Evolution of multidrug resistance in *Plasmodium falciparum*: a longitudinal study of genetic resistance markers in the Greater Mekong Subregion

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Abstract

Increasing drug resistance in *Plasmodium falciparum* to artemisinins and their ACT partner drugs jeopardises effective antimalarial treatment. Resistance is worst in the Greater Mekong Subregion. Monitoring genetic markers of resistance can help to guide antimalarial therapy. Markers of resistance to artemisinins (*PfKelch* mutations), mefloquine (amplification of *P. falciparum* multidrug resistance-1, *PfMDR1*), and piperaquine (*PfPlasmepsins2/3* amplification and specific *P. falciparum* chloroquine resistance transporter, *PfCRT*, mutations) were assessed in 6,722 *P. falciparum* samples from Vietnam, Lao PDR, Cambodia, Thailand, Myanmar between 2007 and 2019. Against a high background prevalence of *PfKelch* mutations, *PfMDR1* and *PfPlasmepsins2/3* amplification closely followed regional drug pressures over time. *PfPlasmepsins2/3* amplification preceded piperaquine-resistance associated *PfCRT* mutations in Cambodia, and reached a peak prevalence of 23/28 (82%) in 2015. This declined to 57/156 (38%) after changing first-line treatment away from dihydroartemisinin-piperaquine to artesunate-mefloquine (ASMQ) between 2014 and 2017. *PfMDR1* amplification increased from 0/293 (0%) between 2012 and 2017 to 12/156 (8%) in 2019. Amplification of *PfMDR1* and *PfPlasmepsins2/3* in the same parasites was extremely rare (4/6,722; 0.06%) and dispersed over time. Mechanisms conferring mefloquine and piperaquine resistance may be counterbalancing. This supports the development of ASMQ plus piperaquine as a triple artemisinin combination therapy.

Keywords: *Plasmodium falciparum*, genetic resistance markers, Greater Mekong Subregion

Introduction

Early diagnosis and treatment together with vector control comprise the cornerstone of effective malaria control. Early effective treatment of *P. falciparum* infections prevents progression of the disease to severe malaria, which still kills over 400,000 lives every year (1). The first-line treatment of uncomplicated falciparum malaria in all malaria endemic countries is with artemisinin combination therapies (ACTs). New compounds are not expected to reach the market before 2026 (2). It is therefore of great concern to regional and global malaria elimination initiatives that artemisinin resistant *P. falciparum* has emerged and spread in the Greater Mekong Subregion (GMS) and, more recently, has emerged independently in Guyana, Papua New Guinea, Ethiopia, Uganda and in particular in Rwanda, where its prevalence has increased over recent years (3, 4). In artemisinin resistant *P. falciparum* infections, the malaria parasites are still cleared after ACT treatment but, because of the loss of ring stage susceptibility, parasite killing is reduced and
clearance is slower. As a result, the artemisinin component of the ACT contributes less to the antimalarial effect and efficacy becomes more dependent upon the partner drug. There are currently six ACTs recommended; artesunate-sulfadoxine-pyrimethamine, artemether-lumefantrine (AL), artesunate-amodiaquine (ASAQ), artesunate-mefloquine (ASMQ), dihydroartemisinin-piperaquine (DHA-PPQ) and most recently artesunate-pyronaridine. When susceptibility to the partner drug declines, ACT efficacy drops significantly, and the proportion of recrudescent infections increases. This has been the pattern observed in the GMS over the last decade, where all six recommended artemisinin-based combination therapies have shown reduced efficacy at some point in time (5).

Increasingly, molecular genetic markers for antimalarial drug resistance have been identified which facilitates monitoring the emergence and spread of resistance. Currently, reliable molecular markers are available for *P. falciparum* resistance to artemisinins (mutations in the propeller region of *PfKelch*), sulfadoxine-pyrimethamine (mutations in dihydrofolate reductase, *PfdHFR*, and dihydropteroate synthase, *PfdHPS*), mefloquine (amplification of multidrug resistance-1, *PfMDR1*), and piperaquine (amplification of *PfPlasmepsin2/3* and specific *P. falciparum* chloroquine resistance transporter, *PfCRT*, mutations). Molecular markers explaining the majority of the variance in susceptibility for the other partner drugs, lumefantrine, amodiaquine and pyronaridine are not well established. For some of the drugs, molecular markers can also monitor the evolution of increasing levels of resistance. For sulfadoxine-pyrimethamine the sequential accumulation of mutations in DHPS and DHFR confer increasing levels of resistance against the two synergistic components (6, 7).

We here describe the evolution of markers of antimalarial drug resistance over time, and the observed combinations of antimalarial resistance markers, in a large set of *P. falciparum* samples obtained from the GMS countries, Vietnam, Lao PDR, Cambodia, Thailand, Myanmar from 2007 to 2019. These observations provide additional insight into the development of mefloquine and piperaquine resistance in the region, and show the very low prevalence of concomitant resistance markers to both mefloquine and piperaquine. This has a direct impact on strategies for drug combinations and deployment.

**Materials and Methods**

**Sample collection and processing**

As part of studies on the treatment, epidemiology and the targeted elimination of artemisinin resistant malaria (Trial registration numbers NCT01350856, NCT02453308, NCT03384498, NCT03355664 and NCT01872702) venous blood samples, filter paper blood spots, and completed rapid diagnostic test strips were collected from patients with microscopy or rapid test confirmed uncomplicated falciparum malaria, and also from healthy subjects in villages where targeted malaria elimination activities were planned. The study sites in Myanmar, Thailand, Cambodia, Lao PDR and Vietnam, were part of large multinational observational and treatment studies in patients with falciparum malaria (TRAC I and TRAC II), or part of large malaria prevalence surveillance as part of malaria elimination studies. Full details of these...
clinical and epidemiological studies have been published previously, and part of the raw data used for this study are included in these publications (8-13). Approvals for the studies were obtained from the Ethical Review Boards of the Faculty of Tropical Medicine, Mahidol University (MUTM 2017-045-03, MUTM 2011-015-01) and University of Oxford Tropical Medicine Ethics Committee Protocol Number: 527-17, 06-11, 1017-13, 1015-13, 32-17; Department of Medical Research, Ministry of Health (Myanmar); the Lao National Ethics Committee for Health Research; and the National Ethical Committee for Health Research, in Cambodia.

DNA was extracted from dried blood spots, completed rapid diagnostic test strips (both stored desiccated at room temperature), and frozen whole blood samples by standard methods at the Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand. DNA purification was performed using a QIAgen kit™ (QIAgen, Germany) according to the manufacturer’s instructions.

Assessment of mutations in PfKelch and PfCRT

Polymorphisms in the PfKelch gene were examined by nested PCR amplification covering the propeller region of the gene as described previously (8), followed by sequencing of the gene using an ABI Sequencer (Macrogen Inc, South Korea). The sequencing results were then aligned against the PfKelch gene of the reference strain 3D7 (putative PF13_0238 NCBI Reference Sequence (3D7): XM_001350122.1). Analysis was performed with Bioedit software (Abbott, CA, USA).

PfCRT was amplified from the DNA template using nested PCR. A PCR-restriction fragment length polymorphism assay was developed to assess PfCRT mutations related to piperaquine resistance identified in a previous study (14). These included the following SNPs: N88K, T93S, H97Y, F145I, I218F, CVMNK72-76CVIET, N326S, M343L, G353V, I356T, and R37I. Digestion fragments were analyzed on a 3% agarose gel. For quality control a random third of all PCR products were sent for DNA sequencing at Macrogen Inc, South Korea.

Assessment of PfPlasmepsin2/3 and PfMDR1 gene amplification

PfPlasmepsin2/3 and PfMDR1 copy number copy number were quantified using relative quantitative real-time PCR using Taqman™ real time PCR on a Corbett Rotor-Gene™ Q (Corbett Research, Australia). Primers and probes have been described previously (15, 16). Amplification was performed in triplicate on a total volume of 10 µL as multiplex PCR using Quantitect Multiplex PCR no ROX (QIagen, Germany). Copy number estimates were calculated using the formula: estimated copy number $= 2^{\Delta \Delta Ct}$; with $\Delta \Delta Ct$ denoting the difference between $\Delta C_t$ of the unknown sample and $\Delta C_t$ of the reference sample. Reactions were repeated whenever the profile did not conform to exponential kinetics, or if the standard deviation of the $\Delta \Delta Ct$ values was >1.5, or the Ct value of the PCR reaction was >35. To confirm amplification and to resolve indeterminate results, samples passing these criteria but with an estimated copy number >1.3 were also re-tested once, the last result counting as final. For the main analysis, a cut-off copy number estimate of 1.5 was used to
distinguish single from multiple copy $PfPlasmepsin2/3$ and $PfMDR1$ gene carriage, as used in previous studies (17-19). In addition, we defined the cut-offs for the 90% probabilities that the copy number estimate denotes a single versus multiple copy number of the gene. These probabilities were based on the distributions of the results obtained by the formula calculating the estimated copy number (Supplementary figure 1). This approach acknowledges that values around the cut-off will include the tail ends of the distributions of copy number estimate values representing $P. falciparum$ samples carrying one versus two copy copies of the $PfPlasmepsin2/3$ or $PfMDR1$ genes. For this assessment of adapted cut-off values, samples carrying multiple $P. falciparum$ clones were excluded. Using this approach, for $PfPlasmepsin2/3$ values between 1.14 and 1.52 and for $PfMDR1$ values between 1.15 and 1.61 were considered indeterminate i.e. distinction of single from multiple copy numbers of the gene was uncertain (Supplementary figure 1). For 2710 samples, $PfPlasmepsin2/3$ amplification was confirmed in 595/675 (88%) using this alternative method. For estimates <1.14, the 90% probability cut-off for a single gene copy number, and for indeterminate values between 1.14 – 1.52, the proportion of $PfPlasmepsin2/3$ amplified parasite according to presence of the breakpoint SNP were 75/1734 (4%) and 114/301 (38%), respectively.

Results

$PfPlasmepsin2/3$ and $PfMDR1$ gene amplification and PfKelch mutations

$PfPlasmepsin2/3$ and $PfMDR1$ copy numbers were assessed in 6,722 $P. falciparum$ samples from the Greater Mekong Subregion between 2007 and 2019; from Cambodia (n=649), Laos (n=1332), Thailand (n=666), Myanmar (n=3925) and Vietnam (n=150). $PfKelch$ genotyping was performed in 3,848 of these samples; the remainder of samples had insufficient parasite DNA left after the copy number assessments. Multiple copy number in $PfPlasmepsin2/3$ was observed in 571 out of 6,722 (8.5%) samples, most of which, 519/2,293 (22.6%), were from the Eastern GMS (Vietnam, Lao PDR, Cambodia, North-Eastern Thailand). The prevalence was substantially lower in the Western GMS (Myanmar and Western Thailand); 52/4,429 (1.2%); p=0.00001. Gene amplification in $PfMDR1$ was observed in 321 out of 6,722 (4.8%) samples, of which 78/2293 (3.4%) were from the eastern GMS. Mutations in the propeller region of $PfKelch$, after amino acid position 440, were found in 2116 out of 3,848 (55.0%) samples, of which 555/761 (72.9%) were in the Eastern GMS. In the Eastern GMS, 432/555 (76.2%) of $PfKelch$ mutants carried the C580Y mutation. In Cambodia between 2007 and 2017, 68/69 (99%) of the $PfPlasmepsin2/3$ amplified parasites also carried a $PfKelch$ propeller region mutation; after the switch to ASMQ as first-line treatment this proportion reduced to 61/88 (69%) (Figure 1).

The prevalence of $PfMDR1$ and $PfPlasmepsin2/3$ amplification in each country over time was associated with the concurrent first-line antimalarial drug treatment in those countries. In Myanmar, where first-line therapy is with AL, the
prevalence of PfMDR1 (144/3925; 3.7%) and PfPlasmepsin2/3 amplification (50/3925; 1.3%) remained low. In Cambodia, the prevalence of PfMDR1 amplification during the period of first-line treatment with ASMQ reached 16/85 (19%) (Figure 1), but PfMDR1 amplified parasites disappeared after ASMQ was replaced with DHA-PPQ. After deployment of DHA-PPQ, the prevalence of PfPlasmepsin2/3 amplified parasites increased to 23/28 (82%) in 2015. After the slow transition back to ASMQ, starting from 2014, this proportion then declined to 57/156 (37%) in 2019. The prevalence of parasites carrying multiple copy number of PfMDR1 increased from 0/293 (0%) between 2012 and 2017 and after full redeployment of ASMQ since 2017 back to 12/156 (8%) in 2019.

Using the conventional cut-off of 1.5 to denote gene amplification, concomitant PfPlasmepsin2/3 and PfMDR1 amplified parasites was very rare (Figure 2) and was observed in only four isolates; 4/6,722 (0.06%): one sample from Pailin collected in year 2008 (1.58 and 1.76 PfPlasmepsin2/3 and PfMDR1 copies, respectively) and one sample from Pursat, Cambodia in year 2019 (2.30 and 1.78 PfPlasmepsin2/3 and PfMDR1 copies, respectively), and another two from Kayin State, Myanmar (2/6722; 0.02%) collected in 2016 (1.51 and 1.55, 2.08 and 2.72 PfPlasmepsin2/3 and PfMDR1 copies, respectively). Applying the stricter cut-off value, corresponding to a 90% chance of a real amplification of these genes, the number of concomitant multiple copy numbers of PfPlasmepsin2/3 and PfMDR1 was reduced to 3 parasite samples (0.04%) collected in 2008, 2016 and 2019.

PfCRT mutations in relation to PfPlasmepsin2/3 and PfMDR1 amplification

The PfCRT mutations associated with piperazine resistance were tested in 536 P. falciparum isolates, collected between 2007 and 2019 from Cambodia (n=478) and Vietnam (n=58). PfCRT mutations were found at positions T93S (12.7%, 68/536), H97Y/L (23.5%, 126/536), F145I (6.0%, 32/536), I218F (4.9%, 26/536), M343I/L (2.6%, 14/536), and G353V (5%, 27/536). In addition, the CVIET haplotype without any other PfCRT mutation was observed in 208/537 (38.81%) parasites; the CVIDT haplotype without other mutations was found in 47/536 (8.77%) and the CVNMN haplotype without other mutations was found in 1/536 (0.2%). Double mutations of PfCRT (not including the CVIET, CVIDT and CVNMN haplotypes) were not observed, except in 13 samples with multiple clone infections (supplementary figure 2.3 and 4).

The prevalence of novel piperazine resistance-associated PfCRT mutations increased over time (Figure 1). In 2007, 11% (5/42) of P. falciparum samples showed PfPlasmepsin2/3 gene amplification, whereas no parasites had one of the novel piperazine resistance-associated PfCRT mutations. After that, the prevalence of PfPlasmepsin2/3 amplification increased to 25/100 (25.0%) in 2012 and 9/9 (100%) in 2017, together with an increase in novel PfCRT mutations to 16/100 (16.0%, of which 8 H97Y/L, 6 I218F, 1 M343I/L, 1G353V) in 2012 and 7/9 (78%, of which 3 T93S, 2 H97Y/L, 2 I218F) in 2017. There was a strong association between an amplification of PfPlasmepsin 2/3 and the presence of the downstream PfCRT mutations ($r^2 =0.89$). Of the parasites carrying one of the novel PfCRT mutation, 216/293 (73.7%) also carried multiple copy numbers of PfPlasmepsin2/3.
Out of 478 samples with complete data, we observed only a single sample (0.21%, 1/478), collected in year 2018 from northern Cambodia, harbouring a piperaquine resistance-associated PfCRT mutation together with amplification of PfMDR1 (Figure 3). This sample showed a single copy number of PfPlasmepsin2/3. In this study, we did not identify a single sample showing a combination of all 4 resistance genes (PfKelch, PfPlasmepsin2/3 and PfMDR1 amplification, and a piperaquine resistance-associated PfCRT mutation, Figure 3).

Discussion

This study in the GMS analysed the temporal trends in the prevalence of molecular markers for mefloquine resistance (PfMDR1 amplification) and piperaquine resistance (PfPlasmepsin2/3 amplification and novel piperaquine resistance associated PfCRT mutations). As most resistance mechanisms confer a fitness disadvantage, the prevalence of resistant parasites and their evolution with the spread of increasingly fit lineages depends on the drug pressures on the parasite population. The results of this molecular epidemiology study thus need to be interpreted in the context of different drug use between countries and changes over time. Cambodia introduced ASMQ as first line antimalarial treatment in 2000, then changed to DHA-PPQ in 2010, and subsequently gradually changed back to ASMQ from 2014 to 2017. Vietnam deployed ASMQ until 2005 and then moved to DHA-PPQ until 2020, when 4 provinces changed to artesunate-pyronaridine and another 2 provinces in 2021. In Myanmar and Lao PDR, AL remains first-line treatment, with limited use of DHA-PPQ in Myanmar. Thailand used ASMQ as their first line treatment until 2015, when it changed to DHA-PPQ. As with adjacent Cambodia, Northeastern Thailand switched back to ASMQ in 2019.

In Myanmar and Lao PDR, with low or absent levels of drug pressure from either mefloquine or piperaquine, the prevalence of parasites carrying either amplified PfMDR1 or PfPlasmepsin2/3 still remains very low in this study. In Western Thailand and Cambodia, the prevalence of PfMDR1 multiple copy numbers increased significantly during deployment of ASMQ, and was associated with high failure rates in patients with combined artemisinin and mefloquine resistant infections (21). In Cambodia, PfMDR1 multiple copy numbers rapidly declined after the change in first-line therapy towards DHA-PPQ in 2010. This has been confirmed in several studies (13, 15, 22, 23) and is explained mainly by the fitness costs associated with PfMDR1 gene amplification in the absence of mefloquine drug pressure (16). This has also been shown in in-vitro cultures (24). In Cambodia and Vietnam amplification of PfPlasmepsin2/3 increased with the deployment of DHA-PPQ, and increased much more rapidly in parallel with the increasingly high treatment failure rates with DHA-PPQ observed since 2013 (25, 26). In addition to PfPlasmepsin2/3 amplification, novel mutations in the PfCRT gene, in addition to the chloroquine resistance related K76T mutation (in the CVIET, CVIDT and CVMNK haplotypes), have been associated with piperaquine resistance. These ‘downstream’ novel mutations are closely linked to, and were preceded by, amplification of PfPlasmepsin2/3 as shown in Figure 1. In patients with P. falciparum infections carrying multiple PfPlasmepsin2/3 copies and one of the PfCRT H97Y, F145I, or G353V mutations, treatment failure rates were higher after treatment with DHA-PPQ than in infections with only PfPlasmepsin2/3 amplified parasites (16). The role of
PFPlasmepsin2/3 amplification in piperaquine resistance is still uncertain. Ex-vivo drug sensitivity testing of
PFPlasmepsin2/3 amplified parasites showed increased resistance to piperaquine in a bespoke piperaquine survival assay
(27). However, gene edited P. falciparum parasites with multiple PFPlasmepsin2/3 copies and over-expression of
PFPlasmepsin2, do not show increased piperaquine resistance (28). In contrast, gene edited parasites with PF CRT H97Y,
F145I, M343L, or G353V mutations are resistant to piperaquine in-vitro (14). PFPlasmepsin2/3 amplification could still
play an indirect role in piperaquine resistance, but this is currently unclear. The strong selective sweep of a single
PFKelch C580Y containing P. falciparum lineage in the eastern GMS under DHA-PPQ drug pressure was likely initially
driven by artemisinin resistance. The predominance of this lineage increased rapidly after 2009 in countries deploying
DHA-PPQ, acquiring amplification of PFPlasmepsin2/3, and only more recently the novel PF CRT mutations (9). This
sequence of event is also supported by a detailed genomic epidemiological study from the same area using whole
genome sequencing showing initial spread of a P. falciparum PFKelch C580Y and PFPlasmepsin2/3 amplified co-lineage,
which then diversified and acquired one of the novel PF CRT mutations (29). All studies thus far found that the novel
piperaquine-associated SNPs in PF CRT are mutually exclusive.

Both artemisinin and piperaquine resistance contributed to the high DHA-PPQ failure rates in Cambodia and Vietnam.
Piperaquine resistance alone is associated with recrudescence rates of approximately 20% assessed 42 days after
treatment with DHA-PPQ, compared to 45% in P. falciparum infections with both artemisinin and piperaquine resistance
(23). Recrudescence resistant infections are overall more transmissible, which drives their spread (30). Artemisinin
resistant infections have high rates of gametocytosaemia and may be more transmissible even before treatment failure
rates begin to rise (8). After withdrawal of DHA-PPQ in Cambodia from 2016, the prevalence of PFPlasmepsin2/3
amplified parasites declined from close to 100% to 38%, but these parasites have not disappeared, in contrast with the
complete disappearance of PF MDR1 amplification after mefloquine withdrawal. This may suggest a lower fitness cost in
parasites carrying multiple PFPlasmepsin2/3 copy numbers, or some other unidentified advantage not associated with
piperaquine resistance. The relative fitness of P. falciparum carrying multiple copies of PFPlasmepsin or the novel PF CRT
mutations has not been established. Alternatively, the currently very low level of multiplicity of malaria infection may
allow relatively unfit parasites to persist in the absence of competition. Concomitant PF MDR1 amplification and PF CRT
mutations associated with piperaquine resistance was not observed, except in one parasite strain with a single
PFPlasmepsin2/3 copy number. The presence of parasites carrying amplified genes of both PF MDR1 and PFPlasmepsin2/3
was also very rare, with only single cases observed in 2008, 2016 and 2019, and an overall prevalence of 0.06% (4/6,722)
or 0.04% (3/6,722) when applying the stricter cut-off value. Assuming free mixing between parasite populations, a
rough estimate of the total expected parasite strains harbouring both PFPlasmepsin2/3 and PF MDR1 amplified genes
would be around 17, a number obtained by simply multiplying the proportions of each amplified gene by year and the
total number of parasite samples assessed per year. Also, in Cambodia between 2014 and 2016, when both ASMQ and
DHA-PPQ were deployed, not a single parasite carrying amplification of both genes was observed. The observations
both suggest that amplification of both genes in the same P. falciparum parasite may confer a fitness disadvantage or
compromised transmissibility. Interestingly, the increase in *PfMDR1* gene amplification in Cambodia after re-deployment of ASMQ has been less pronounced than in the first decade of the millennium when deployment of this ACT in Cambodia followed nearly two decades of mefloquine monotherapy in adjacent Thailand. Despite the increasing use of ASMQ since 2014, with full deployment since 2017, the prevalence of parasites with multiple *PfMDR1* copies increased to only 8% (12/160) in 2019, and ASMQ remains to date an effective treatment for uncomplicated falciparum malaria in Cambodia (3). The much lower levels of transmission and thus lower level of competition may again be a contributor. It may also be that amplification of both *PfMDR1* and *PfPlasmepsin2/3* within the same parasite renders the parasite very unfit. The continued relatively high prevalence of *PfPlasmepsin* amplification would then be a barrier for a rapid increase in *PfMDR1* amplification and thus the re-emergence of mefloquine resistance.

These data support continued deployment of ASMQ in Cambodia. The very low prevalence of concomitant mefloquine and piperaquine resistance also supports combining both drugs in triple artemisinin combination therapies (TACT), in which an artemisinin derivative is combined with two well-matched existing partner drugs (5). This provides a more effective treatment for multidrug resistant falciparum malaria, but could also extend the life span of existing antimalarial drugs by slowing or preventing the emergence of resistance. The TACT DHA-PPQ+MQ was recently studied in a large randomised trial in uncomplicated falciparum malaria (12). This TACT was shown to be well tolerated, safe and highly effective, including in areas of multidrug resistant malaria such as Cambodia and Vietnam. TACT could therefore be one of the few remaining treatment options in the GMS. However, sustained efficacy will depend on the absence of fit parasites which are resistant to both partner drugs, since these parasites would be readily selected with the deployment of a TACT containing DHA-PPQ+MQ.

Other studies confirm the absence of concomitant *PfPlasmepsin2/3* and *PfMDR1* amplification (14, 31). However, a retrospective study from 2017 from Cambodia reported a much higher prevalence; up to 30% of parasites had amplification of both *PfMDR1* and *PfPlasmepsin2/3*, although this was not associated with increased rates of treatment failure with ASMQ (32). We believe that the different methodology to assess gene copy numbers in this study, using dye-based qPCR assays, might have resulted in a substantial over-estimation of gene amplifications. Although dye-based qPCR assays, including EvaGreen® and SYBR® Green, have certain advantages over probe-based qPCR assays in terms of cost effectiveness and time efficiency, these dyes can bind non-specifically to double stranded DNA outside the targeted qPCR product. This causes increased background signal and false positive results (33). In the current study, in parasites with a copy number read-out above 1.52, the statistically determined cut-off for a >90% chance of genuine *PfPlasmepsin2/3* amplification, the proportion of parasites carrying the characteristic SNP at the duplication breakpoint for *PfPlasmepsin2/3* was 88%. This reassures the reliability of our results, but also shows that in a minority of around 10% of parasites samples, *PfPlasmepsin2/3* amplification might have been assigned wrongly.

A shortcoming of our study is that gene amplification in a minor *P. falciparum* clone in patients with multiple clone infections might not have been detected by using the current cut-off for the estimated copy number. This cannot be a
large confounder as multiple clone infections were identified in only 263 out of 2710 samples (9.7%). Another caveat is that absence of PfMDR1 amplification might not exclude mefloquine resistance, since the drug is thought to have several targets (34). In earlier studies of mefloquine resistance in Thailand PfMDR1 amplification only accounted for two thirds of the variance in susceptibility (16). Concomitant resistance to piperaquine and mefloquine might then not be detected by the current resistance markers. This emphasizes the importance of continuing to test in-vitro drug susceptibility -particularly in treatment failures.

In conclusion, our study shows that the molecular genetic markers for mefloquine and piperaquine resistance have evolved differently in the Western compared to the Eastern GMS. This appears to have resulted from the differences in antimalarial drug pressure. In contrast to the disappearance of PfMDR1 amplification after discontinuation of ASMQ, the prevalence of PfPlasmepsin2/3 amplification in Cambodia remains high after discontinuation of DHA-piperaquine. Concomitant amplification of PfMDR1 and PfPlasmepsin2/3 in the same P. falciparum parasite, as well as simultaneous occurrence of the novel PfCRT mutations and PfMDR1 amplification, are extremely rare. Mechanisms conferring mefloquine and piperaquine resistance may counteract each other. This can be evaluated in the laboratory with gene edited P. falciparum strains, and through continued genetic epidemiological surveillance. These results provide support for the development and evaluation of a TACT containing artesunate, mefloquine and piperaquine.

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Author Contributions Statement

MI, NJW and AMD contributed to study design. RV, CP, AS, APP, SP, TP, CN, OM, RT, NHC, DL, NHDT, TJP, JJC, RWP, CA, LS, MM, NTN, PNN, NJP, EAA, FHN, FMS, MD, and AMD collected clinical samples and data. KS, SS, and MI prepared DNA
and genotyped and sequenced. MI, KS, MM, NJW and AMD analysed the data. MI, NJW and AMD wrote the report. All authors read and approved the final manuscript.

Competing financial interests

All authors declare no competing financial interests.

Availability of data and materials

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

References


Figure legends.

Figure 1 Change in the frequency (95% C.I.) of PfPlasmepsin2/3, PfMDR1 gene amplification and novel piperaquine resistance related PfCRT mutations during 2007-2019 in Cambodia.

Figure 2. Distribution of PfPlasmepsin2/3 and PfMDR1 copy number estimates in 6,722 P. falciparum samples obtained from Greater Mekong Subregion countries between 2007 and 2019, coded according to country (Figure 1a). The shaded areas represent PfPlasmepsin2/3 and PfMDR1 estimates with indeterminate result, defined as <90% chance of representing a single versus multiple copy number of the gene.

Figure 3 Relation between PfMDR1 amplification, PfPlasmepsin2/3 amplification and novel piperaquine resistance associated PfCRT mutations.