Multidrug-Resistant *Plasmodium falciparum* Parasites in the Central Highlands of Vietnam Jeopardize Malaria Control and Elimination Strategies

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ABSTRACT *Plasmodium falciparum* resistance to dihydroartemisinin-piperaquine has spread through the Greater Mekong Subregion to southwestern Vietnam. In 2018 to 2019, we collected 127 *P. falciparum* isolates from Dak Nong (36), Dak Lak (55), Gia Lai (13), and Kon Tum (23) provinces in Vietnam's Central Highlands and found parasites bearing the *Pfkelch13* C580Y mutation and multiple *plasmepsin 2/3* genes (mean prevalence, 17.9%; range, 4.3% to 27.8%), conferring resistance to dihydroartemisinin-piperaquine. This information is important for drug policy decisions in Vietnam.

KEYWORDS *Pfcr*, *Pfkelch13*, Vietnam, dihydroartemisinin, falciparum malaria, piperaquine, *plasmepsin 2/3*, pyronaridine resistance

Artemisinin-based combination therapies (ACTs), such as dihydroartemisinin-piperaquine (DHA-PPQ), have been recommended by the World Health Organization (WHO) for first-line treatment of uncomplicated *Plasmodium falciparum* worldwide since 2006 (1, 2). In the Greater Mekong Subregion (GMS), the epicenter of resistance to all antimalarial drugs (3–6), the spread of resistance to DHA and its partner drug PPQ has markedly impacted malaria control and elimination strategies (7). Artemisinin resistance, which manifested as delayed parasite clearance (DPC), was first reported in western Cambodia in early 2008 (8, 9), the same year that DHA-PPQ was introduced in response to a rapid increase in artesunate-mefloquine treatment failures (5, 10).

Genomewide association studies linked artemisinin resistance or DPC to a locus on chromosome 13, containing at least 20 single-nucleotide polymorphisms (SNPs) in the propeller domain of the *Pfkelch13* gene (11), of which C580Y and R539T were the most prevalent (12–15). Of multiple *Pfkelch13* SNPs found in parasite populations in western Cambodia (14), over time, C580Y became the dominant haplotype with the strongest association with DPC (15). As the use of DHA-PPQ increased between 2008 and 2013, these artemisinin-resistant parasites quickly acquired resistance to PPQ via amplification of the *plasmepsin 2/3* genes on chromosome 14 and a polymorphism in the exonuclease gene on chromosome 13 (*exo-E415G*) (16). Both markers were strongly correlated with DHA-PPQ failures in Cambodia, and *plasmepsin 2/3* amplification showed the strongest correlation with high survival rates in *ex vivo* and *in vitro* piperaquine survival assays (17).

One colineage containing *Pfkelch13* C580Y allele (KEL1) and amplified *plasmepsin 2/3* (PLA1), termed KEL1/PLA1, that emerged in western Cambodia in 2008 had become dominant and spread to northern Cambodia in 2012, northeastern Thailand and southern Laos in 2014 to 2015, and then southwestern Vietnam in 2016 (Fig. 1) (18–21). As a result, DHA-PPQ cure rates fell to 12.7% in northeastern Thailand, 38.2% in western Cambodia, 73.4% in northeastern Cambodia, and 47.1% in southwestern

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FIG 1 Location of the four provinces studied and the sweep of the KEL1/PLA1 colineage from western Cambodia through the GMS (red arrow) and the likely spread from Binh Phuoc to the Central Highlands of Vietnam (blue arrow).

Vietnam by 2018 (21, 22). Furthermore, several novel and mutually exclusive mutations (T93S, H97Y, F145I, and I218F) in the *Pfcr* gene, harboring chloroquine-resistant allele CVIET at positions 72 to 76, have emerged within the KEL1/PLA1 colineage, further enhancing resistance to PPQ and fueling the spread of these subgroups of superresistant parasites (21–23).

In Vietnam, DHA-PPQ has been used since early 2000 (24, 25), with resistance first reported in the southwestern province of Binh Phuoc in 2009 and soon after in the Central Highlands provinces of Gia Lai in 2010 and Dak Nong and Quang Nam in 2012 (26). In

Binh Phuoc, treatment failures increased from 0% to 2.7% from 2012 to 2013, to 26% by 2015 (27), and to 52.9% by 2018 (22), necessitating a drug policy change, with mefloquine added to DHA-PPQ for the treatment of patients in Binh Phuoc (27, 28). Notably, in 2015, the *Pfkelch13* C580Y and *exo-E415G* polymorphisms and amplification of *plasmepsin 2/3* were observed in 94%, 93%, and 80% of recrudescing parasites, respectively, and the prevalence of the KEL1/PLA1 lineage in Binh Phuoc reached >80% by 2018 (21).

Until recently, DHA-PPQ treatment has been mostly efficacious in Central Vietnam (27, 29). Limited data have been reported on the presence and spread of parasites with the KEL1/PLA1 colineage into Central Vietnam. Thus, there is an urgent need to monitor the spread of antimalarial drug resistance in Central Vietnam.

The aim of this study was to characterize molecular markers responsible for resistance to DHA-PPQ (*Pfkelch13*, *plasmepsin 2/3*, *exo-E415G*, *Pfmdr1*, and *Pfcr1*) in samples collected from patients with symptomatic *P. falciparum* malaria in Dak Nong, Dak Lak, Gia Lai, and Kon Tum provinces in the Central Highlands of Vietnam to provide data for guiding policy changes for the treatment of multidrug-resistant parasites in the Central Highlands of Vietnam.

Recruitment of symptomatic malaria patients took place between June 2018 and February 2019 at selected district hospitals and health stations in Dak Nong (Cu Jut and Tuy Duc districts), Dak Lak (Cu Mgar and Lak districts), Gia Lai (KBang and Kong Chro districts), and Kon Tum (Ia Hdrai and Ngoc Hoi districts) provinces in the Central Highlands of Vietnam (Fig. 1). All four provinces border on eastern Cambodia, and Kon Tum also borders on southern Laos.

Patients aged 1 to 75 years who presented with malarial symptoms and were diagnosed with uncomplicated *P. falciparum* malaria by blood film microscopy were invited to participate in the study. All participants provided a finger-prick blood sample collected on Whatman 31 ET chromatography filter paper. Parasite DNA was extracted using the QIAamp DNA minikit (Qiagen GmbH, Germany). Malaria species were confirmed by seminested PCR as previously described (30). For artemisinin resistance, clinically relevant SNPs in codons 441 to 675 of the *Pfkelch13* gene were characterized using a previously described method (14). For PPQ resistance, mutation in the *exo-E415G* gene and amplification of the *plasmepsin 2/3* genes were evaluated as previously described (16, 17). Codons 72 to 76, 93, 97, 145, 218, and 220 in the *Pfcr1* gene were analyzed by PCR amplification and sequencing of the respective fragments as previously described (31), with modified D2b primer 5'-AACAAATAAGAACATAATCATAC-3' used instead of D2. The copy number of the *P. falciparum* multidrug resistance protein 1 (*Pfmdr1*), associated with lumefantrine and mefloquine resistance (32, 33), was determined by quantitative PCR assay using SYBR green chemistry (34).

Of the 127 *P. falciparum* patients recruited into the cross-sectional molecular epidemiological survey, 36 were from Dak Nong, 55 from Dak Lak, 13 from Gia Lai, and 23 from Kon Tum. The mean age of participants was 32.0 ± 10.9 years, with 1 patient aged <18 years, and 85% (108/127) of the patients were male. The mean body weight of the patients was 56.4 ± 7.6 kg, and their mean axillary body temperature was $38.7 \pm 0.7^\circ\text{C}$, with 86% (109/127) of them febrile with a temperature $\geq 37.5^\circ\text{C}$. Geometric mean parasitemia before DHA-PPQ treatment was 6,439 parasites/ μl (range, 80 to 224,889 parasites/ μl). These patients were subsequently treated with DHA-PPQ per Vietnam Ministry of Health guidelines by the health station/district hospital staff with no involvement with the study team.

Analysis of *Pfkelch13* polymorphism showed that Dak Nong and Dak Lak had similarly high prevalences of C580Y mutants (80.6% [29/36] and 83.6% [46/55], respectively), with no statistically significant difference between these areas (χ^2 , $P = 0.9236$). Kon Tum had significantly fewer parasites ($P < 0.05$) carrying *Pfkelch13* C580Y-resistant alleles than Gia Lai (30.4% [7/23] and 38.5% [5/13], respectively). There was no statistically significant difference ($P = 0.9024$) in the prevalence of C580Y mutants between the sites in Gia Lai and Kon Tum. Two patients from Kon Tum (8.7% [2/23]) were infected with parasites containing the R539T mutation. Parasites bearing this mutation had been widespread in western

TABLE 1 Molecular markers of drug resistance in blood samples collected from symptomatic patients with *Plasmodium falciparum* malaria from four provinces in Central Highlands of Vietnam

Gene polymorphism	Prevalence (% [n ^a])			
	Dak Nong (N ^b = 36)	Dak Lak (N = 55)	Gia Lai (N = 13)	Kon Tum (N = 23)
<i>Pfkelch13</i> C580Y	80.6 (29)	83.6 (46)	38.5 (5)	30.4 (7)
<i>Pfkelch13</i> R539T	0	0	0	8.7 (2)
<i>exo-E415G</i>	66.7 (24)	85.5 (47)	61.5 (8)	30.4 (7)
PM2/3 CN ^c (>1.5)	30.6 (11)	20.0 (11)	38.5 (5)	8.7 (2)
<i>Pfkelch13</i> C580Y+PM2/3 CN (>1.5)	27.8 (10)	16.4 (9)	23.1 (3)	4.3 (1)
<i>Pfcrtr</i> (72-76 CVIET)	100 (36)	92.7 (51)	100 (13)	95.7 (22)
<i>Pfcrtr</i> (72-76 CVIDT)	0	3.6 (2)	0	4.3 (1)
<i>Pfcrtr</i> (72-76 CVVET)	0	1.8 (1)	0	0
<i>Pfcrtr</i> (72-76 WGIET)	0	1.8 (1)	0	0
<i>Pfcrtr</i> T93S	13.9 (5)	27.3 (15)	7.7 (1)	13.0 (3)
<i>Pfcrtr</i> H97Y	19.4 (7)	9.1 (5)	0	4.3 (1)
<i>Pfcrtr</i> F145I	22.2 (8)	3.6 (2)	0	0
<i>Pfcrtr</i> I218F	2.8 (1)	7.3 (4)	0	0
<i>Pfcrtr</i> A220S	100 (36)	100 (55)	100 (13)	100 (23)
<i>Pfmdr1</i> CN (>1.5)	19.4 (7)	23.6 (13)	15.4 (2)	8.7 (2)

^an, number of isolates from each province.^bN, total number of isolates from each province.^cPM2/3, plasmepsin 2/3; CN, copy number.

Cambodia and Binh Phuoc before the recent hard selective sweep occurred, which resulted in complete domination of C580Y in those areas. The prevalence of C580Y mutations observed in this study was lower than that reported in a recent multicenter therapeutic efficacy study (TES) of pyronaridine-artesunate (Pyramax) conducted from 2017 to 2018, with 96.2% (25/26) in Dak Nong and 57.8% (37/64) in Gia Lai (35).

The prevalence of PPQ-resistant mutations in *exo-E415G* was also high in Dak Nong and Dak Lak, at 66.7% (24/36) and 85.5% (47/55), respectively, but significantly lower ($P < 0.05$) in Kon Tum, at 30.4% (7/23) (Table 1). In Gia Lai, the prevalence of the *exo-E415G* mutation was higher, at 61.5% (8/13), but not statistically different from that in Kon Tum ($P = 0.1426$).

Analysis of *plasmepsin 2/3* copy variation showed that the prevalence of parasites with multiple copies of the gene was higher for Dak Nong, Dak Lak, and Gia Lai (30.6% [11/36], 20.0% [11/55], and 38.5% [5/13], respectively) but lower in Kon Tum (8.7% [2/23]) (Table 1). However, the differences between the sites were not statistically significant ($P > 0.05$). These values were lower than those reported in the TES of pyronaridine-artesunate for Dak Nong (76.9% [20/26]) but comparable with those for Gia Lai (35.9% [23/64]) (35).

In this study, the prevalence of parasites with both drug-resistant markers, *Pfkelch13* C580Y and *plasmepsin 2/3* amplification, was 27.8% (10/36), 16.4% (9/55), and 23.1% (3/13) in Dak Nong, Dak Lak, and Gia Lai, respectively, but only 4.3% (1/23) in the *P. falciparum* isolates from Kon Tum, with a significant difference only between the prevalence in Dak Nong and Kon Tum ($P = 0.0375$). In contrast, the prevalence of resistant alleles reported in the TES of pyronaridine-artesunate was markedly higher in the *P. falciparum* isolates from Dak Nong (76.9% [20/26]) and Gia Lai (32.8% [21/64]) (35). These discrepancies may be attributed to relatively low numbers of subjects surveyed in both studies in Dak Nong and in the present study in Gia Lai, which results in wide confidence intervals for the mean population prevalence estimates. Furthermore, the present study was conducted during a different period (2018 to 2019) than the TES of pyronaridine-artesunate (2017 to 2018). Even though 23 of 36 *P. falciparum* isolates collected in the present study were from the Tuy Duc district in Dak Nong, the same district as in the TES of pyronaridine-artesunate (35), the local population there is highly mobile, with many people routinely travelling to the forest areas near the border with Cambodia. These factors may explain the different picture of prevalence of resistant parasites. Evidently, there may be focal areas within the

provinces with different levels of resistance to DHA-PPQ. This presents a significant challenge for drug policy and reinforces the need for surveillance to implement a prompt change in antimalarial treatments to slow the spread of DHA-PPQ resistance.

The prevalence of drug resistance-mediating polymorphisms in the transporter gene *Pfcr*t revealed that most (>92%) *P. falciparum* isolates from the four provinces had mutations at codons 72 to 76 and 220 in the *Pfcr*t gene (Table 1), confirming a high level of resistance to chloroquine. Twenty-two percent of isolates from Dak Nong had the F145I mutation associated with resistance to PPQ and DHA-PPQ treatment failures (36). The newly emerging mutations in *Pfcr*t associated with increased resistance to PPQ and DHA-PPQ at positions T93S, H97Y, and I218F were also detected (Table 1).

Note that for *P. falciparum* isolates from Dak Nong, Dak Lak, Gia Lai, and Kon Tum, the prevalence of multiple copies of the *Pfmdr*1 gene was 19.4% (7/36), 23.6% (13/55), 15.4% (2/13), and 8.7% (2/23), respectively, suggesting that a moderate number of isolates from these provinces have reduced sensitivity to lumefantrine and mefloquine. It has been shown that PPQ and mefloquine are exerting opposing selection pressure, and PPQ-resistant parasites are more likely to have a single copy of *Pfmdr*1 (16, 37). A triple combination of DHA-PPQ with mefloquine has been evaluated (38). However, the implications of applying selective pressure on “triple mutants,” if this combination is to be considered for a first-line treatment, will require further evaluation.

Our data and those from the TES of pyronaridine-artesunate (35) clearly show that the most significant mutations in *Pfkelch*13, *exo-E415G*, and *Pfcr*t, and amplification of *plasmepsin* 2/3, all characteristic of KEL1/PLA1 colineage subgroups, are prevalent in Dak Nong, Gia Lai, and Dak Lak. Kon Tum appears to have the lowest prevalence of resistant parasites. This is not surprising because, in this study, Kon Tum was the province most distant from Binh Phuoc, where the KEL1/PLA1 colineage likely entered Vietnam as it was spreading through the eastern GMS. It is likely that the KEL1/PLA1 colineage is spreading northward from Binh Phuoc and has already reached Kon Tum. The presence of resistance markers associated with lumefantrine and mefloquine, neither of which is a frontline drug in Vietnam, also suggests the possibility of cross-border movement of resistant parasites from Cambodia, where artesunate-mefloquine was used previously (10, 39), or Lao People's Democratic Republic, where artemether-lumefantrine is being used for first-line treatment of *P. falciparum* malaria (40).

Because continuous use of DHA-PPQ would only strengthen selective pressure on *P. falciparum* parasites in the Central Highlands of Vietnam, resulting in complete domination of resistant parasites throughout the country and potential spread to southern Laos, this ACT should no longer be used to treat falciparum malaria, and other options should be evaluated as soon as possible. In Vietnam, the recent TES of pyronaridine-artesunate showed the ACT to be efficacious, with PCR-adjusted adequate clinical and parasitological response at day 42 of 96.1% (147/153), in treating DHA-PPQ-resistant parasites (35). However, the proportion of patients with parasitemia at day 3 was 24%, indicating delayed parasite clearance and suggesting greater reliance on the partner drug pyronaridine to kill artemisinin-resistant parasites. It remains to be seen whether this ACT will sustain the efficacy above 90%. If used as an alternative to DHA-PPQ, pyronaridine-artesunate could be protected with a third drug to delay treatment failure (41). In the meantime, there is a need for systematic longitudinal genetic surveillance in Vietnam and elsewhere in the GMS to guide the control and elimination of multidrug-resistant *P. falciparum* parasites.

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The order of the first two authors was determined by scientific contribution, with Huynh Hong Quang responsible for conceptualization, clinical procedures, leading and

supervising the research team, and reviewing and editing the manuscript and Marina Chavchich responsible for experimental design, molecular methodologies, data curation and analysis, writing the original draft, and reviewing and editing the manuscript.

K.A.E. and N.J.M. are military service members. This work was prepared as part of their official duties.

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The study was ethically approved by the Vietnam MoH, Institute of Malarology, Parasitology and Entomology Quy Nhon (IMPE-QN) Institutional Review Board (IRB) (approvals: N-679/VSR-LSDT, 7 March 2018; and N-119/VSR-LSDT, 4 March 2019), with mutual recognition of the approved protocol by the Australian Government Departments of Defense and Veterans' Affairs Health Research Ethics Committee (DDVA HREC 2018/1029403).

We have no conflicts of interest to declare.

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