), statistical comparison used the log-rank test (Mantel-Cox). Abbreviations: ACPR, adequate clinical and parasitological response; IC_{50}, half-maximal inhibitory concentration.

cytocidal activity [4, 21, 27]. This study validated the AQSA, a novel amodiaquine parasite survival assay, which correlated well with clinical outcome, and was sufficiently sensitive and specific to use as a resistance phenotype to investigate potential amodiaquine resistance molecular markers. Consequently, we propose AQSA ≥45% parasite survival as a novel definition for amodiaquine resistance.

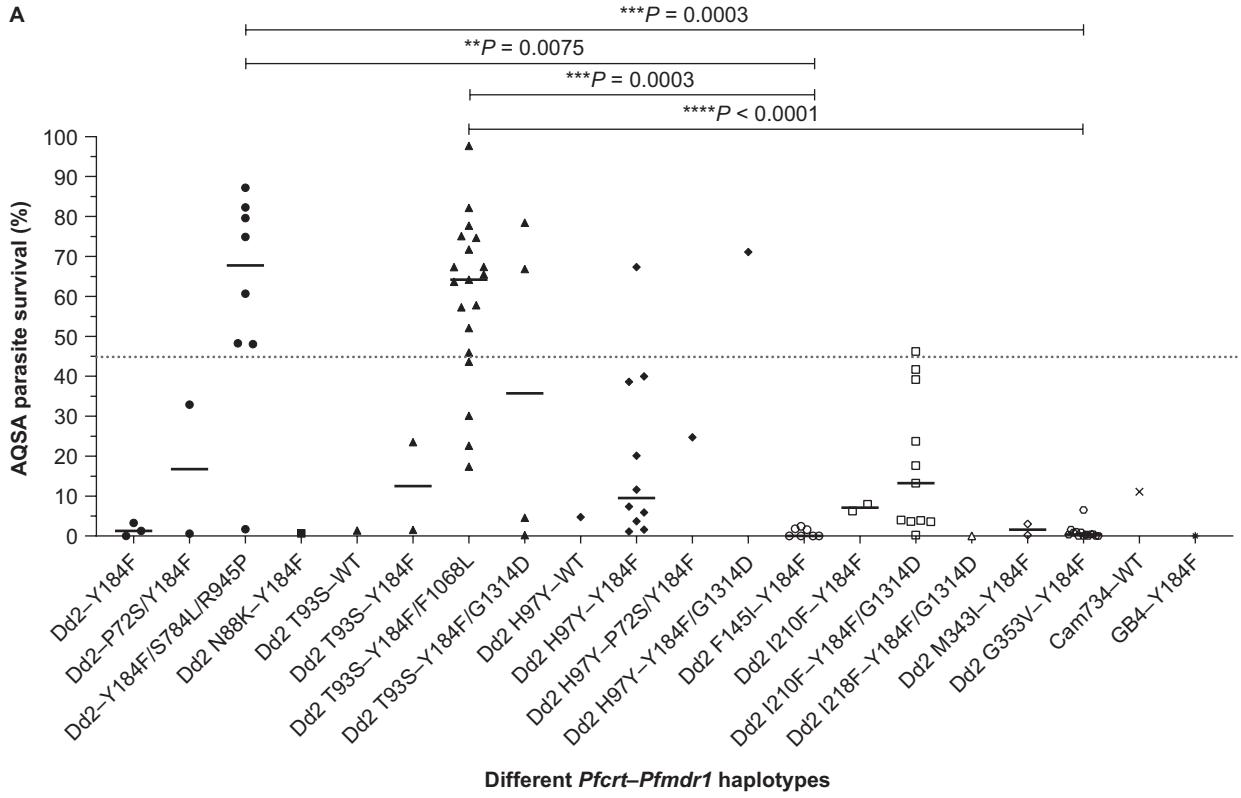
In Africa, *Pfmdr1* N86 and D1246 selection following artemether-lumefantrine improves clinical outcomes with artesunate-amodiaquine in *P. falciparum* malaria [28]. This suggests that an artemether-lumefantrine-amodiaquine triple therapy could counterselect for resistance, with ongoing initiatives to develop the combination [29]. However, the rationale for artemether-lumefantrine-amodiaquine in Cambodia has not been demonstrated. Our data show that amodiaquine resistance in Cambodia exists in the absence of the African amodiaquine-resistant haplotype *Pfmdr1* 86Y/Y184/1246Y [10], as all mutant *Pfmdr1* haplotypes were N86/184F/D1246. Also, mutations common in South American *P. falciparum Pfmdr1* (S1034C, N1042D and D1246Y) were absent. Molecular markers for lumefantrine resistance are not validated for Southeast Asia, and our data do not support counter selection for amodiaquine susceptibility. Rather, resistance to both amodiaquine and lumefantrine appears possible in Cambodian *P. falciparum*.

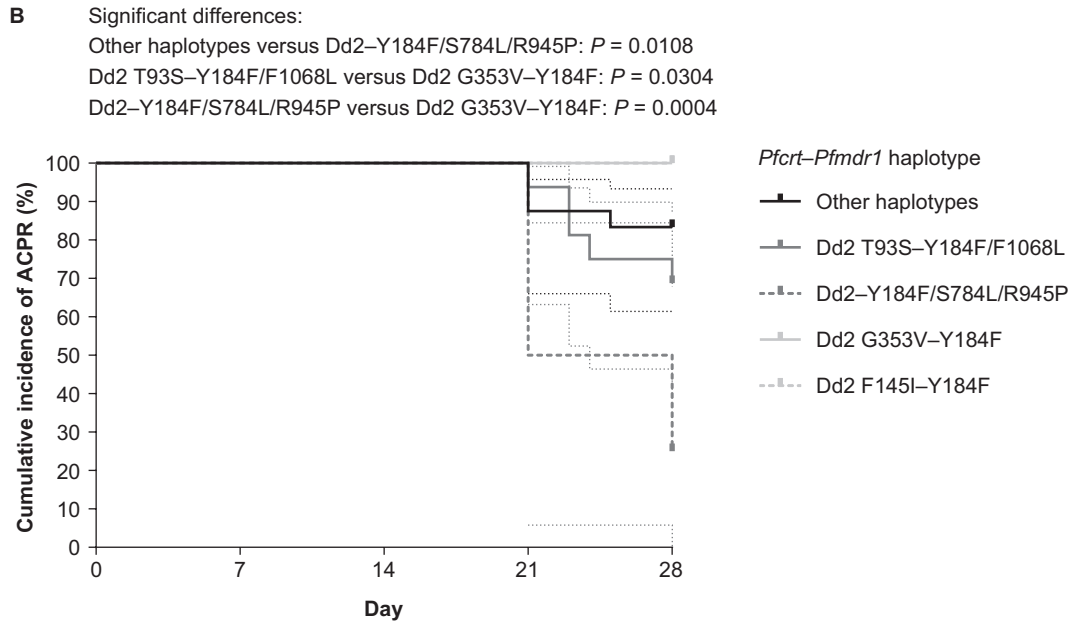
Table 2. *Pfcrt* or *Pfmdr1* Haplotype and Parasite Survival in the Amodiaquine Survival Assay (AQSA)

Haplotype	n (%)	Median Parasite Survival, % (IQR)
Pfcrt	**N = 94**	
Dd2[a]	13 (13.8)	48.1 (1.5–77.2)
Dd2 F145I	7 (7.4)	0.0 (0–1.8)
Dd2 G353V	13 (13.8)	0.4 (0–1.0)
Dd2 G367C	1 (1.1)	0.4
Dd2 H97Y	14 (14.9)	15.9 (4.5–46.8)
Dd2 I210F	13 (13.8)	8.0 (3.8–31.5)
Dd2 I218F	1 (1.1)	0.0
Dd2 M343I	2 (2.1)	1.6 (0.2–3.0)
Dd2 N88K	1 (1.1)	0.7
Dd2 T93S	27 (28.7)	57.8 (22.6–71.7)
Cam734[a]	1 (1.1)	11.1
GB4[a]	1 (1.1)	0.0
Pfmdr1	**N = 94**	
Wild type	3 (3.2)	4.8 (1.4–11.1)
Y184F	43 (45.7)	1.3 (0–6.3)
P72S/Y184F	3 (3.2)	24.7 (0.6–32.9)
Y184F/F1068L	19 (20.2)	64.2 (46.0–74.7)
Y184F/G1314D	18 (19.1)	13.7 (3.6–42.8)
Y184F/S784L/R945P	8 (8.5)	67.8 (48.1–81.6)

Abbreviations: IQR, interquartile range; *Pfcrt, P. falciparum* chloroquine resistance transporter; *Pfmdr1*, *P. falciparum* multidrug resistance 1.

[a]Mutations for *Pfcrt* D2d: M74I/N75E/K76T/A220S/Q271E/N326S/I356T/R371I; *Pfcrt* Cam734: M74I/N75D/K76T/A220S/Q271E/T333S; *Pfcrt* GB4: 74I/75E/76T/A220S/Q271E/R371I.





Study day	0	21	23	24	25	28
Number at risk other haplotypes	24	24	21	21	21	20
Number at risk Dd2 T93S–Y184F/F1068L	16	16	15	13	12	12
Number at risk Dd2–Y184F/S784L/R945P	4	4	2	2	2	2
Number at risk Dd2 G353V–Y184F	13	13	13	13	13	13
Number at risk Dd2 F145I–Y184F	3	3	3	3	3	3

Figure 4. Effect of *P. falciparum Pfcrt–Pfmdr1* haplotype on resistance phenotype. *A*, Parasite survival in the amodiaquine survival assay (AQSA) for Cambodian clinical isolates (N = 92), the horizontal gray dotted line indicates the AQSA resistance phenotype (≥45% parasite survival). Symbols represent *P. falciparum* isolates, and black horizontal bars and I bars indicate the median and interquartile range; statistical comparison used the Kruskal-Wallis test. *B*, Kaplan-Meier plots for day-28 ACPR in 60 patients with *P. falciparum* malaria treated with artesunate-amodiaquine for amodiaquine-resistant and -susceptible *Pfcrt–Pfmdr1* haplotypes. Abbreviation: ACPR, adequate clinical and parasitological response.

In the expanded parasite data set, all except 2 *Pfcrt* haplotypes were on the Dd2 background (ie, C72/V73/74I/75E/76T/220S/271E/326S/356T/371I). The amodiaquine-resistant 72–76 SVMNT haplotype reported in South American parasites was absent, consistent with previous data [10].

In Asia, novel *Pfcrt* mutations have emerged on the Dd2 chloroquine-resistant allelic background, in contrast to Africa where 3D7, GB4, and Cam783 haplotypes predominate [30]. In clinical isolates and gene edited parasites, *Pfcrt* Dd2 decreases in vitro susceptibility to amodiaquine relative to *Pfcrt* 3D7 and thus can be considered an amodiaquine-tolerant background [30, 31]. In the current study, 85.9% (79/92) of the Dd2-based haplotypes had additional mutations. The *Pfcrt* mutations G353V and F145I were significantly associated with amodiaquine sensitivity in the AQSA, and G353V was associated with ACPR. These findings are consistent with data in gene-edited parasites showing that these mutations confer resistance to piperaquine but sensitize parasites to amodiaquine, chloroquine, and quinine [32, 33]. Notably, in gene-edited parasites neither G353V nor F145I had any impact on lumefantrine susceptibility, and counterselection between lumefantrine and amodiaquine appears unlikely [32]. In previous studies, *Pfcrt* T93S has been associated with piperaquine resistance, and its prevalence has been increasing in Cambodia [33–35]. In the current study, *Pfcrt* T93S was the most common *Pfcrt* haplotype and was associated with both amodiaquine-susceptible and amodiaquine-resistant haplotypes. Thus, elevated AQSA values were not associated with an identified polymorphism in *Pfcrt*. Rather, key mutations in this gene appear to be associated with sensitization to amodiaquine.

Pfmdr1 showed 6 haplotypes in this study, and 51.1% (48/94) were Y184F plus another mutation. Of these, S784L/R945P and F1068L were associated with clinical and in vitro resistance. *Pfmdr1* S784L has previously been noted from several locations in Cambodia at frequencies between 0.5 and 29.8% but was not associated with R945P [10]. *Pfmdr1* F1068L was reported from Pailin at a low frequency (4.2%) [10], versus 20.2% (19/94) in the current study. This study is the first report to our knowledge associating these *Pfmdr1* haplotypes with amodiaquine resistance. However, our data set is insufficient to show causality or to perform multivariate analysis. Thus, extended genome-wide association studies and genome editing are required to validate our findings. The single amodiaquine-susceptible *Pfmdr1* S784L/R945P mutant in the AQSA was the only isolate with *Pfmdr1* amplification. Conclusions cannot be drawn from one isolate, but additional investigations may be valuable.

The origin of amodiaquine resistance in Cambodia is unclear. This drug was not used recently and saw only limited implementation in the 1990s. Piperaquine resistance selection is unlikely to be associated with amodiaquine resistance emergence, as the associated genotype, that is, *Pfpm2* amplification and *Pfcrt* mutations have either no effect or sensitize parasites to amodiaquine [32]. We could thus hypothesize that amodiaquine resistance emerged in Cambodia in the past consecutively to extensive chloroquine use and since then has been perpetuated by an unidentified mechanism.

While confirming artesunate-amodiaquine resistance in Cambodia, this was a small study conducted in a limited geographical region. Although significant relationships between SNPs in key resistance genes and amodiaquine resistance phenotypes were observed, causality cannot be determined. For example, the associations could result from the close relatedness of parasites in this study. Further investigations are required to confirm the putative amodiaquine resistance markers and assess their relevance to other malaria endemic areas. In the absence of molecular markers, the AQSA provides a novel methodology to assess clinically relevant amodiaquine resistance. However, AQSA specificity and sensitivity were determined according to the limited study size and location, and the $\geq$ 45% parasite survival AQSA resistance phenotype may require revision with additional data.

This study highlights the need for careful assessment of therapeutic outcomes and molecular markers before introducing a new antimalarial treatment in Cambodia. Resistance to amodiaquine was unexpected and was not associated with any known resistance genotype from other malaria endemic areas. Our findings indicate that clinical resistance was linked to the acquisition of high-level resistance against an amodiaquine-tolerant background. Thus, any amodiaquine-based combination would place partner compounds under a high selective pressure and be inappropriate in Cambodia.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Author contributions. R. L., P. R., F. A., and B. W. contributed to the concept and design of the study. M. M.-K., C. M., N. K., S. K., S. Ke, C. K., N. Kl, R. E., S. C., and B. I. were involved in data acquisition and M. M.-K., R. L., C. M., N. K., D. M. B., M. D. B., P. R., F. A., and B. W. in data analysis and interpretation. All authors critically reviewed the manuscript, approved the final version, and take full responsibility for the publication.

Acknowledgments. Naomi Richardson of Magenta Communications Ltd developed a first draft of this article from the statistical output, collated author contributions, provided graphic services, and was funded by the World Health Organization.

Disclaimer. The funding source was not involved in the design and conduct of the study, the interpretation of the results, and the development of this publication. The funders of this study had no role in study design, data collection, data analysis, data interpretation, writing of the report, and the decision to submit. The corresponding author had full access to all data in the study and final responsibility for the decision to submit for publication.

Financial support. This work was supported by the Bill & Melinda Gates Foundation and United State Agency for International development-President's Malaria Initiative through the World Health Organization. M. M.-K. and B. W. were supported by 5% initiative (MIVS-ACT, grant number 15SANIN211).

Potential conflicts of interest. M. D. B., D. M. B., and P. R. are staff members of the World Health Organization. The authors alone are responsible for the views expressed in this publication and they do not necessarily represent the decisions, policy, or views of the World Health Organization. All other authors have no conflicts of interest to report. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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