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Prevalence and characterization of piperaquine, mefloquine and artemisinin derivatives triple-resistant *Plasmodium falciparum* in Cambodia

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Background: In early 2016, in Preah Vihear, Northern Cambodia, artesunate/mefloquine was used to cope with dihydroartemisinin/piperaquine-resistant *Plasmodium falciparum* parasites. Following this policy, *P. falciparum* strains harbouring molecular markers associated with artemisinin, piperaquine and mefloquine resistance have emerged. However, the lack of a viable alternative led Cambodia to adopt artesunate/mefloquine countrywide, raising concerns about a surge of triple-resistant *P. falciparum* strains.

Objectives: To assess the prevalence of triple-resistant parasites after artesunate/mefloquine implementation countrywide in Cambodia and to characterize their phenotype.

Methods: For this multicentric study, 846 samples were collected from 2016 to 2019. Genotyping of molecular markers associated with artemisinin, piperaquine and mefloquine resistance was coupled with phenotypic analyses.

Results: Only four triple-resistant *P. falciparum* isolates (0.47%) were identified during the study period. These parasites combined the *pfk13* polymorphism with *pfmdr1* amplification, *pfpm2* amplification and/or *pfcrt* mutations. They showed significantly higher tolerance to artemisinin, piperaquine and mefloquine and also to the mefloquine and piperaquine combination.

Conclusions: The use of artesunate/mefloquine countrywide in Cambodia has not led to a massive increase of triple-resistant *P. falciparum* parasites. However, these parasites circulate in the population, and exhibit clear resistance to piperaquine, mefloquine and their combination *in vitro*. This study demonstrates that *P. falciparum* can adapt to more complex drug associations, which should be considered in future therapeutic designs.

Introduction

The initial hypothesis, according to which the emergence of resistance to artemisinin-based combination therapies was unlikely, has been contradicted by several reports from South East Asia, particularly in the framework of dihydroartemisinin/piperaquine use in Cambodia. Resistance to the dihydroartemisinin/piperaquine combination was first linked to the concomitant presence of the K13 C580Y haplotype (a marker of artemisinin resistance) and *pfpm2* amplification (piperaquine resistance).[1,2,3] In addition, the extended piperaquine pressure led to a secondary resistance mechanism through the selection of specific *pfcrt* polymorphisms.[4,5] This resistance context led to the withdrawal of the dihydroartemisinin/piperaquine combination for malaria

treatment in 2016. However, due to the lack of viable alternatives, the artesunate/mefloquine combination was reintroduced first in the Preah Vihear province (Northern Cambodia) in 2016. Shortly afterwards, a study showed the significant emergence of *P. falciparum* strains that concomitantly harboured resistance markers to artemisinin (K13 C580Y), piperaquine (*pfpm2* amplification) and mefloquine (*pfmdr1* amplification).[6] This suggested that *P. falciparum* can become resistant to artesunate/mefloquine and dihydroartemisinin/piperaquine, a major challenge for malaria control in the Greater Mekong subregion. This rapid evolution of the parasites also brought a major concern regarding the following countrywide introduction achieved in 2017. However, this study was limited to only one Cambodian district and did not include any phenotypic characterization of these triple-mutant *P. falciparum* parasites.[6] This left many open questions about the emergence and expansion dynamics of such triple-mutant parasites and their resistance profile. Therefore, we carried out a longitudinal and multicentric study in Cambodia to determine the dynamics of triple-mutant *P. falciparum* emergence and to phenotypically and genotypically characterize their resistance to artemisinin, mefloquine and piperaquine in the framework of artesunate/mefloquine reintroduction.

Methods

P. falciparum clinical isolates

P. falciparum isolates were from patients with uncomplicated *P. falciparum*-caused malaria enrolled in WHO therapeutic efficacy studies (2016–19). Venous blood was collected in acid-citrate-dextrose tubes (Becton-Dickinson, Franklin Lakes, NJ, USA). An aliquot (200 µL) of fresh blood was frozen at −20°C for DNA extraction, and the remaining blood was adapted to *in vitro* culture at 2% haematocrit (O^+ human blood, Centre de Transfusion Sanguine, Phnom Penh, Cambodia) in RPMI 1640 supplemented with 0.5% AlbuMAX II, 2.5% human plasma (mixed serogroups), 5% CO_2 and 5% O_2, at 37°C. The *P. falciparum* 3D7 reference clone was obtained from the Malaria Research and Reference Reagent Resource Center and grown in the same conditions. In addition to the 2016–19 field isolates, three *P. falciparum* strains collected in 2012 and later characterized for harbouring the K13 C580Y mutation, and *pfpm2* and *pfmdr1* amplifications were included in the analyses.[7]

Determination of molecular resistance markers

The presence of the *P. falciparum* K13 (PF3D7_1343700 from codon 445 to 680) polymorphisms in the propeller domain was determined by dideoxy sequencing according to Ariey *et al.*[2] *Pfmdr1* and *pfpm2* copy number variations were determined by quantitative PCR according to Witkowski *et al.*[3] with modification of the hybridization temperature to 63°C. The presence of *pfcrt* (PF3D7_0709000) polymorphisms (codons 93, 97, 145, 343 and 353) was determined by dideoxy sequencing using the primers described (Table S1, available as Supplementary data at *JAC* Online) or WGS data.

Pfpm2 and pfmdr1 gene expression

RNA was extracted from 100 µL of infected RBCs (5%–10% with *P. falciparum* rings 24–27 h post-invasion) with 1 mL of TRIzol followed by 200 µL of chloroform. After centrifugation at 12 000 g for 15 min, the aqueous phase was extracted and mixed with 100% ethanol. RNA was purified at 4°C using the RNeasy kit (QIAGEN), following the manufacturer's instructions. The remaining genomic DNA was digested with DNase I (Promega). After reverse transcription of RNA to cDNA (Promega GoScript Reverse Transcriptase Kit), quantitative PCR was performed to measure *pfmdr1* and *pfpm2* gene expression, using fructose-bisphosphate aldolase (PF3D7_1444800) as housekeeping gene.[3]

Antibody production and western blot analysis

Three synthetic peptides, GKEQKEKKDGNLSIKEEV-Cys, Cys-HGTHDELL SAQDGIYKKYVKLAK and Cys-EAMENYYEENTNDT, which correspond to residues 2–19, 486–499 and 1398–1419 (C-terminus) of PfMDR1 primary sequence, respectively, were coupled to keyhole limpet haemocyanin (KLH) via the additional cysteine residue to immunize rabbits. The PepR3 antibody used in this study corresponds to a mix of the purified antibodies against the three peptides (ProteoGenix).

Protein extracts were prepared from the three *pfk13/pfpm2/pfmdr1* triple-mutant strains collected in 2012 and two WT strains. Synchronized, 24–30 h post-invasion free trophozoites were prepared following two rounds of incubation with 0.002% saponin (Sigma), washed three times in PBS, and resuspended in sterile water supplemented with protease inhibitors (Complete, Roche). Soluble and insoluble *P. falciparum* proteins were obtained through two freezing/thawing cycles, and centrifugation at 16 100 g at 4°C for 30 min. Protein amounts equivalent to 0.25×10^6 and up to 4×10^6 parasites were separated and transferred, as previously described.[8] Blots were incubated with primary antibodies followed by horseradish peroxidase (HRP)-conjugated secondary antibodies (Promega, 1/25000) and developed by chemiluminescence (Pierce). Primary antibodies were against rabbit Pf-PM2 (Ab737, 1/1000),[9] Pf-MDR1 (purified PepR3, 1/1000; this study) and Pf-aldolase (HRP-coupled; Abcam, 1/10000), used as loading control. ChemiDoc and Image Lab (BioRad) were used to evaluate the relative amounts of the corresponding proteins.

Mefloquine, piperaquine and artemisinin *in vitro* susceptibility assays

Mefloquine was provided by the Worldwide Antimalarial Resistance Network (WWARN) and prepared in dimethyl sulphoxide (Sigma–Aldrich, Singapore). The *in vitro* susceptibility of cultured parasites to mefloquine was determined using the H^3-hypoxanthine uptake inhibition assay, as previously described, with modifications.[10] Parasites were synchronized at the ring stage by incubation with 5% D-sorbitol (0–12 h post-invasion) and exposed to mefloquine at different concentrations (2 to 1500 nM) in the presence of 0.5 µCi of H^3-hypoxanthine (PerkinElmer, Waltham, USA) for 48 h. Tritium incorporation was measured with a β-counter (MicroBetaTriLux; PerkinElmer Waltham, USA). The IC_{50} was determined using the ICEstimator software (http://www.antimalarial-icestimator.net).

Piperaquine tetraphosphate was provided by WWARN and prepared in 0.5% lactic acid. The piperaquine survival assay was performed according to Witkowski *et al.*[3] Briefly, *P. falciparum* parasites at the ring stage, 0–3 h post-invasion, were obtained by centrifugation in 75% Percoll and their concentration adjusted to between 0.5% and 1% of parasitaemia. Parasites were incubated with 200 nM piperaquine or 0.5% lactic acid (drug-free control). The drug was washed off after 48 h of exposure. Parasitaemia was measured in 10 000 RBCs under a microscope, after 72 h of culture. For each *P. falciparum* strain tested, the survival rate was determined as the ratio between the parasitaemia in the drug-exposed condition and in the drug-free control condition. The *in vitro* susceptibility of *P. falciparum* strains to artemisinin was assessed using the ring-stage survival assay (RSA) as previously described.[11]

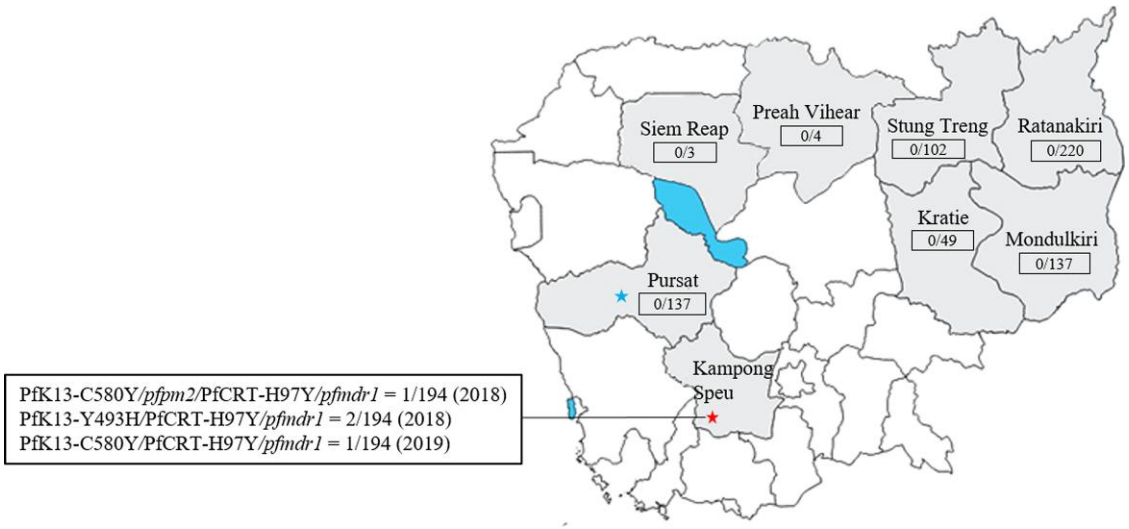

Figure 1. Prevalence and geographical location of triple-resistant isolates in Cambodia during the 2016–19 period. *Pfk13* polymorphisms and co-amplification of *pfpm2* and *pfmdr1* genes were assessed in 846 *P. falciparum* isolates collected in Cambodia during therapeutic efficacy studies between 2016 and 2019. *Pfcrt* polymorphisms in codons 93, 97, 145, 343 and 353 were assessed in 39/45 isolates presenting *pfmdr1* amplification. The prevalence of triple mutants and the year of collection are indicated. The red star indicates the province where triple mutants were collected during this study. The blue star indicates the province where three *pfk13/pfpm2/pfmdr1* triple-mutant *P. falciparum* isolates were collected in 2012.[7] The resistance phenotype of these isolates was determined in the present study. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

Piperaquine and mefloquine co-susceptibility in vitro assay

P. falciparum parasites at the ring stage, 0–3 h post-invasion, were obtained by centrifugation in 75% Percoll followed by incubation with 5% D-sorbitol and adjusted to between 0.5% and 1% of parasitaemia. Parasites were incubated with seven increasing doses of a piperaquine +mefloquine mixture (from 12.5 to 800 nM for each drug) for 48 h. After washes to eliminate the drugs, parasites were maintained in culture for 24 h. After a total of 72 h in culture, blood smears were prepared, and *P. falciparum* survival was determined as the ratio between the proportion of viable parasites in the drug-exposed condition and in the drug-free control.

Ethics

All *P. falciparum* isolates were collected during therapeutic efficacy studies performed in accordance with the Cambodian National Ethical Committee for Human Research (identifier: NECHR #086, NECHR #087, NECHR #092 and NECHR #106).

Statistical analyses

Statistical analyses were performed with the GraphPad Prism 7.0 software. A *P* value of <0.05 was considered significant. The Mann–Whitney *U*-test was used to compare medians. The IC_{50} values of the piperaquine +mefloquine combination were determined for each strain and compared using the non-linear regression analysis tool in GraphPad 7.0 and the ICEstimator software.

Results

Prevalence of triple-mutant P. falciparum *parasites*

Molecular analysis of 846 *P. falciparum* field isolates collected at eight centres in Cambodia between 2016 and 2019 (Figure 1, Table 1) detected *pfmdr1* amplification in 45/846 isolates, of which only 1 presented *pfpm2* amplification. *Pfcrt* sequencing (codons 93, 97, 145, 343 and 353) was possible in 39 of the 45 isolates with *pfmdr1* amplification. Finally, four isolates (0.47% of 846) harboured markers of resistance to artemisinin, to piperaquine (*Pfcrt* polymorphism and/or *Pfpm2* amplification) and mefloquine. Specifically, these four samples harboured the K13 C580Y ($n=2/4$) or Y493H ($n=2/4$) mutation, the CRT H97Y mutation and *pfmdr1* amplification. Only one sample presented *pfpm2* co-amplification in addition to the other markers, resulting in a quadruple-mutant profile. These four triple- and quadruple-mutant isolates were all collected at Kampong Speu (Western Cambodia) in 2018 ($n=3$) and 2019 ($n=1$). The prevalence of piperaquine and mefloquine co-resistance profiles at this site was 1.34% ($n=3/223$) in 2018 and 0.90% ($n=1/111$) in 2019 (Table 1). This lower prevalence in 2019 was not statistically significant ($P=0.72$, chi-squared).

Pf-PM2 and Pf-MDR1 gene and protein expression

mRNA expression of *pfpm2* and *pfmdr1* was measured in five WT isolates (*pfk13*-WT, single copy of *pfmdr1* and *pfpm2*) and in seven triple-resistant isolates (the four identified in the present study and three collected in 2012 [Figure 2(a)]. The median relative *pfmdr1* expression level was higher in the triple-resistant than in WT *P. falciparum* [0.77 (IQR: 0.68–1.02) versus 0.38 (IQR: 0.32–0.50); $P=0.0025$, Mann–Whitney *U*-test]. The median *pfmdr1* expression levels in the triple-resistant isolates and in three *pfmdr1*-amplified isolates were not significantly different [0.62 (IQR: 0.57–0.77); $P=0.0714$, Mann–Whitney *U*-test).

The median relative expression of *pfpm2* was significantly higher in the co-amplified isolates than in WT *P. falciparum* isolates [1.42 (IQR: 1.06–1.46) versus 0.59 (IQR: 0.56–0.62);

Table 1. Prevalence of triple-resistant *P. falciparum* isolates collected in Cambodia between 2016 and 2019

Sites	2016 % (*n*/*N*)	2017 % (*n*/*N*)	2018 % (*n*/*N*)	2019 % (*n*/*N*)	Total % (*n*/*N*)
Kampong Speu	0 (0/44)	0 (0/55)	4.9 (3/61)	2.9 (1/34)	2.1 (4/194)
Pursat	0 (0/29)	0 (0/50)	0 (0/42)	0 (0/16)	0 (0/137)
Siem Reap	0 (0/3)	n/a	n/a	n/a	0 (0/3)
Kratie	0 (0/18)	n/a	0 (0/23)	0 (0/8)	0 (0/49)
Stung Treng	0 (0/43)	0 (0/59)	n/a	n/a	0 (0/102)
Rattanakiri	0 (0/56)	0 (0/59)	0 (0/53)	0 (0/52)	0 (0/220)
Mondulkri	0 (0/32)	0 (0/60)	0 (0/44)	0 (0/1)	0 (0/137)
Preah Vihear	0 (0/4)	n/a	n/a	n/a	0 (4)
Total	0 (0/229)	0 (0/283)	1.34 (3/223)	0.9 (1/111)	0.47 (4/846)

n/a, not available.

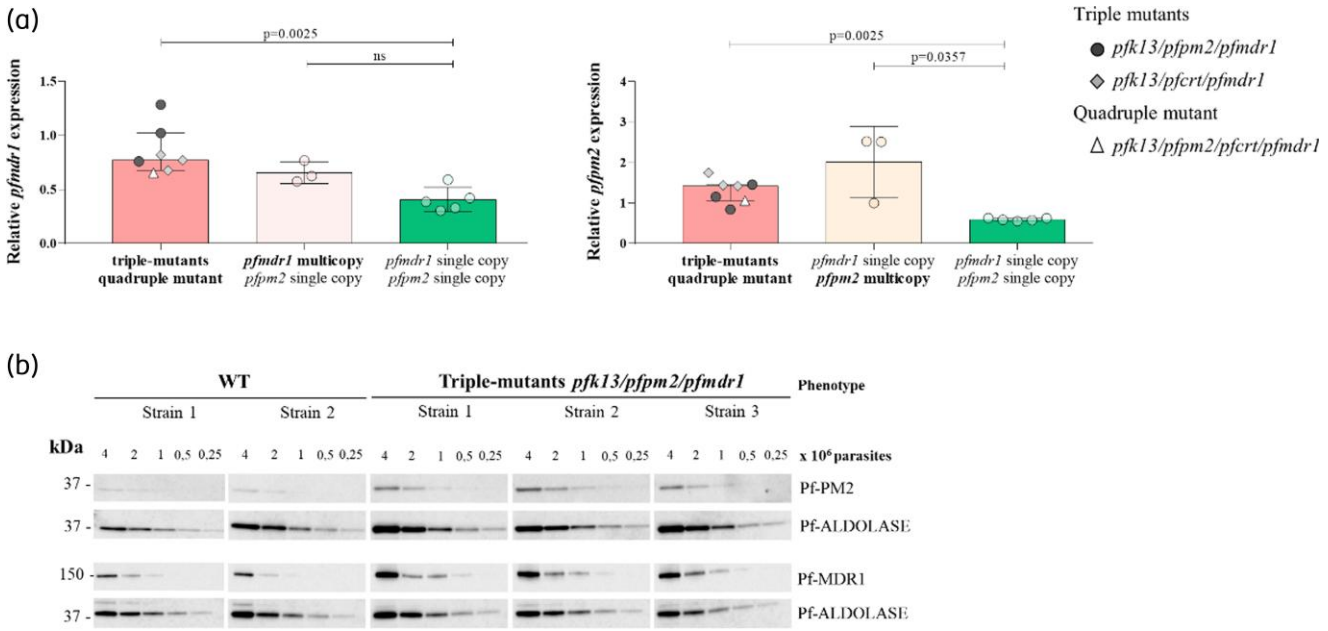

Figure 2. (a) *Pfmdr1* and *pfpm2* expression in triple- and quadruple-mutant *P. falciparum* isolates. *Pfmdr1* and *pfpm2* normalized expression levels were quantified in seven triple-mutant isolates (left histogram bar red bar and black dots, light grey diamonds and white triangle according to genotypes), five WT strains (right histogram bar green bar) and three *pfk13*/*pfmdr1* and three *pfk13*/*pfpm2* double-mutants isolates (middle histogram bar pink or orange bar). Each symbol corresponds to the mean value in one isolate (three replicates). Each bar corresponds to the median expression in quadruple, triple or double mutants and WT isolates (compared with the Mann–Whitney *U*-test). (b) Western blot analysis of Pf-PM2 and Pf-MDR1 expression in three *pfk13*/*pfpm2*/*pfmdr1* triple mutants and two WT isolates. Pf-PM2 and Pf-MDR1 expression levels were assessed in synchronized *P. falciparum* trophozoites (24–30 h post-invasion). Pf-aldolase was used as loading control. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

$P = 0.0025$, Mann–Whitney *U*-test]. However, the median *pfpm2* expression in three *pfpm2*-amplified isolates [2.5 (IQR: 1.00–2.52); $P = 0.0357$, Mann–Whitney *U*-test] was significantly higher than in the co-amplified isolates.

To evaluate the differences in protein expression, Pf-PM2/aldolase and the Pf-MDR1/aldolase ratios were compared in two WT *P. falciparum* isolates and three *pfk13/pfpm2/pfmdr1* co-amplified isolates. Pf-PM2 and Pf-MDR1 showed a trend of overexpression (but not significant) in the co-amplified isolates compared with WT controls (by 2–5.4-fold and 2.8–5.6-fold, respectively), suggesting functional gene amplification [Figure 2(b)].

Phenotypic characterization

Susceptibility to mefloquine was measured in five *P. falciparum* WT isolates and the six triple mutants and one quadruple mutant with markers of resistance to piperaquine, mefloquine and artemisinin. The median mefloquine IC_{50} values were 52.36 (IQR: 28.08–54.68) for WT and 84.45 (IQR: 78.16–116.20) for triple-resistant isolates ($P = 0.025$, Mann–Whitney *U*-test) [Figure 3(a)].

Susceptibility to piperaquine showed a median survival rate of 0% (IQR: 0–0) for WT isolates and of 14.59% (IQR: 12.85–19.24)

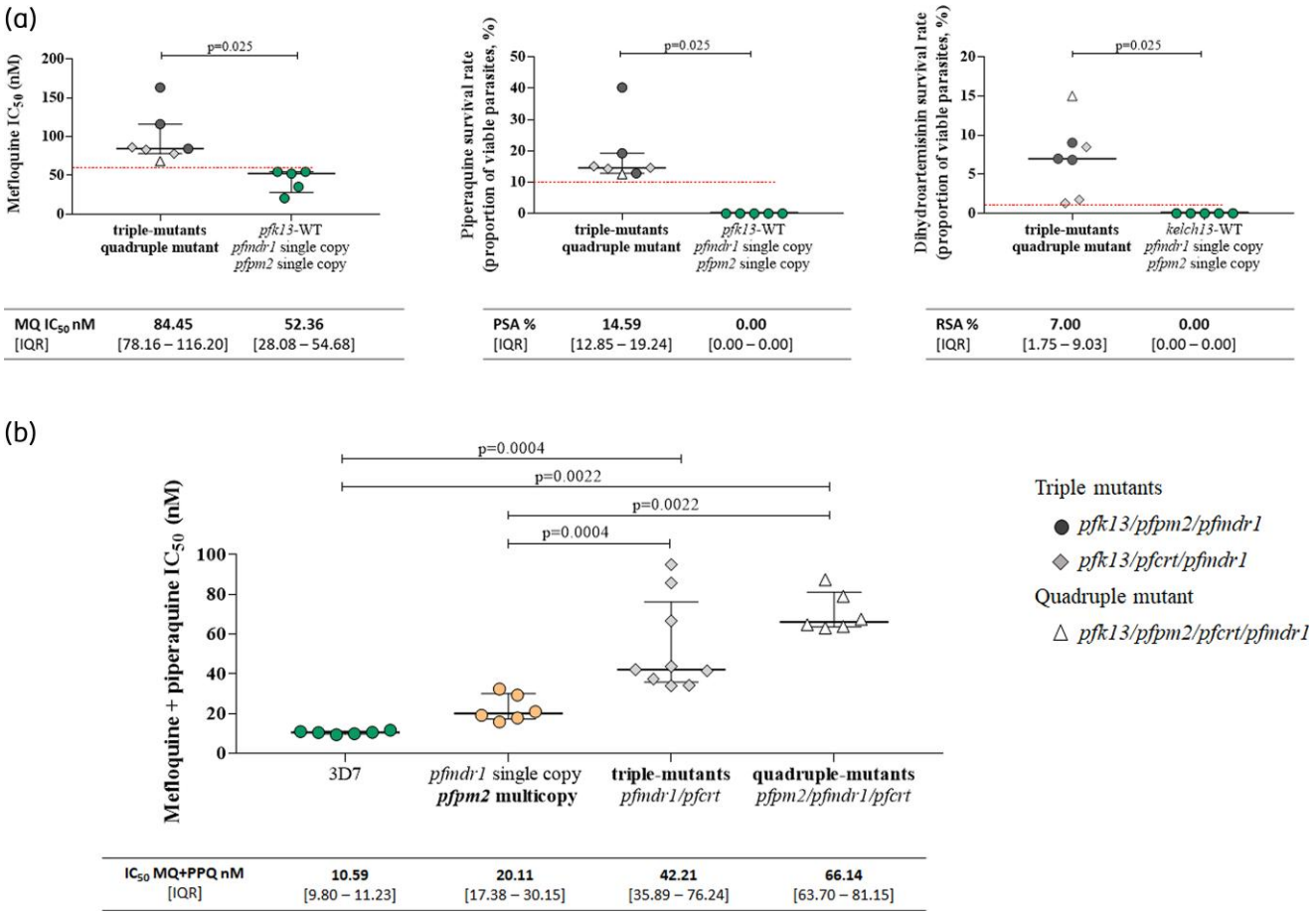

Figure 3. Phenotypic characterization of triple- and quadruple-mutant *P. falciparum* isolates. (a) Five WT (green dots) and six triple mutants in addition to one quadruple mutant with markers of resistance to piperaquine, mefloquine and artemisinin (identified by black dots, diamonds and triangle) collected from 2012 to 2019 were adapted to *in vitro* culture and their susceptibility to mefloquine, piperaquine and dihydroartemisinin was tested. The red dotted line represents the resistance threshold for each drug. Between-group differences were assessed using the Mann–Whitney *U*-test. (b) The 3D7 reference clone (green dots, six replicates), a dihydroartemisinin/piperaquineresistant isolate (yellow dots, six replicates), three *pfk13/pfcrt/pfmdr1* triple mutant isolates (light grey diamonds, three replicates/each) and the *pfk13/pfpm2/pfcrt/pfmdr1* quadruple-mutant isolate (white triangles, six replicates) were exposed at the ring stage 0–3 h post-invasion to eight doses (from 0 to 800 nM) of the piperaquine+mefloquine combination for 48 h. Survival was calculated relative to the drug-free control after 72 h of culture. The (piperaquine+mefloquine) IC50 data were compared with the Mann–Whitney *U*-test. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

for the triple-resistant isolates ($P=0.0025$) [Figure 3(b)]. The median survival rates to dihydroartemisinin (tested with the RSA) were 0% (IQR: 0–0) for the WT isolates and 7.00% (IQR: 1.75–9.03) for the triple-resistant isolates ($P=0.0025$, Mann–Whitney *U*-test).

Susceptibility to the mefloquine+piperaquine combination was measured in the *P. falciparum* 3D7 reference clone, one dihydroartemisinin/piperaquine-resistant isolate collected in Cambodia, one *pfk13/pfpm2/pfcrt/pfmdr1* quadruple mutant and three *pfk13/pfcrt/pfmdr1* triple-mutant isolates. The median (piperaquine+mefloquine) IC_{50} values were 10.59 nM (IQR: 9.80–11.23) for the 3D7 clone, 20.11 nM (IQR: 17.38–30.15) for the dihydroartemisinin/piperaquine-resistant isolate, 42.21 nM (IQR: 35.89–76.24) for the *pfk13/pfcrt/pfmdr1* triple mutants and 66.14 nM (IQR: 63.70–81.15) for the *pfk13/pfpm2/pfcrt/pfmdr1* quadruple mutant. The IC_{50} values of the WT 3D7 sensitive clone, the triple and quadruple mutants, and dihydroartemisinin/piperaquine-resistant strains were significantly different ($P<0.01$, Mann–Whitney *U*-test).

Discussion

Our multicentric monitoring showed an overall prevalence of 0.47% of triple-resistant *P. falciparum* parasites in the first 3 years of artesunate/mefloquine implementation. This prevalence rate is much lower than the 30% detected in Northern Cambodia after less than 12 months following artesunate/mefloquine reintroduction.[6] In line with recent literature data, our finding shows that the scenario of triple-resistant *P. falciparum* surge did not occur in Cambodia.[12] Nevertheless, our study and previous reports confirm their circulation in Cambodia and also in other neighbouring countries, such as Vietnam, with a similar low

prevalence.[13] At this stage, we cannot explain the geographic discrepancies in triple-resistant parasites prevalence within Cambodia. Several factors, such as parasite genetic background, local entomological inoculation rate, immunity, treatment compliance and treatment policy, may contribute to this distribution heterogeneity, but their implication needs to be experimentally demonstrated. In terms of genotype, the main association was between *pfmdr1* amplification, *pfcrt* polymorphisms and artemisinin resistance.

Co-amplification of *pfmdr1* and *pfpm2*, initially observed in Northern Cambodia, was detected only in one strain, and was associated with increased transcription and protein expression, confirming the functional impact of this genotype. This unique strain also harbours *pfcrt* polymorphisms, thus leading to a quadruple-mutant profile. Several reasons could lead to the low representation of this profile; although complex, the fitness costs associated with resistance to piperaquine and mefloquine is well documented and might participate in the low frequency of a parasite combining these resistances.[14,15] In addition, piperaquine, mefloquine and artemisinin derivatives have not been used together at the same time in Cambodia. Thus, whether or not this quadruple-mutant profile would have been selected using this tri-therapy is unknown, our data only demonstrating the viability of this complex multiresistant genotype.

The major added value of this study was the phenotypic characterization of triple- and quadruple-mutant parasites. Our data showed that these parasites are phenotypically resistant to artemisinin, mefloquine and piperaquine *in vitro*, with susceptibility levels compatible with clinical resistance to artesunate/mefloquine and dihydroartemisinin/piperaquine. Moreover, our results showed that these parasites partially lose their susceptibility to the mefloquine/piperaquine combination *in vitro*. This indicates that mefloquine/piperaquine-resistant *P. falciparum* strains may be selected due to the current use of the mefloquine/piperaquine combination, thus questioning the recently proposed triple artemisinin-based combination strategy, in which an artemisinin derivative is combined with mefloquine and piperaquine.

In summary, we observed that *P. falciparum* parasites resistant to artemisinin, piperaquine and mefloquine circulate at a low rate in Cambodia. The genotypic and phenotypic data showed that *P. falciparum* can overcome genetic constraints thought to preclude the selection of resistance to both piperaquine and mefloquine. This genetic plasticity suggests that future therapeutic strategies should not only rely on leveraging drug resistance antagonisms to prevent malaria parasite multiresistance.

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Transparency declarations

P.R. is a staff member of the World Health Organization. The authors alone are responsible for the views expressed in this publication that do not necessarily represent the decisions, policy or views of the World Health Organization. There are no other conflicts of interest to report. None of the authors declared financial conflict of interest.

Author contributions

Data acquisition: M.M.K., C.R., S.S., A.B., N.K., S.K., C.K., N.K., R.E., C.K., C.K., S.C. Data analyses: M.M.K., C.R., N.K., R.L., P.R., J.C.B., B.W. Sampling and data management: S.K. Study design: J.C.B., B.W. Funding acquisition: J.C.B., B.W. Manuscript redaction: M.M.K., C.R., P.R., J.C.B. and B.W.

Supplementary data

Table S1 is available as Supplementary data at *JAC* Online.

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