Plasmodium Falciparum Phenotypic and Genotypic Resistance Profile During the Emergence of Piperaquine Resistance From Royal Thai Army and Civilian patients in Northeastern Thailand

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Abstract

Malaria remains a public health problem in Thailand, especially along its borders where highly mobile populations can contribute to persistent transmission. This study aimed to determine resistant genotypes and phenotypes of 112 *Plasmodium falciparum* isolates from patients along the Thai-Cambodia border during 2013-2015. The majority of parasites harbored *pfmdr1*-Y184F mutation. A single *pfmdr1* copy number, had CVIET haplotype of amino acids 72-76 of *pfcr1* and no *pfcytb* mutations. All isolates had a *pfk13* point mutation (R539T, R539I, and C580Y), and increased ring-stage survival assay (except R539I). Multiple copies of *pfpm2* and *pfcr1*-F145I were first detected in 2014 (12.8%) and increased to 30.4% in 2015. Parasites containing either multiple *pfpm2* copies with and without *pfcr1*-F145I or a single *pfpm2* copy with *pfcr1*-F145I exhibited the elevated IC₉₀ values of piperazine. Antimalarials were detected in 21 samples (18.8%) by mass spectrometer, and 12 samples (10.7%) by bioassay. Collectively, the emergence of these resistance patterns mirrored the reports of dihydroartemisinin-piperazine treatment failures in the adjacent province of Cambodia, Oddar Meanchey, suggesting a migration of parasites across the border. As malaria elimination efforts ramp up in Southeast Asia, host nations militaries and other groups in border regions must be a focus for interventions

Introduction

An estimate of 229 million malaria cases occurred worldwide in 2019¹ and artemisinin-based combination therapies (ACT) have been the first-line drug treatment for uncomplicated *Plasmodium falciparum* infection for most malaria endemic areas; however, resistance to both artemisinin (ART) and its partner drugs has increased over the years at a pace requiring intensive surveillance and monitoring. The first case reports of ART resistance emerged in Thailand in the late 1990s² and in 2003-2005. Thai Ministry of Public Health (MoPH) and Armed Forces Research Institute of Medical Sciences (AFRIMS) studies began to suggest that first-line ACT regimens (2-day artesunate (AS) plus mefloquine (MQ)) were failing along both sides of the Thai-Cambodia border near Trat, Thailand and Pailin, Cambodia³. Militaries and other mobile forest goers in these border areas constitute a population at risk for succumbing to multi-drug resistant malaria as well as facilitating transmission, necessitating targeted control strategies if the Greater Mekong Subregion (GMS) is to achieve malaria elimination. Much of the research conducted at AFRIMS has mil-to-mil engagement with Thai and Cambodian armed forces, which allows for clinical and surveillance studies to be conducted in these high risk populations, resulting in interventions tailored to the military and shared with the MoPH

Following the case reports of ACT failure, the Artemisinin Resistance: Confirmation, Characterization and Planning for Containment Project (ARC) in 2006 showed a delayed clearance resistance phenotype in Cambodia along the Thai border which continued to spread across mainland Southeast Asia⁴⁻⁹. But it was not until 2014 that an ART resistance molecular marker was identified with mutations in the propeller domain of *P. falciparum* Kelch-13 gene (*pfk13*) (PF3D7_1343700)¹⁰⁻¹³, which can be confirmed by the ring-stage survival assay (RSA)^{10,14} and/or delayed parasite clearance times in clinical trials^{4,5}. Multiple copies of *P. falciparum* multi drug resistance 1 (*pfmdr1*) (PF3DF_0523000) is a well-established marker for MQ resistance^{15,16}, while the *pfmdr1* single-nucleotide polymorphisms (SNPs) have been associated with modulation of parasite tolerance or susceptibility to several antimalarial drugs including quinine (QN), amodiaquine (AQ), chloroquine (CQ), MQ and lumefantrine (LUM)¹⁷. The main *pfmdr1* SNPs associated with drug resistance include N86Y, Y184F, S1034C, and N1024D¹⁸⁻²³. The wild-type N86 in *pfmdr1* has been associated with increased tolerance to the artemether (ATM) and LUM^{18,23,24}, while parasites harboring the N86Y mutation exhibit an increased susceptibility to MQ²⁵. An ACT alternative to AS-MQ is dihydroartemisinin-piperazine (DHA-PPQ) and the first PPQ resistance markers identified were multiple copies of *P. falciparum* plasmepsin 2 (*pfpm2*) (PF3D7_1408000)²⁶⁻²⁹, followed by mutations in *P. falciparum* chloroquine resistance transporter (*pfcr1*) (PF3D7_0709000) downstream of the 4-aminoquinoline resistance locus (positions 72-76 with K76T)³⁰⁻³³, and the E415G mutation in *P. falciparum* exonuclease (*pfexo*) (PF3D7_1362500)^{26,27,34}. PPQ resistance can be characterized by the elevated IC₉₀ values³⁵ and PPQ survival assay (PSA) with a survival rate of more than 10%, defining a PPQ resistant phenotype³⁰. Atovaquone-proguanil (ATQ-PG) is not an ACT but ATQ resistance subsequently emerged which was linked to specific mutations in *P. falciparum* mitochondrial cytochrome B (*pfcytb*) (AY282930.1) in particular the mutation at positions 258 and 268³⁶⁻³⁸.

Twenty years after the first ACT failures, there are now descriptions of the spread of a single multidrug resistant malaria parasite lineage (PfPailin) across the eastern GMS^{39,40}. Efforts in mapping parasite population structure and gene flow can assist in understanding and predicting the spreading pattern of resistance. Military populations at border areas who are deployed to endemic areas should be integrated in surveillance and monitoring efforts as they are on the front-lines of malaria transmission. Here we report resistance characteristics of *P. falciparum* clinical isolates collected from Thai soldiers and civilian patients between 2013 and 2015

and show the trends in drug resistance and spread. At that time, the national treatment guidelines in Thailand recommended AS-MQ for *P. falciparum* malaria. During this same period, we reported dramatic loss of efficacy of DHA-PPQ (54% treatment failure) in a study that was conducted by AFRIMS in Anlong Veng, 12 km from the Thai border of Sisaket Province⁴¹. As the neighboring countries strive to achieve malaria elimination, there is ongoing concern about the risk of malaria transmission across borders, where treatment guidelines are different.

While there is a pressing need for new classes of antimalarials, intensive malaria surveillance to track drug resistance is required in high risk military populations to achieve malaria control so that these border foci of infections no longer pose a threat to elimination. Here, the investigation into *in vitro* drug susceptibility and molecular drug resistance marker profiling of *P. falciparum* isolates in patients presenting to Thai military health facilities is reported.

Materials And Methods

Study setting, protocol and subjects

This minimal risk surveillance study was open between July 2013-September 2015, enrolling Thai adults (aged 18 years and over) presenting with uncomplicated *P. falciparum* or *P. falciparum/P. vivax* mixed-infections at military health facilities. The enrollment population included soldiers, border police, or their family members and other villagers located near the Royal Thai Army (RTA) health clinics in Sisaket and Surin Provinces, located in northeastern Thailand on the Thai-Cambodia border. Inclusion criteria were asexual parasitemia per rapid diagnostic test (RDT) or blood film, and no malaria infection or antimalarials taken within the past seven days. The protocol was approved by the Walter Reed Army Institute of Research Institutional Board, Institute for Development of Human Research Protection, and RTA Institutional Review Board. All research was performed in accordance with relevant guidelines and regulations. All study subjects provided written informed consent prior to participation. Goal enrollment was 50 malaria cases per year, balancing the number of isolates needed to characterize resistant genotypes/phenotypes with the local population size and expected incidence of cases that could be enrolled.

Sample collection and preparation

Patients diagnosed with *P. falciparum* infection at RTA clinics were subjected to peripheral venipuncture prior to treatment. Two microscopists examined Giemsa-stained peripheral blood smears for each volunteer to determine malaria species infection and parasite densities for blood stages. Venous blood samples were collected in EDTA tubes for DNA extraction and molecular characterization and in sodium-heparin tubes for *ex vivo* bioassay and *in vitro* drug sensitivity assay. Plasma was separated from blood, frozen at -80 °C, and infected packed red blood cells were cryopreserved. All blood and processed blood samples were stored at -80C and transported in dry ice to AFRIMS for molecular characterization, *ex vivo* bioassay, and *in vitro* culture adaptation and drug sensitivity testing.

Molecular markers of malaria drug resistance

Parasite genomic DNA was extracted from 200 µL of EDTA whole blood by using EZ1 DNA blood kit with an automated EZ1 Advanced XL purification system as per the manufacturer's instructions. The DNA was stored at -20 °C. T100TM Thermal Cycler (Bio-Rad Laboratories) was employed to evaluate resistance makers including, *P. falciparum* kelch13 propeller domain (*pfk13*) (PF3D7_1343700), *pfcr* SNP F145I (KM288867.1), and *pfcytb* SNPs (AY282930.1)^{10,42}. ABI 7500 Real-time PCR system (Applied Biosystems) was employed to characterize *pfcr* SNPs (72-76) and *pfmdr1* SNPs (Positions 86, 184, 1034, 1042). Primers used to identify *pfkelch13*, *pfcytb*, *pfcr* and *pfmdr1* SNPs are shown in Tables S1 and S2.

Copy number variation assay

To determine copy numbers of *pfmdr1* and *pfpm2* genes, real-time quantitative PCR (qPCR) was performed on genomic DNA as previously described^{15,28,43} with some modifications. For *pfmdr1*, amplification reactions were performed according to the TaqMan Real-time PCR methods using ABI 7500 Real-time PCR system (Applied Biosystems) with 200 nM of each forward and reverse primer (Table S2) and 2 ng of DNA template while Rotor-Gene Q (QIAGEN, Valencia, CA) using Type-it® HRM™ kit was employed for *pfpm2*²⁸. The primers and probes (Table S2) used were as previously described to amplify the following loci: *pfmdr1* (PF3D7_0523000) and *pfpm2* (PF3D7_1408000), respectively²⁸. For the housekeeping gene, *β-tubulin* (PF3D7_1008700), *β*-tubulin forward and reverse primers were designed and used as a reference control for all experiments with the same validated PCR conditions as target primers. *P.*

falciparum 3D7 and Dd2 were used as references for single and multiple copy numbers of *pfmdr1*, respectively. All samples including the references clones were performed in duplicate. The average copy number values for each genes were calculated using $2^{-\Delta\Delta Ct}$ method. Parasites with copy number greater than 1.5 copies for *pfmdr1*¹⁵ and 1.6 copies for *pfpm2*²⁸ were interpreted to contain multiple copies of each gene.

***In vitro* Culture adaptation and maintenance of parasites**

Of 112 collected samples, 86 samples were subjected for *in vitro* culture adaptation. The exclusion criteria of the *in vitro* culture adaptation were *P. falciparum*/*P. vivax* mixed infections, % parasitemia < 0.05, and *ex vivo* bioassay activity > 250 nM (DHA equivalent). Culture adaptation of the parasites was performed using the modification method of Trager and Jensen⁴⁴. Parasites were maintained in fresh human erythrocytes (O⁺) in RPMI-1640 medium (Sigma), containing 15% AB⁺ human serum (heat inactivated and pooled) and supplemented with 25 mM HEPES, 25 mM sodium bicarbonate, and 0.1 mg/mL gentamicin. Human blood products (erythrocytes and serum) were obtained from the Thai Red Cross. Culture flasks were gassed with 5% CO₂, 5% O₂, 90% N₂ and incubated at 37 °C.

***In vitro* 72 hours drug susceptibility by Histidine rich protein 2 (HRP2)**

Drug susceptibility test using HRP2-ELISA to measure 50% or 90% inhibitory concentration (IC₅₀ and IC₉₀) was performed following previously published methods⁴⁵⁻⁴⁷. Dried drug-coated plates containing antimalarial drugs as described in Chaorattanakawee *et al*,^{35,46} were used and *in vitro* drug susceptibility testing was carried out for control reference clones (W2, D6, C2B) (Malaria Research & Reference Reagent Resource, Manassas, Vermont, USA) as described previously³⁵. IC₅₀s and IC₉₀s were estimated by nonlinear regression analysis using GraphPad Prism version 6.0.

Ring-stage survival assay (RSA)

In vitro RSA_{0-3 h} was performed on 0-3 h post-invasion rings obtained from selected culture-adapted clinical isolates following published methods with slight modifications^{14,47}. Parasite cultures were synchronized using 5% D-sorbitol and 75% Percoll to obtain 0 to 3-h post-invasion rings which were adjusted to 0.5-1% starting parasitemia with a 2% hematocrit in culture media (0.5% Albumax RPMI 1640 with 2.5% AB serum), and cultured in a 48-well microplate with 700 nM DHA and 0.1% DMSO in separate wells. The culture plate was then incubated for 6 hours at 37 °C in modular incubator chambers and gassed with 5% CO₂, 5% O₂ and 90% N₂. Cells were then washed once, resuspended in a drug-free medium, and cultured further for 66 hours. Susceptibility to DHA was assessed microscopically on thin films by estimating the percentage of viable parasites, relative to control (% survival rate). For the controls, the RSA_{0-3h} was also performed on *P. falciparum* reference clones W2, IPC-4884 and IPC-5202 (BEI Resources, NIAID, NIH, USA). A survival rate > 1% was deemed resistant for RSA.

***Ex vivo* bioassay**

P. falciparum-based bioassay was carried out to measure the antimalarial activity of patient plasma identifying if study volunteers were likely to have recently taken antimalarial drugs. Plasma was prepared from blood collected on the screening day for evaluation according to previously published methods^{48,49}. In brief, the complement-inactivated samples were serially diluted and applied in one column to 96-well microplate at 50 µL/well. Two columns with serial dilutions of spiked plasma were added to each plate as controls. In addition to the plates with unknown samples, one plate was dosed with six serial dilutions in duplicate of DHA (from 100 to 2.5 ng/mL). A suspension of 200 µL of malaria parasite-infected red blood cells (W2 clone; 0.05% parasitemia with > 80% rings at a 1.7% hematocrit) was added to all wells. The microplates were placed into a chamber, flushed with a mixture of gas consisting of 5% CO₂, 5% O₂ and 90% N₂, and plated into an incubator at 37°C for 72 h. HRP2-ELISA was performed as described above.

Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) Analysis

To detect baseline antimalarials prior to treatment in the study population, plasma samples were extracted by using protein precipitation 2-fold volume of acetonitrile containing internal standard, then 1-minute vortex mixing, 10 minute-centrifuged, filtered supernatant with 0.22 µm PTFE filter and then transferred to HPLC vial. The LC separation was performed on ACQUITY UPLC (Waters) coupled with tandem mass spectrometer (Xevo TQ-S, Waters) and eluted on Waters Acquity UPLC® BEH C18, 2.1 x 50 mm, 1.7 µm

column at a flow rate of 0.5 mL/minute in 8 minutes run time. Mobile phase consisted of (A) 5 mM ammonium acetate pH 4.5 in water and (B) 5 mM ammonium acetate pH 4.5 in acetonitrile:methanol (50:50 v/v). The gradient started with 10% B, raised to 98% B in 6 minutes and held at this composition for 0.5 minute, decreased to 10% B and re-equilibrated for 1 minute. Column temperature was set at 40 °C. Selective mass to charge (m/z) transition for each compound was monitored as follow: AS (407.4 > 261.23), DHA (307.13 > 261.26), MQ (379.1 > 321.0), carboxy primaquine (cMQ) (310.22 > 290.05), PG (256.1 > 171.9), cycloguanil (CYC) (251.95 > 194.9), primaquine (PQ) (260.07 > 85.95), carboxy primaquine (cPQ) (275.34 > 175.11), orthoquinone (OQ) (260.2 > 147.08), stable isotopic labeled artesunate (SIL-AS) (411.3 > 261.18), and stable isotopic labeled primaquine (SIL-PQ) (264.35 > 86.15).

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6.0 (GraphPad Software, Inc., San Diego, CA, USA). The difference of data between groups was assessed by nonparametric Kruskal-Wallis, Mann-Whitney or Dunn’s multiple comparison tests, as appropriate. Statistical significance was defined as a *P* value< 0.05.

Results

Study population, demographic, and parasitological parameters

In total, 117 individuals were enrolled but 5 individuals were excluded from analysis due to lack of *P. falciparum* on a blood smear in 4 patients and *P. vivax* monoinfection in 1 patient. Therefore, 112 individuals with uncomplicated *P. falciparum* were included in the analysis (Table 1). The median age of the participants was 23 years (IQR: 22-30.75) and most were male (98.2%), in the military (83%), and from Sisaket Province (96.4%). Among rapid diagnostic test and/or microscopy positive malarial isolates, 108 samples (96.4%) were confirmed by PCR as *P. falciparum* infections and 4 samples (3.6%) as mixed *P. falciparum* and *P. vivax* infections. The geometric mean of parasitemia of the participants was 12,097 parasites/μL.

Table 1. Patient and parasitological characteristics of 112 participants.

	2013	2014	2015	<i>P</i> value
Number of cases	27	39	46	-
Age (y), median (IQR)	22 (22-24)	23 (22-31)	23 (22-44)	0.1210
Male:female, n (%)	27:0 (100:0)	39:0 (100:0)	44:2 (96:4)	-
Civilian:Military, n (%)	1:26 (4:96)	5:34 (13:87)	13:33 (28:72)	-
Weight (kg), median (IQR)	60 (56-67)	62 (60-71)	60 (58-67)	0.698
Site location, Sisaket:Surin, n (%)	27:0 (100:0)	35:4 (90:10)	46:0 (100:0)	-
Parasitemia (no./μL), geometric mean (95% CI)	11,963 (6,698-17,944)	14, 960 (8,940-25,035)	10,704 (7,158-16,008)	0.320
<i>P. falciparum</i> , n (%)	27 (100%)	36 (92%)	45 (98%)	-
Mixed <i>P. falciparum</i> / <i>P. vivax</i> , n (%)	0	3 (8%)	1 (2%)	-

P values calculated by the Kruskal-Wallis test

Mutations in *Pfmdr1*, *Pfcr*, *Pfk13*, and *Pfcy* gene

112 samples were evaluated for SNPs of *pfmdr1*, *pfcr* (positions 72-76), *pfcr* (position 145), *pfk13*, and *pfcy* as molecular markers of drug resistance associated with MQ, CQ, PPQ, ART, and ATQ, respectively. Of four point mutations on *pfmdr1*, only the Y184F mutation (97.3%) was detected (Table 2), suggesting the presence of two *pfmdr1* haplotypes in the samples: NYSN and NFSN (representing amino acids positions 86, 184, 1034, and 1042, respectively) with NFSN as the most prevalent genotype. Genotyping of *pfcr* showed all mutant haplotypes at positions 72-76 (CVIET), which is associated with CQ resistance. Regarding the novel *pfcr* mutation (F145I) associated with PPQ resistance, no F145I mutation was identified in 2013, but the F145I mutation of *pfcr* was detected in 5 (12.8%) samples and 14 (30.4%) in 2014 and 2015, respectively. Concerning *pfk13* as associated with ART resistance,

three nonsynonymous mutations (R539T, R539I, and C580Y) in the *k13* propeller domain were observed in 112 samples and C580Y was the predominant *pfk13* mutation, accounted for 84.8% of all *pfk13* mutations. The number of C580Y mutants increased from 2013 (63%) to 2015 (100%). There were no mutations detected in *pficytb* for amino acid positions 258 and 268.

Copy Number Variation of *pfmdr1* and *pfpm2*

Single *pfmdr1* copies were present in nearly all of evaluable cases (107/112), only 5 samples had multiple copies of *pfmdr1*, found in samples collected in 2013 and 2014. Multiple *pfpm2* copies were found in about half of evaluable cases (58/112), and the number of parasites harboring multiple *pfpm2* copies steadily rose from 7.4% to 78.3% from 2013 to 2015. Of 5 samples containing multiple *pfmdr1* copies, all had single *pfpm2* copy and *pfcrf* F145I wild-type. All parasites harboring multiple *pfpm2* copies contained *pfpk13*-C580Y mutation and of those samples, 13 samples (22.4%) contained the novel *pfcrf* F145I mutation.

Haplotype and Copy number variation (CNV) of *P. falciparum* isolates

The parasite isolates were categorized into nine groups according to their genotypes of *pfmdr1*, *pfk13*, and *pfcrf* in combination with their CNV of *pfmdr1* and *pfpm2* (Table S3). Overall, the most prevalent parasites were those in group III, containing *pfk13*-580Y alleles, multiple *pfpm2* copies, *pfcrf*-F145I alleles and *pfmdr1* 184F alleles with a single copy number. This was followed by parasites in group II which was the same as Group 3 except with *pfpm2* single copy number.

The number of parasites in group II decreased from 55.6% to 13%, while those in group III increased from 7.4% to 56.5%. Parasites in group VI, containing the *pfcrf*-145I and multiple *pfpm2* copies, were also found to be increasing over the study period. It was noted that parasites in group VII to IX, harboring the *pfk13*-539I/T alleles with no novel mutation on *pfcrf*, and *pfmdr1*-184F only held a single *pfpm2* copy.

Prevalence of molecular markers for ART-, MQ- and PPQ-Resistance

With limited number of ACT options, we assessed if any parasites had all mutations for ART-, MQ-, and PPQ-resistance (*pfk13* SNPs, *pfmdr1* CNV, *pfpm2* CNV, and *pfcrf*-F145I mutation). No tested parasites in this study carried all the aforementioned markers, although this is largely driven by having only 5 isolates with multiple copies of *pfmdr1*. If that marker is excluded, only 11.6% of the isolates (13/112) harbored *pfk13*-C580Y, multiple *pfpm2* copies, and *pfcrf*-F145I (3 isolates in 2014 and 10 isolates in 2015). All of the 16 parasites with *pfk13*-R539T carried a single *pfpm2* copy number with no *pfcrf*-F145I mutation but four of them had multiple *pfmdr1* copies.

Table 2. Mutations of malaria resistance molecular markers of *P. falciparum* in Thailand between 2013 and 2015

Year	No. of tested	<i>pfmdr1</i>				<i>pfcrf</i>		<i>pfk13</i>			<i>pficytb</i>	
		N86Y	Y184F	S1034C	N1042D	CVIET (72-76)	F145I	R539T	R539I	C580Y	I258M	Y268S
2013	27	0	27 (100%)	0	0	27 (100%)	0	9 (33.3%)	1 (3.7%)	17 (63.0%)	0	0
2014	39	0	36 (92.3%)	0	0	39 (100%)	5 (12.8%)	7 (17.9%)	0	32 (82.1%)	0	0
2015	46	0	46 (100%)	0	0	46 (100%)	14 (30.4%)	0	0	46 (100%)	0	0
Total	112	0	109 (97.3%)	0	0	112 (100%)	19 (16.9%)	16 (14.3%)	1 (0.9%)	95 (84.8%)	0	0

Year	No. of tested	<i>pfmdr1</i> CNV		<i>pfpm2</i> CNV	
		single copy	multiple copy (>1.5)	single copy	multiple copy (>1.6)
2013	27	24 (88.9%)	3 (11.1%)	25 (92.6%)	2 (7.4%)
2014	39	37 (94.8%)	2 (5.2%)	19 (48.7%)	20 (51.3%)
2015	46	46 (100%)	0	10 (21.7%)	36 (78.3%)
Total	112	107 (95.5%)	5 (4.5%)	54 (48.2%)	58 (51.8)

Fig. 1 shows the prevalence of *pfpm2* copy number variation, *pfmdr1* copy number variation, *pfprt*-F145I, *pfk13*-C580Y and *pfk13*-R539T mutations over time. The prevalence of parasites with multiple *pfpm2* copies associated with PPQ resistance increased from 2013 to 2015, similar to the prevalence of parasites harboring *pfprt*-F145I and *pfk13*-C580Y mutations. In contrast, the prevalence of parasites with multiple *pfmdr1* copies associated with MQ resistance and *pfk13*-R539T mutation decreased after 2013 through 2015.

Table 3. *In vitro* susceptibility of *P. falciparum* isolates to dihydroartemisinin (DHA), artesunate (AS), artemisone (ATM), chloroquine (CQ), piperaquine (PPQ), cycloguanil (CYC), mefloquine (MQ), quinine (QN), lumefantrine (LUM), doxycycline (DOX), atovaquone (ATQ).

		Geometric Mean IC ₅₀ (nM) (95% CI)						<i>P</i> value
Drug	W2 clone	N	2013	N	2014	N	2015	
DHA	3.8 (2.9-4.8)	11	10.8 (8.8-13)	11	7.2 (5.8-8.9)	18	7.1 (5.4-9.5)	0.057
AS	4.8 (3.7-6.1)	11	8.2 (6.7-10)	11	4.8 (3.9-5.9)	18	4.9 (3.8-6.2)	0.005 ^{1,2}
ATM	1.5 (0.8-2.9)	11	1.9 (1.1-3.1)	11	0.7 (0.5-1.0)	18	0.9 (0.6-1.6)	0.021 ¹
CQ	234 (189-289)	11	89.3 (42-190)	11	157.4 (101-244)	18	228.4 (145-359)	0.025 ²
PPQ	63 (50-78)	11	58.5 (43-79)	10	75.1 (54-104)	16	406.9 (137-1206)	0.006 ²
CYC	3,314 (1,945-5,648)	8	1399 (855-2290)	6	1566 (790-3102)	18	8642 (5086-14684)	0.0008 ^{2,3}
MQ	62.1 (52-74)	11	18.3 (9.9-34)	11	35.9 (20-63)	18	67.4 (48.9-93.0)	0.0027 ²
QN	277 (232-331)	11	68.2 (42-110)	11	136.8 (99-190)	18	144.9 (113-185)	0.023 ²
LUM	3.6 (2.4-5.4)	11	3.4 (2.3-5.0)	11	6.5 (4.4-9.8)	18	4.7 (3.5-6.4)	0.052
DOX	7,580 (5,858-9,808)	11	23092 (19205-27767)	11	18491 (15206-22485)	18	14008 (10737-18276)	0.025 ²
ATQ	12.5 (7.8-20)	10	1.7 (0.9-3.2)	11	2.5 (1.6-3.8)	17	3.3 (1.8-6.0)	0.253

P values calculated by the Kruskal-Wallis test

^{1,2,3} Significant difference calculated by Dunn's multiple comparisons test of data in 2013/2014, 2013/2015, and 2014/2015, respectively

In vitro drug susceptibility of *P. falciparum* isolates.

Of 86 *P. falciparum* mono-infection samples that met the parasitemia level criteria for *in vitro* culture, *in vitro* drug susceptibility of only 40 samples (11, 11 and 18 samples from 2013, 2014, and 2015, respectively) against antimalarial drug panel could be obtained by HRP2-ELISA. (Table 3). In viewing the geometric mean (GM) IC₅₀ data for all drugs by year, CQ, PPQ, CYC, MQ, and QN all exhibited statistically significant decreases in drug susceptibility. Compared to the ART- and MQ-sensitive W2 reference clone, isolates had greatly reduced sensitivity to PPQ, CYC, and DOX, with PPQ and CYC having a noticeable drop in effectiveness between 2014 and 2015. In contrast, while there was a steady decline in CQ and MQ sensitivity from 2013 to 2015 but GM-IC₅₀ values remained lower than that of CQ-resistance W2 reference (IC₅₀-CQ = 234 nM) and MQ-resistance D6 reference (IC₅₀-MQ = 118 nM), respectively. There

was a modest increase in DHA and AS susceptibility, which did not change dramatically over the 3-year period. All parasite isolates had the IC₅₀-ATQ lower than that of the ATQ-resistance C2B reference (IC₅₀-ATQ = 13,240 nM), suggesting no ATQ-resistant phenotype. No significant changes in drug susceptibility of DHA, LUM and ATQ were observed during the period of this study.

***In vitro* Ring stage Survival Assay (RSA) and *pfk13* mutations**

To get a better understanding of ART-resistance, 40 isolates from 2013 to 2015 were tested in *in vitro* RSA_{0-3h} to measure % survival rate against DHA, and the association with *pfk13* mutations assessed (Fig. 2A). One isolate was excluded due to the growth rate between 0 and 72 hours being less than 1.5, leaving 39 isolates evaluable by RSA_{0-3h}. Survival rates for the 11 evaluable isolates from 2013 ranged from 0.84 to 44.5% (median 19.9, IQR of 3.4-30.3), for the 11 evaluable 2014 isolates the range was 3.7 to 82.8% (median 16.1, IQR of 9.9-60.9) and for the 17 evaluable isolates in 2015 ranged from 1.4 to 22.8% w (median 8.3, IQR of 2.8-22.8), whereas reference clone survival rate was as expected with medians of 0.4, 2.5, and 23.2 % for ART-sensitive W2, ART-resistant IPC-4884 and IPC-5202, respectively. In 2013, 90.9% (10/11) were deemed ART-resistant, all of which contained either *pfk13*-R539T or -C580Y mutations, while the parasite harboring *pfk13*-R539I was sensitive to ART. In 2014 and 2015, all tested parasites containing either *pfk13*-R539T or -C580Y mutation were deemed ART resistant. No significant difference in the proportion of resistant isolates between 2013 and 2014 were found ($P > 0.05$) but there was a significant decrease in median survival rate for 2015 (8.3), compared to those from 2014 (16.1) and 2013 (20.9), Mann-Whitney test, $P = 0.004$ and $P = 0.02$, respectively. It was noted that % survive rate of the parasite harboring *pfk13*-R539I was 0.84%, closer to the cut-off value.

PPQ-susceptibility of parasites with PPQ molecular markers

With the emergence of PPQ resistance associated with DHA-PPQ treatment failures identified in Cambodia from 2010-2013⁴¹, the distribution of PPQ-IC₉₀ values for parasites from Thailand (with newly emerged *pfcr*-F145I mutation with or without amplified *pfpm2*) is displayed in Fig. 2B. It was noted that PPQ-IC₉₀ results were evaluable in 11 isolates in 2013, 10 isolates in 2014, and 18 isolates in 2015. In 2013, there was only one isolate with multiple *pfpm2* copies and it had an elevated IC₉₀ value. In 2014, no significant differences in IC₉₀ values in the isolates with single and multiple *pfpm2* copies were observed ($P > 0.05$). In 2015 there was a significant difference in parasites with single (226 nM) and multiple *pfpm2* copies (62,347 nM) without *pfcr*-F145I ($P = 0.036$) whereas no significant differences in IC₉₀ values in parasites with single (53,930 nM) and multiple *pfpm2* copies (77,882 nM) with *pfcr*-F145I ($P > 0.05$). Isolates with single *pfpm2* and wild type *pfcr* had more than 10-fold lower IC₉₀s than parasites with multiple *pfpm2* and/or *pfcr*-F145I. With the exception of 1 isolate, parasites harboring *pfcr*-F145I mutation exhibited higher IC₉₀ values than those without the mutation (PPQ-IC₉₀s for standard clone W2, D6 and C2B: 124, 157, and 232 nM, respectively).

In the light of an association between PPQ resistance and haplotypes, Table S4 shows PPQ-IC₉₀s of the parasite isolates in different haplotype subgroups. It can be clearly seen that parasite isolates containing multiple *pfpm2* copies alone (group III), the *pfcr*-F145I alone (group V), or the combination of both markers exhibited noticeably high IC₉₀ values, indicative of PPQ resistance phenotype.

Preexisting antimalarial treatment

During the enrollment process, participants were queried about recent malaria infection or antimalarial drug use and also past medical history of malaria. Of 112 participants, 27 (24.1%) had reported prior malaria infection: 15 (55.6%), 7 (25.9%), 3 (11.1%), and 2 (7.4) had 1, 2, 3, and 4 episodes of malaria in the last 12 months. Thirty participants (26.8%) admitted that they had previously taken antimalarials more than 1 week. The most taken antimalarials was PQ (26.8%), followed by AS and MQ (16.1%) and CQ (10.7%). 18 participants (16.1%) had taken AS, MQ, and PQ, while 12 participants (10.7%) had taken PQ and CQ.

Many antimalarials are long-acting or participants may not disclose recent receipt of antimalarial drug; therefore antimalarial drug levels in plasma samples were determined by two independent techniques, *ex vivo* bioassay and LC-MS/MS, to assess potential effects on study results. In the *ex vivo* bioassay, 12 of 112 patient plasma samples (10.7%) evaluated against *P. falciparum* W2 clone had significant antimalarial activity (>17.6 nM DHA equivalents) (Table 4), while 89.3 % of evaluable samples were considered negative (≤ 17.6 nM). The positive results were measured in DHA activity equivalents ranging from 49.8 to 3,855 nM with a median activity of 234.2 nM (IQR of 94.3 to 751.4 nM). When LC-MS/MS was employed to detect antimalarial drugs in patient plasma samples, 21 samples (18.8%) were found to contain some antimalarial drug (Table 4). Sixteen of the 21 had MQ, first-line therapy for *P. falciparum* at the time of the study and with a half-life of 8 to 20 days. Six had PQ or cPQ; in 2013-2015 a single dose of PQ had not yet been widely used for *P. falciparum* anti-gametocyte activity but it was first-line for *P. vivax* infections. Interestingly four people had

evidence of DHA or AS which has a very short half-life (2-3 hours). When comparing both methods, 12 patient plasma samples demonstrated positivity for antimalarial drugs in both *ex vivo* bioassay and LC-MS/MS method while another 9 samples showed negative result in *ex vivo* bioassay but were positively detected under LC-MS/MS, in which 3 and 6 samples contained PQ and MQ, respectively. It was important to note that only 8 samples (38.1%) from the positive samples could be *in vitro* cultured and evaluated for their drug susceptibility, which showed no significant difference to those without the baseline medicines.

Table 4. Comparison preexisting antimalarial activity in the sample obtained by *ex vivo* bioassay and liquid chromatography-mass spectrometry (LC-MS/MS).

Sample ID	<i>Ex vivo</i> Bioassay (nM DHA equivalents)	Drugs detected by LC-MS
BA-005	79.4	MQ, cMQ
BA-006	90.6	MQ, cMQ
BA-028	105.6	MQ, cMQ
BA-032	-	MQ, cMQ
BA-046	3,855	DHA, MQ
BA-047	-	PQ, cPQ
BA-048	207.5	MQ, cMQ
BA-053	-	PQ, cPQ
BA-055	-	MQ, cMQ
BA-057	49.8	MQ, cMQ
BA-059	260.9	AS
BA-064	758.1	AS,DHA, PQ, cPQ
BA-066	731.3	AS, DHA, MQ
BA-068	-	MQ, cMQ
BA-072	-	PQ, cPQ
BA-078	191.4	MQ, cMQ
BA-084	840.4	DHA,PQ, cPQ, MQ, cMQ
BA-087	468.4	PQ, cPQ, MQ, cMQ
BA-095	-	MQ, cMQ
BA-103	-	MQ, cMQ
BA-110	-	MQ, cMQ

Discussion

Even though the Greater Mekong Subregion (GMS) has long been the epicentre of antimalarial drug resistance, these countries are aiming to achieve malaria elimination by 2030⁵⁰. At the time this study was enrolling volunteers, the only validated molecular markers for ACTs were *pfmdr1* but by time of writing this manuscript we developed assays to delineate markers and *in vitro* assays to ART, PPQ, and ATQ resistance. As the number of malaria cases in the GMS continues to decline following aggressive malaria control efforts, conducting *P. falciparum* therapeutic efficacy studies has become more difficult. Therefore, current efforts have to rely more on the surveillance of molecular markers in concert with *in vitro* data. This type of intensive surveillance of molecular and *in vitro* data with a minimum of 6 week patient follow up is effective and easily adapted to mobile populations such as military where the majority of volunteers enrolled. Similar approaches can be applied to migrants and occupations where follow up post treatment is difficult,

including workers in forestry, agriculture or animal husbandry and refugee populations. Despite the limitations of the study, where clinical treatment outcomes were not readily available, we were able to characterize the changes in drug resistance overtime in this difficult to reach population. Since most cases of malaria in Thailand occur in border regions, areas where a military presence is required, Thai soldiers stationed at these locations are at increased risk of developing multidrug resistant malaria. There has been limited information published on drug resistance in the military cohorts and this provides new insights.

Chloroquine resistance was first reported in the GMS since the 1950s⁵¹, and several groups⁵²⁻⁵⁶ have characterized CQ resistant parasite lineages as one of four mutant *pfprt* genotypes at positions 72-76 (CVIET, SVMNT, CVMNT, and CVMET; mutation underlined), with the SVMNT genotype being detected in Brazil/Peru and Melanesia lineages^{57,58}. Mutations in *pfprt* are associated with CQ, AQ and LUM resistance; especially, the *pfprt* 72-76 CVIET and SVMNT haplotype. The 76T point mutation is the key marker, while SVMNT haplotype is required for AQ resistance^{53,55}. The present results show that all collected parasite isolates harbored the *pfprt* 72-76 CVIET haplotype similar to previous reports⁵⁹⁻⁶¹. Even though CQ sensitivity of the parasite isolates was still slightly higher than that of the CQ-resistance W2 reference clone, when the geometric mean IC₅₀s of the CQ-sensitive isolates of 30.1 nM⁶² was applied, all the parasites collected under this study would be deemed CQ-resistant. Decreased CQ sensitivity was observed from 2013 to 2015 that can likely be attributed to continued CQ use in Thailand for treatment of *P. vivax*.

Parasite isolates carrying the artemisinin resistant gene have been reported in Thailand^{61,63}. Some *pfk13* mutations (F446I, N458Y, M476I, Y493H, R539T, I543T, P553L, R561H and C580Y) were validated for conferring artemisinin resistance⁶⁴, while several other mutations may be deemed candidates. The current study showed that the *pfk13*-C580Y mutation was predominant, rising from 63% in 2013 to 100% in 2015. These findings are in good agreement with those reported by others^{42,61} in that several *pfk13* mutations developed in Sisaket (62% 580Y)⁴² but by 2015 the C580Y mutation became the sole predominant mutation found⁶¹. This pattern was not followed by other regions of Thailand. In southern Thailand (Yala Province) very few isolates with *pfk13* mutations have been detected^{61,65}. The *pfk13*-R539T has been previously identified in Thailand, Cambodia, Laos, and Vietnam⁶⁶⁻⁶⁹; however, the *pfk13*-R539I found in this study has not yet been reported in Thailand. The *pfk13*-R539I mutation was previously observed at 0.9% in Ghanaian isolates⁷⁰. As assessed by the RSA_{0-3h}, the R539I mutation appears not to be associated with ART resistance. The R539I mutation provided the lower stabilization score, implying an effect on the protein structure and function⁷⁰. All the collected isolates also had a wild-type cytochrome b gene (*pfcytb*) at both codons 258 and 268 (those associated with atovaquone/proguanil treatment failure^{36,38,71}) which is consistent with other isolates collected along Thai-Myanmar and Thai-Cambodia borders during 1998-2005⁷². Additionally, no noticeable increase in atovaquone IC₅₀ of parasites from this study was observed in comparison to the previous report, suggesting that atovaquone/proguanil can still be utilized for treatment and prophylaxis of multidrug-resistant *P. falciparum* malaria in Thailand. Of all the *pfmdr1* SNPs analyzed, allelic variation was only observed in *pfmdr1* position 184. This is in good agreement with the previous report by Thita *et al.*⁷³, in which 89% of *P. falciparum* isolates from Thai-Cambodia border from Chanthaburi and Trat province from 1988 to 2016 had the *pfmdr1* 184F allele. Previous studies in Uganda and Bioko Island suggest that this allele may play a certain role in mediating resistance to some antimalarials^{74,75}, and while in the current study, decreased susceptibility to QN, CQ, and MQ was observed, causality cannot be established. Different results were observed from the *P. falciparum* isolates from the southern part of Thailand, with the *pfmdr1* 86Y allele significantly more common⁷⁶.

In addition to SNPs in established malaria resistance markers, *pfpm2* and *pfmdr1* copy numbers were quantified as associated with PPQ and MQ resistance, respectively. The increased trend in multiple *pfpm2* copy numbers were clearly seen with a decline in multiple *pfmdr1* copy numbers. Novel mutations of the *pfprt* gene downstream of the 4-aminoquinoline resistance locus (positions 72 to 76), including T93S, H97Y, F145I, I218F, M343L, or G353V, were recently shown to be associated with PPQ resistance^{30,32,33}. From this study, there was a relatively rapid rise in the F145I mutation, from 0% in 2013 to 30% in 2015. It cannot be ruled out that other novel *pfprt* mutations may have been present in the RTA samples presented here, since they were not studied. In this study, IC₉₀ of PPQ were employed to investigate the PPQ resistant phenotypes associated with the tested genetic markers. Parasite isolates holding either multiple *pfpm2* copies with *pfprt*-F145 or the combination of single or multiple *pfpm2* copy numbers with *pfprt*-F145I mutation exhibited the elevated IC₉₀ values of PPQ. Some parasite isolates harboring multiple *pfpm2* copy numbers without *pfprt*-F145I mutation seemed to have a broad IC₉₀ values (203 to 5,376 nM), while parasite isolates with a single *pfpm2* copy number had the IC₉₀ values around 71 to 1,183 nM. The high IC₉₀ values of parasite isolates with only multiple *pfpm2* copy numbers could be stemmed from other novel *pfprt* mutations that were not detected in this study. Previous studies have shown that the overexpression of *pfpm2* and *pfpm3* in the 3D7 genetic background did not alter the sensitivity of *P. falciparum* to PPQ, suggesting that the increase in *pfpm2*

copy number alone is not the sole modulator of PPQ resistance^{47,77}, the initial selection of *pfpm2* and *pfmdr1* copy number variations developed a genetic background important for novel *pfcr*t mutations to emerge⁷⁸. The increased *pfpm2* copy numbers was also accompanied with the escalated numbers of *pfk13*-C580Y and *pfcr*t-F145I mutations.

Overall, from 2013-2015, *P. falciparum* isolates collected from the military population demonstrated progression from 62% to 100% 580Y *pfk13* mutations, all with increased survival rates by RSA. Regarding ACT partner drugs, there was a decrease in multiple *pfmdr1* copy numbers from 11% to 0% and a corresponding increase of multiple *pfpm2* copy numbers or multiple *pfpm2* copy numbers with *pfcr*t-F145I mutation from 7.4% to 78.3%. The first-line antimalarial used during this time was AS-MQ, so the drive toward increasing PPQ resistance likely came from cross-border mixing of parasite populations from Cambodia. In 2013, an AFRIMS study in Anlong Veng, Cambodia, 12 km from the Cambodia-Thai border at Sisaket province, showed a similar rate of 65% C580Y *pfk13* mutant⁴¹. When the 54% failure rate of DHA-PPQ was observed in Anlong Veng in 2013⁴¹, the molecular markers for neither ART nor PPQ were available so it is only retrospectively that the association of molecular markers and *in vitro* data can be seen. Similarly in Sisaket, the 87% failure rate of DHA-PPQ from the TRACII study (2015-2018)⁶³ may have been predicted from these surveillance data.

The benefits of having information about past medical history of malaria can help interpret *ex vivo/in vitro* drug testing results obtained from study samples. It also helps national programs better understand how often anti-malarial drugs are being used within communities and if this use is outside regulated dispensing of drugs by health care providers. The use of previous antimalarial drugs or preexisting antimalarial activity in patients can have an effect on malaria treatment⁴⁶. A bioassay method was developed for the measurement of antimalarial activities of ART and its derivatives in either the plasma or sera of patients^{79,80}, while bioanalytical LC-MS/MS has widely been used to detect several drugs^{81,82}. In this study, results from bioassay demonstrated a good correlation to LC-MS/MS determination for blood stage antimalarial activities. The factors that limit the detection of the two techniques may lie on the drug metabolites and the amount of the drugs in the samples. For example, 3 samples found positively in LC-MS/MS method contained PQ, the erythrocytic activity of which is little, hence not detected in *ex vivo* bioassay. Another 6 samples, on the other hand, were found negative in *ex vivo* bioassay but positive in LC-MS/MS method with the presence of MQ and cMQ at the concentration lower than 150 ng/ml. These results suggested that bioassay was able to screen blood stage antimalarial activity in certain levels but could not specify which drugs or metabolites are present in the patient plasma samples whereas LC-MS/MS method could. Therefore, it will be fruitful to develop LC-MS/MS method to simultaneously screen multiple antimalarial drugs and their metabolites in a single run.

The difficulty of choosing a national first-line therapy in the GMS due to differing patterns of antimalarial resistance as illustrated. High rates of AS-MQ failures along the Thai-Myanmar border^{16,83} resulted in the first-line treatment being changed to DHA-PPQ in 2015. However, the parasites from this high risk mobile military population had patterns developed over three years which were more similar to isolates in Cambodia, and then manifested a similar drop in DHA-PPQ efficacy. The Thai government responded to that regional challenge and since 2019, the first-line treatment for *P. falciparum* has been changed to artesunate-pyronaridine (AS-PND) in Sisaket and Ubon Ratchathani Provinces. Recent reports suggest there are two lineages within the GMS. For *pfk13* genotypes, the C580Y mutation predominates in the eastern GMS and the F466I mutation has spread along the Myanmar border⁶¹. Similarly, *pfpm2* gene amplification has been found in northeastern Thailand near Cambodia border, but not in western and southern Thailand⁶¹. It is interesting to note that *P. falciparum* isolates from eastern Greater Mekong has different mechanism of *pfpm2* amplification from those in Myanmar as judged by the sequences flanking the amplified region⁶¹. While there may be predominant lineages which have emerged, continued local surveillance will be important to determine if and how these patterns shift. The emergence of the novel *pfcr*t-F145I in 2014 from this study followed the emergence and DHA-PPQ treatment failures in Cambodia^{41,46,84}. Shrestha, *et al.*⁸⁵ showed that more than 98% of the parasites collected from northern Cambodia carried newly emerged *pfcr*t mutations and after 2014, the prevalence of parasites with *pfcr*t-F145I mutation started to decrease, being out-competed by other less resistant, but more fit *pfcr*t variants such as *pfcr*t-T93S and I218F³³. Recently, Imwong, *et al.*⁶¹ has reported that *pfcr*t-G353V was detected in 7 of 142 isolates and 7 of 38 isolates after mass drug administration with DHA-PPQ in Cambodia. Understanding the movement of human populations especially along border areas in migrants and militaries, with ongoing surveillance programs can help predict drug efficacy or first detect any introduction of novel mutations which may interrupt the path to malaria elimination in the GMS.

Conclusion

Altogether, data from the molecular, *in vitro* drug susceptibility and survival assay indicate the increased trend of *pfk13*-C580Y mutation, multiple *pfpm2* copy numbers and *pfcrf*-F145I which is indicative of ART and PPQ resistance of Thai *P. falciparum* isolates collected near the Cambodia border, at the same time when DHA-PPQ treatment failures in Cambodia were on the rise. The sharp rise of IC₉₀ values was found to be linked to the presence of multiple *pfpm2* copies and *pfcrf*-F145I mutation, whereas the *pfk13*-R539I mutation was not associated with ART resistance according to RSA. The observed drop in PPQ, MQ, and CYC sensitivity could be an alarming issue for areas where treatment options remain limited.

Declarations

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Author's contributions

DLS, KJ and JG conceived, designed and executed the study. NB, PV, PL, PG, DU, WR, CL, DW, NS, SC2, WK, BAV, MDS, MMF, DLS, PLS, MW, and NCW conceived, designed and supported the study. NU and MA were in charge of sample collection and transportation. CT, SS, SC1, CP, WF performed cell culture, drug susceptibility assay, survival assay and bioassay. KK, CC and PS performed molecular genotyping. WT performed LC-MS/MS. NB and PV initially analyzed the data and all authors assisted in interpreting the data. NB wrote the first draft. All authors edited, reviewed and approved the final manuscript.

Disclaimer

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Ethics approval and consent to participate

All participants or guardians provided written consent and samples were collected under approval Royal Thai Army Institutional Review Board (RTA IRB) and Walter Reed Army Institute of Research Institutional Review board.

Consent for publication

Not applicable

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Competing Interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Prevalence of antimalarial drug resistance mutations over time in Thailand from 2013-2015. Bars indicate the prevalence of parasites with single (blue) and amplified (grey) pfpm2 copy number as a marker of PPQ resistance. The red line indicates the prevalence of parasites harboring pfcr1-F145I mutation associated with PPQ resistance. The orange line indicates the prevalence of amplified pfmdr1 copy number, as a marker of MQ resistance. The black and green lines indicate the prevalence of parasites harboring pfk13-C580Y and pfk13-R539T mutations as markers of ART resistance. Noted that the national treatment guidelines in Thailand from 2013 to 2015 recommended AS-MQ for *P. falciparum* malaria

Figure 2

In vitro characterization. (A) RSA and pfk13 mutations. RSA0-3h survival rate for standard laboratory-adapted clones (W2 for ART-sensitive control, IPC-4884 and IPC-5202 for ART-resistance control) and culture-adapted clinical isolates. The dashed line represents the 1% survival rate cut-off that differentiates ART-resistance ($\geq 1\%$ red-dashed line) from ART-sensitive ($< 1\%$) parasites in RSAs. Median and interquartile ranges are shown. White, green, blue, orange, and pink circles represent parasites harboring pfk13-WT, Y493H, C580Y, R593T, and R593I, respectively. (B) IC₉₀ to PPQ in parasites with pfcr-F145I mutation, with and without pfpm2 amplification. Copy number variations of pfpm2 are shown on the x-axis, and PPQ IC₉₀ is shown on the y-axis (log₁₀ scale). Red circles represent parasites harboring pfcr-F145I mutation. Bars represent median and interquartile range.