Impact of the first-line treatment shift from dihydroartemisinin/piperaquine to artesunate/mefloquine on *Plasmodium vivax* drug susceptibility in Cambodia

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**Background:** Cambodia is the epicentre of the emergence of *Plasmodium falciparum* drug resistance. Much less is known regarding the drug susceptibility of the co-endemic *Plasmodium vivax*. Only *in vitro* drug assays can determine the parasite's intrinsic susceptibility, but these are challenging to implement for *P. vivax* and rarely performed.

**Objectives:** To evaluate the evolution of Cambodian *P. vivax* susceptibility to antimalarial drugs and determine their association with putative markers of drug resistance.

**Methods:** *In vitro* response to three drugs used in the past decade in Cambodia was measured for 52 clinical isolates from Eastern Cambodia collected between 2015 and 2018 and the sequence and copy number variation of their *pvmdr1* and *pvcrt* genes were analysed. *pvmdr1* polymorphism was also determined for an additional 250 isolates collected in Eastern Cambodia between 2014 and 2019.

**Results:** Among the 52 cryopreserved isolates tested, all were susceptible to the three drugs, with overall median IC₅₀s of 16.1 nM (IQR 11.4–22.3) chloroquine, 3.4 nM (IQR 2.1–5.0) mefloquine and 4.6 nM (IQR 2.7–7.0) piperaquine. A significant increase in chloroquine and piperaquine susceptibility was observed between 2015 and 2018, unrelated to polymorphisms in *pvcrt* and *pvmdr1*. Susceptibility to mefloquine was significantly lower in parasites with a single mutation in *pvmdr1* compared with isolates with multiple mutations. The proportion of parasites with this single mutation genotype increased between 2014 and 2019.

**Conclusions:** *P. vivax* with decreased susceptibility to mefloquine is associated with the introduction of mefloquine-based treatment during 2017–18.

**Introduction**

Malaria remains one of the most common infectious diseases in the world and the rapid selection of *Plasmodium falciparum* parasites resistant to antimalarial drugs is threatening control efforts. Southeast Asia has been the hotspot of the emergence of *P. falciparum* resistance against virtually all antimalarials used. Regarding *Plasmodium vivax*, inherent constraints associated with parasite biology limit the capacity to investigate this phenomenon. Indeed, *P. vivax* cannot be continuously cultivated *in vitro* and only tedious *in vitro* short-term cultures are possible to determine the intrinsic susceptibility of the parasites. Besides, *P. vivax* parasites are typically asynchronous and multiple developmental stages can be present within an infected patient, which can affect the *in vitro* drug susceptibility of the parasites. Therefore, drug efficacy for *P. vivax* is mainly evaluated through clinical studies that are confounded by a number of biases of host origin (immunity, drug adsorption, metabolism and so on) and parasite factors (reinfection and relapses). Molecular markers of resistance are useful tools to rapidly assess changes in drug susceptibility within a given parasite population.
population. While widely used for the surveillance of *P. falciparum* resistance to many antimalarials, there are still no validated molecular markers of resistance for *P. vivax* clinical isolates. Candidate markers have been proposed in some studies and, notably, polymorphism, amplification or expression of pvcr1 and pvmdr1 are suspected to be involved in chloroquine and/or mefloquine resistance; however, formal evidence of their involvement in natural populations of *P. vivax* remains to be found.3–6

The treatment for *P. vivax* malaria in Cambodia until 2012 was chloroquine. In order to provide a unified drug policy for all species, the treatment changed to a combination of dihydroartemisinin/piperaquine in 2012. Following the emergence of *P. falciparum* resistance to piperaquine, the first-line treatment for all malaria species changed again in 2016 to a combination of artesunate/mefloquine, which was gradually cultured into schizont stage (42–48 h) in Iscove's Modified Dulbecco's Medium (IMDM)-based medium following established protocols.8,10 Incubation was stopped when >40% of mature schizonts in the drug-free control wells was reached and thick blood films were stained with 5% Giemsa (Merck, Germany). The number of schizonts (>4 nuclei visible) per 300 asexual parasites (free merozoites and gametocytes excluded) was determined and normalized to the no-drug controls. IC50 values were determined using ICEstimator online software (http://www.antimalarial-icestimator.net).

**DNA extraction and PCR detection of *P. vivax***

DNA was extracted from 200 µL of whole blood using the QIAamp DNA Blood Mini Kit (QIAGEN, Courtaboeuf, France), according to the manufacturer’s instructions. Molecular detection and identification of *Plasmodium* parasites were performed by real-time PCR as previously described.9

**Drug plate preparation and in vitro susceptibility assay**

*P. vivax* susceptibility to drugs was measured using a protocol modified from Suwanarusk et al.7 in which 96-well plates of drugs were prepared in advance and stored at −20°C. Chloroquine, mefloquine and piperaquine (obtained from the WorldWide Antimalarial Resistance Network (WWARN)) were tested, each at eight different concentrations. Cryopreserved isolates associated with PCR-confirmed *P. vivax* monoinfected, containing more than 75% of ring stages determined by microscopy, were thawed and cultured into schizont stage (42–48 h) in Iscove’s Modified Dulbecco’s Medium (IMDM)-based medium following established protocols.8,10 Incubation was stopped when >40% of mature schizonts in the drug-free control wells was reached and thick blood films were stained with 5% Giemsa (Merck, Germany). The number of schizonts (>4 nuclei visible) per 300 asexual parasites (free merozoites and gametocytes excluded) was determined and normalized to the no-drug controls. IC50 values were determined using ICEstimator online software (http://www.antimalarial-icestimator.net).

**Gene copy number determination of pvmdr1 and pvcr1**

The number of copies of pvmdr1 and pvcr1 was measured by quantitative PCR relative to the single-copy β-tubulin gene using a CFX96 real-time PCR instrument (Bio-Rad) (primers listed in Table 1). PCRs were conducted in 20 µL volumes in a 96-well plate containing 1 x HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne, Tartu, Estonia), 0.5 µM each forward and reverse primers and 2 µL of template DNA. Amplifications were performed under the following conditions: 95°C for 15 min, followed by 45 cycles of 95°C for 15 s, 60°C for 20 s and 72°C for 20 s. The number of pvmdr1 copies for each sample was measured in triplicate relative to a standard curve made of synthet-ic β-tubulin and pvmdr1 genes, each cloned in a pEX-A2 vector (Eurofins Genomics, Greece) and mixed at different ratios from 1:1 up to 1:5 (one copy of β-tubulin and up to five copies of pvmdr1) (Table 1). The number of pvcr1 copies for each sample was measured in triplicate relative to an

### Materials and methods

**P. vivax clinical isolate collection**

Samples were collected from symptomatic patients seeking treatment in Eastern Cambodia between 2014 and 2019. Venous blood was collected in EDTA tubes for molecular analysis and immediately stored at −20°C. Additionally, for *in vitro* assays a heparin tube was collected from which leucocytes were depleted using non-woven fabric (NWF) filters7 (Zhi Xing Bio S&T Co. Ltd, China) and RBCs were immediately cryopreserved in Glycerolyte 57 (Baxter Healthcare Corporation, USA) following published procedures.8

| Name                          | Sequence (5’→3’)                                                                 | Reference |
|-------------------------------|---------------------------------------------------------------------------------|-----------|
| Gene copy number determination |                                                                                 |           |
| pvmdr1 synthetic gene         | GCAACTCCATAAAGACAAACACATCAAGTAGTATGGTACAGCTGAAAGATTTAGACCGCTTACGG               | this work |
|                               | AGGAGTGCAAGGAAGATGTTTTTCTTTCAAA                                                 |           |
| β-TubulinPv synthetic gene    | CAGGAGTTACATGTTGTTAGATTTCTTGTGTTACATTAATTGTGATTGTGAAATTGACCGCTTACATT           |           |
|                               | TATTTCTCCCATACAGACTCCCTTTTATGTTGTTTGGTACCCACTAAAAGACCCAGAGGG                   |           |
| PvMDR1_F                       | GCAACTCCATAAAGACAAACACATCA                                                     | this work |
| PvMDR1_R                       | TTTGAGAAGAAAAACACATCTTCG                                                        | this work |
| PvCRT_F                       | GGAGTCTCCCTAAAATTCCCC                                                         | this work |
| PvCRT_R                       | GTTGCTGCTCCCTACCTCCTG                                                           |          |
| CN_β-tubulin_F                | CATGTGCTGTTAAGATTTCCTG                                                         |           |
| CN_β-tubulin_R                | GTTAGTGCTGCAAAAACCATCA                                                         |           |
| Sequence polymorphism          |                                                                                 |           |
| Pvmdr976 F                    | GGAAGAGTTGCTGCAAAGCCGCCGATGC                                                   |           |
| Pvmdr976 R                    | CATCAACCTCCGCGGTGTCG                                                          |           |
| Pvcr1-o F1 (sense)            | AAGAGCCTGCTAGCCTTCC                                                          |           |
| Pvcr1-o R3                    | AGTTTCCCTCTACCCCC                                                             |           |

**Table 1.** List of primers and synthetic genes used for pvcr1 and pvmdr1 gene copy number determination and sequencing

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**References**

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internal calibrator control. The ΔΔCT method (where CT is the cycle threshold) was used to determine the number of copies in each sample.

Sequence polymorphism of pvmdr1 and pvcrt genes

PCR and Sanger sequencing (Macrogen, Seoul, South Korea) were used to determine pvmdr1 and pvcrt sequences using the following conditions. For pvmdr1, the PCR was conducted in 30 μl reactions consisting of 3 μl of DNA, 0.15 μM primers and 1× HOT FirePol Blend MasterMix (Solis BioDyne) under the following conditions: 94 °C for 10 min, followed by 40 cycles of 94 °C for 30 s, 64 °C for 90 s, 72 °C for 45 s and a final extension at 72 °C for 10 min. For pvcrt, the PCR was conducted in 30 μl reactions consisting of 3 μl of DNA, 0.25 μM primers and 1× HOT FirePol Blend MasterMix under the following conditions: 94 °C for 10 min, followed by 35 cycles of 94 °C for 50 s, 65 °C for 60 s, 72 °C for 90 s and a final extension at 72 °C for 10 min. Primers used are described in Table 1. Expected amplicon sizes are 604 bp and 1186 bp, respectively, for pvmdr1 and pvcrt. Nucleotides and corresponding amino acids were analysed using MEGA 7 software (Beckman). The sequences generated were compared with AY571984.1 for pvmdr1 and with AF314649.1 for pvcrt.

Statistical analysis

Comparison of mean of non-Gaussian data was done by the Mann-Whitney test. Multiple comparisons of means of non-Gaussian data were performed by Kruskal-Wallis and Dunn’s post hoc tests. Proportions were compared by Fisher’s exact test. All analyses were performed using GraphPad Prism (v7.00). A P value of <0.05 was considered significant.

Ethics

The research was conducted in accordance with the Declaration of Helsinki and national and institutional standards. Ethics clearance for the samples used in this study was obtained from the Ministry of Health National Ethics Committee in Cambodia (364NECHR, 478NECHR, 270NECHR and 317NECHR). All patients or their parents/guardians provided informed written consent.

Results

Evolution of Cambodian P. vivax in vitro susceptibility to three antimalarials

The susceptibility of 52 P. vivax cryopreserved clinical isolates from Eastern Cambodia collected between 2015 and 2018 was assessed by a schizont maturation assay validated using susceptible and resistant strains of P. falciparum (Table S1, available as Supplementary data at JAC Online). The IC50 values for all 52 isolates were low and homogeneous for the three drugs, indicating the absence of high-grade resistant parasites. Median IC50 values of 16.1 nM (IQR 11.4–22.3), 3.4 nM (IQR 2.1–5.0) and 4.6 nM (IQR 2.7–7.0) were observed for chloroquine, mefloquine and piperquine, respectively (Figure 1).

The susceptibility data for the three drugs were analysed according to the time of collection of the isolates. While for mefloquine there was no significant difference in IC50 depending on the collection period, the response of parasites to chloroquine and piperquine was significantly different. The IC50 of chloroquine decreased between 2015–16 [median 20.7 nM (IQR 16.0–33.44)] and 2017–18 [median 14.3 nM (IQR 10.1–19.4), P = 0.0023] in a fashion similar to the IC50 of piperquine [median 6.7 nM (IQR 4.3–8.1) versus median 4.1 nM (IQR 2.6–5.3), P = 0.0213].

Polymorphisms of pvcrt and pvmdr1 and association with drug susceptibility of isolates

Sequences of pvmdr1 were interpretable for the 52 cryopreserved isolates. All isolates had the T958M mutation. Two other mutations were detected: Y976F (in 28/52, 54% of isolates) and F1076L (in 45/52, 86.5% of isolates). Three different pvmdr1 genotypes were observed, the most frequent being the triple T958M-Y976F-F1076L mutant (28/52, 54%), followed by the double T958M-F1076L mutant (17/52, 33%) and the single T958M mutant (7/52, 13%) (Table S2). All isolates with the Y976F mutation had also the F1076L mutation. No isolate had the WT pvmdr1 Sal-1 sequence. Sequences of pvcrt were interpretable for 45 isolates, of which 69% (31/45) had a lysine insertion at codon 10 (K10insert). In addition, two newly described mutations were observed, albeit at low frequency: one isolate (1/45, 2%) had an A107V mutation and four (4/45, 9%) an S163N mutation. Overall, four different pvcrt genotypes were detected, the most frequent being the K10insert alone (noted as genotype 1, 27/45, 60%), followed by the Sal-1 WT (13/45, 29%), the K10insert/S163N (genotype 2, 3/45, 7%) and one isolate (1/45, 2%) for each of the S163N (genotype 3) and K10insert/A107V (genotype 4) genotypes. All isolates had a single copy of both pvmdr1 and pvcrt genes.

The in vitro susceptibility of isolates to all drugs was analysed according to their polymorphisms in pvmdr1 and pvcrt. IC50 values were compared between the different genotypes. Among all the pvmdr1 and pvcrt polymorphisms, the only significant association was between pvmdr1 and the response of isolates to mefloquine (Figure S1).

Multiple comparison analysis of the susceptibility to mefloquine between the three pvmdr1 genotypes showed a significantly lower IC50 for the triple T958M-Y976F-F1076L mutants [median 2.9 nM (IQR 2.1–4.3)] compared with the single T958M mutants [median 7.0 nM (IQR 5.0–10.6), P = 0.0072] (Figure 2). The double T958M-F1076L mutant parasites had a median IC50 of 4.4 nM (IQR 2.0–4.9), intermediate between single and triple mutants, although the differences did not reach statistical significance.

Evolution of pvmdr1 polymorphism in Eastern Cambodia following successive changes in national treatment guidelines

The polymorphism of pvmdr1 was then analysed according to the therapeutic strategy implemented in Eastern Cambodia. To increase the sample size and identify significant changes in frequency, we included a larger number of isolates (total N = 301 isolates) collected in the same area between May 2014 and September 2019 for which DNA only was available. No other genotypes were observed among those additional isolates. During the period of dihydroartemisinin/piperaquine deployment (2014–16), the frequency of the single mutant (T958M) was 8% (N = 9/113). A significant increase (P = 0.005) to 20% (N = 38/188) was observed after adoption of artesunate/mefloquine (Figure 3). No significant difference in frequency over time was observed for the double and triple mutants. The copy number of the pvmdr1 gene could be
determined for 241 clinical isolates and all had a single copy of the gene.

Discussion

The aim of this study was to assess the evolution of P. vivax in response to antimalarials used in Cambodia following the successive changes in first-line treatment. We therefore determined the in vitro susceptibility to drugs of cryopreserved parasites collected in Eastern Cambodia between 2015 and 2018 and tested for associations between susceptibility and polymorphisms in the pvmdr1 and pvcrt genes. Overall, no isolate showed susceptibility above the assumed resistance threshold of 220 nM chloroquine. Currently there is no established cut-off for piperaquine or mefloquine resistance in P. vivax. Future studies that associate in vitro drug response of parasites with clinical outcomes (adequate clinical and parasitological response versus treatment failure) are needed to determine clinically relevant thresholds of resistance. However, the IC50 values observed in this study are very low (median <5 nM for both mefloquine and piperaquine). It is thus highly unlikely that those susceptibilities are of any therapeutic concern. Additionally, the values obtained here are among the

Figure 1. Overall susceptibility of 52 cryopreserved Cambodian P. vivax isolates to chloroquine, mefloquine and piperaquine measured by in vitro schizont maturation assays (top panel). Black circles represent individual IC50 values of P. vivax isolates (the horizontal line shows the median and the whiskers show the IQR). For comparison, plain light blue circles represent IC50 values of the susceptible P. falciparum 3D7 reference strain and plain dark blue circles the chloroquine or mefloquine IC50 values of resistant P. falciparum clinical isolates. Susceptibility to chloroquine, mefloquine and piperaquine of isolates collected when the first-line treatment was dihydroartemisinin/piperaquine (2015–16) or artesunate/mefloquine (2017–18) (bottom panels). The susceptibility of isolates to chloroquine and piperaquine significantly decreased over time (Mann-Whitney test, *P = 0.0023 and **P = 0.0213, respectively). This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.
Increased significantly between 2015 and 2018, with the average susceptibility profiles. First, freeze–thaw procedures on the drug response of parasites. Cryopreservation should be conducted to assess any effect of biases in the IC50 measurements. Future studies examining differences in IC50 between fresh parasites, which could, in theory, have introduced some biases in the IC50 measurements. Future studies examining differences in IC50 between paired isolates determined before and after cryopreservation should be conducted to assess any effect of freeze–thaw procedures on the drug response of parasites. Even though these parasites cannot be categorized as resistant, this study demonstrates an interesting evolution in their drug susceptibility profiles. First, P. vivax susceptibility to chloroquine increased significantly between 2015 and 2018, with the average IC50 decreasing by almost half within 4 years. This observation is consistent with the withdrawal of chloroquine from guidelines since 2012. Indeed, in a study conducted between 2008 and 2010, up to 18% of treatment failure following chloroquine treatment was reported in Eastern Cambodia, where the cryopreserved isolates used in our work originated. However, another therapeutic efficacy study conducted in the same area, but 2 years after the replacement of chloroquine with dihydroartemisinin/piperaquine, did not find any sign of treatment failure. Given these previous clinical results and the decrease in IC50 reported here, we hypothesize that there was indeed some chloroquine resistance before the change in the treatment guidelines, following which parasites gradually regained susceptibility. Importantly, none of the polymorphisms in pvcr1 or pvmdr1 detected in this work could be linked to the changes in susceptibility to chloroquine observed over time. This suggests that determinants other than pvcr1 and pvmdr1 are involved in the modulation of chloroquine susceptibility in P. vivax.

Because we did not identify any isolate highly resistant to chloroquine, pvcr1 and pvmdr1 polymorphisms detected in our work can definitely be excluded as markers of resistance. Other mutations in these genes (or their amplification) not observed in our study could, however, still be related to chloroquine resistance. Besides, our work cannot exclude a link between increased pvcr1 expression and chloroquine resistance. Similar genotype–phenotype association studies in areas where high-grade chloroquine-resistant P. vivax are reported are needed to provide conclusive data on the basis of chloroquine resistance for this species.

Interestingly, the same trend for piperaquine susceptibility evolution was observed, as IC50 were significantly lower after artesunate/mefloquine implementation. This increase in piperaquine susceptibility is probably due to the consequence of the withdrawal of dihydroartemisinin/piperaquine as first-line treatment in the country and could also result from mefloquine pressure on parasite populations. Indeed, in P. falciparum, piperaquine and mefloquine exert opposite pressure on parasites and increased susceptibility to piperaquine is linked to decreased susceptibility to mefloquine. Of note, only normal dose–response curves for piperaquine (and other drugs) and no paradoxical parasite growth at high drug concentrations were observed (as seen with P. falciparum piperaquine-resistant strains), further confirming the absence of resistance of P. vivax in Cambodia.

No significant evolution of mefloquine susceptibility over time was observed but polymorphism in pvmdr1 was associated with differences in susceptibility to mefloquine. Indeed, the single T958M mutant was significantly less susceptible than the triple T958M–F1076L mutant, though none of the isolates had an IC50 high enough to indicate high-grade mefloquine resistance. Additionally, the frequency of this less-susceptible T958M genotype doubled between 2015 and 2019 in Eastern Cambodia following the introduction of artesunate/mefloquine. Altogether, these results suggest ongoing selection of decreased susceptibility to mefloquine. Notably, high IC50 values of mefloquine were previously associated with pvmdr1 gene amplification and parasites with multiple copies of pvmdr1 did not carry the Y976F mutation, suggesting a fitness cost associated with having both the mutation and the gene amplification. In our current work, none of the isolates tested had pvmdr1 amplification and none had high mefloquine resistance. We believe we are currently observing changes in genotypes and phenotypes predating the emergence of clinically relevant resistance to mefloquine with parasites currently losing the mutations within pvmdr1 prior to acquiring multiple copies of the gene.

The results of this work indicate that the susceptibility of P. vivax parasites to antimalarials evolves in response to the drugs used in the field.
the country. The difference with *P. falciparum* parasites from the same area is, however, striking. Indeed, while for *P. vivax* only modest changes in susceptibility were observed, for *P. falciparum* in the same time frame and the same locations, emergence of fully resistant parasites was thoroughly documented. This difference most probably results from several different factors and among these, the production of gametocytes by *P. vivax* (and its transmission to the vectors) before patients become symptomatic (and thus exposed to the drugs) certainly limits the selective pressure exerted on the parasite populations. In addition, a significant proportion of individuals infected by *P. vivax* are asymptomatic and therefore undetected and untreated. Despite these factors, changes in susceptibility over time and selection of specific genotypes are observed, indicating that some selective pressure does occur. This selection is probably driven by the pressure exerted by lingering antimalarials after treatment on reactivating hypnozoites during relapsing episodes. Decreased susceptibility to mefloquine would allow parasites to survive lingering subtherapeutic drug concentrations upon relapses.

In conclusion, this work warrants further monitoring of the evolution of *P. vivax* susceptibility to mefloquine and *pvmdr1* polymorphism in Cambodia in order to allow early detection of the emergence of fully resistant mefloquine isolates.

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Transparency declarations
None to declare.

Supplementary data
Figure S1 and Tables S1 and S2 are available as Supplementary data at JAC Online.

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