Molecular Surveillance and in vitro Drug Sensitivity Study of *Plasmodium falciparum* Isolates from the China–Myanmar Border

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Abstract. The emergence and spread of resistance in *Plasmodium falciparum* to the frontline treatment artemisinin-based combination therapies in Southeast Asia require close monitoring of the situation. Here, we collected 36 clinical samples of *P. falciparum* from the China–Myanmar border in 2014–2016, adapted these parasites to continuous culture, and performed in vitro drug assays on seven antimalarial drugs. Data for 23 parasites collected in 2010 and 2012 from the same area reported in an early study were used to assess longitudinal changes in drug sensitivity. Parasites remained highly resistant to chloroquine (CQ) and pyrimethamine, whereas they were generally sensitive to mefloquine (MFQ), lumefantrine (LMF), naphthoquine (NQ), and pyronaridine (PND). Parasites showed a similar temporal trend in sensitivity to CQ, NQ, and PND, with gradual reduction in the half-maximal inhibitory concentrations (IC₅₀s) after 2012. The IC₅₀s to the aminoalcohol drugs MFQ, LMF, and quinine (QN) all significantly declined in 2014, followed by various degrees of increase in 2016. Pyrimethamine displayed a continuous increase in IC₅₀ over the years. The Dd2-like *P. falciparum* chloroquine-resistant transporter mutations were fixed or nearly fixed in the parasite population. The *P. falciparum* multidrug resistance 1 F1226Y mutation was detected in 80% parasites in 2016 and associated with reduced sensitivity to LMF and QN ($P < 0.05$). The N51I in *P. falciparum* dihydrofolate reductase and K540E/N and A581G in *P. falciparum* dihydropteroate synthase that are associated with antifolate resistance were either fixed or were approaching fixation in recent years. This study provides an updated picture and temporal trend of antimalarial drug resistance in the China–Myanmar border region, which will serve as a reference for antimalarial treatment.

INTRODUCTION

Malaria is one of the most devastating vector-borne diseases and still threatens the lives of hundreds of millions of people in tropical and subtropical countries. Despite the significant decline in global malaria incidence, there were still 228 million malaria cases in 2018.¹ The Greater Mekong Subregion (GMS) in Southeast Asia, consisting of Cambodia, Vietnam, Laos, Thailand, Myanmar, and China's Yunnan and Guangxi provinces, has made a great stride in malaria control and is pursuing regional malaria elimination with an aim to achieve this goal by 2030. China is the first in this region to achieve zero indigenous malaria cases in 2017, but imported malaria, especially through its porous international borders with other countries in the GMS, threatens the maintenance of the malaria-free status.² The GMS is an epicenter of antimalarial drug resistance, and the emergence of artemisinin resistance in *Plasmodium falciparum* in the last decade poses a great challenge to malaria elimination.³ Artemisinin resistance, first detected in western Cambodia,^{4,5} has been found in most parts of the GMS.^{6–9} Artemisinin resistance, together with resistance to the partner drugs, has led to increased clinical failures of artemisinin combination therapies (ACTs) such as artesunate–mefloquine (AS–MFQ) and dihydroartemisinin–piperaquine (DHA–PPQ).^{10–14} There is an urgent need to strengthen resistance surveillance on *P. falciparum*, prevent the spread of resistant strains, and implement effective

treatments to ensure timely achievement of malaria elimination.

Drug resistance in malaria parasites is monitored through clinical efficacy studies of frontline drugs, ex vivo or in vitro drug assays, and molecular epidemiological studies of markers associated with resistance. Each method has its own pros and cons, and in combination, they offer a comprehensive picture of antimalarial drug resistance in a region. Although molecular markers are convenient for monitoring the evolution of drug resistance, the resistance mechanisms for many drugs are not understood. Mutations in the *P. falciparum* chloroquine-resistant transporter (*pfcr*) are the major determinants of chloroquine (CQ) resistance.^{15,16} Recently, some new *pfcr* mutations in the GMS were associated with resistance to PPQ, another 4-aminoquinoline drug.^{17,18} The *P. falciparum* multidrug resistance 1 gene (*pfmdr1*) is associated with resistance to multiple antimalarial drugs.¹⁹ In particular, the N86Y mutation contributes to the enhancement of CQ resistance. *Plasmodium falciparum* multidrug resistance 1 amplification has been associated with resistance to mefloquine (MFQ) and artemisinin derivatives in vitro.²⁰ The mechanisms of resistance to the antifolate drugs sulfadoxine and pyrimethamine (PY) are well understood and associated with mutations in the *P. falciparum* dihydropteroate synthase (*pfdhps*) gene (e.g., S436A, A437G, K540E, A581G, and A613S) and *P. falciparum* dihydrofolate reductase (*pfdhfr*) gene (e.g., N51I, C59R, S108N, and I164L), respectively. Mutation combinations in the *pfdhps* and *pfdhfr* genes mediate the failures of the drug sulfadoxine–PY.²¹ Recent studies have revealed that mutations in the propeller domain of the *P. falciparum* Kelch 13 (*Pfk13*) gene are associated with artemisinin resistance, which is manifested as delayed parasite clearance.²² A global map of *Pfk13* mutations showed that resistance-conferring mutations were restricted to the GMS.²³ The recently observed resistance to PPQ in Cambodia was

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associated with gene amplification of the hemoglobin digestive enzyme *plasmepsin 2/3*.^{24,25}

To track the evolution of drug resistance in malaria parasites in eastern and northeastern Myanmar along the China–Myanmar border, we collected longitudinal clinical samples from this region and performed in vitro drug assays and molecular studies on the archived parasite samples.²⁶ Our earlier studies demonstrated high prevalence of resistance to CQ and antifolate drugs, despite these drugs have been withdrawn from treating *P. falciparum* for a long time. For several drugs, our studies also revealed significant variations in sensitivity between years. As a continuation of the resistance monitoring efforts, we collected *P. falciparum* clinical isolates in 2014 and 2016 and adapted them to continuous culture. Through in vitro drug assays and genotyping of drug resistance–related genes, we wanted to obtain an updated drug sensitivity profile and understand how the parasite population evolves in the face of intensified control efforts.

MATERIALS AND METHODS

Parasite sample collection. Clinical isolates of *P. falciparum* were collected in 2014 and 2016 from malaria clinics located at the Nabang township in west Yunnan Province, China, and the Laiza town, Kachin State, Myanmar, along the China–Myanmar border. Diagnosis by microscopy was performed initially by field medical staff and later, confirmed by PCR in the laboratory. Patients, after signing the written informed consent, donated up to 5 mL of venous blood, which was used to establish continuous parasite culture. The human subject protocol for this study was approved by the Institutional Review Boards of Kunming Medical University and Pennsylvania State University.

Genetic polymorphisms of drug resistance genes. DNA extraction from in vitro–cultured *P. falciparum* blood samples was performed by using the High Pure PCR Template Preparation Kit (Roche Life Science, Indianapolis, IN). Parasites were first genotyped at the *P. falciparum* merozoite surface proteins *msp1* and *msp2* and *glutamate-rich protein (glurp)* genes.²⁷ Only the monoclonal infections of the *P. falciparum* isolates were used for genotyping drug resistance genes and for in vitro drug assays. Polymorphisms in resistance-related genes were determined by PCR and sequencing of the amplified fragment.^{28,29} The *pfprt* fragments covered codons 72–76, 220, 271, 326, 356, and 371; two *pfmdr1* fragments covered codons 86, 184, 1240, and 1246; a *pfdhfr* fragment included codons 51, 59, 108, and 164; and two *pfdhps* fragments contained the codons 436, 540, and 581.

In vitro culture of *P. falciparum* and drug assays. Parasite isolates were cultured in type O⁺ human red blood cells (RBCs) in a complete medium under an atmosphere of 92% N₂–3% O₂–5% CO₂. Parasites in the ring stage were synchronized by 5% sorbitol and diluted with complete medium to a 0.5% parasitemia and 2% hematocrit. We used the SYBR® green I-based assay to determine the sensitivities of *P. falciparum* isolates to seven antimalarial drugs using a 96-well plate format. Drugs were added to each well of a 96-well microplate at an initial concentration of 3.75 μ M for CQ and PY, 256 nM for naphthoquine (NQ) and MFQ, 160 nM for pyronaridine (PND), 10.24 μ M for quinine (QN), and 800 nM for lumefantrine (LMF), which were serially diluted. In each plate, the 3D7 reference

strain was included as an internal control. Three technical repeats and two biological replicates were performed for each parasite isolate.

Statistical analyses. All data were analyzed with SPSS 20 and GraphPad Prism 6.0. The half-maximal inhibitory concentrations (IC₅₀s) were calculated by fitting the drug response data to a sigmoid curve. Geometric mean and SD were calculated based on the replicates of each isolate. Median and interquartile range were used because the data were not normally distributed. The IC₅₀ values between the years were compared using the Mann–Whitney U test. Mutation frequencies of drug resistance genes were analyzed using the Chi-square test.

RESULTS

In vitro drug sensitivity profiling of parasite isolates. We collected and culture-adapted 16 and 20 monoclonal *P. falciparum* isolates from the China–Myanmar border in 2014 and 2016, respectively. Using a standard SYBR® green-I–based method, we measured their in vitro IC₅₀ values to seven antimalarials (Figure 1, Table 1). Previously published data of 23 parasite isolates collected in 2010 and 2012 from the same study area were used for constructing the trends.²⁶ For CQ, 96.4% of the parasites were resistant with IC₅₀ values higher than the 100 nM cutoff value (Figure 1).³⁰ For MFQ, 42.4% parasite isolates were considered resistant with IC₅₀ above the 30 nM cutoff value proposed in an earlier study.³⁰ The median IC₅₀ of QN was significantly lower than the cutoff value of 600 nM as proposed earlier,³⁰ with 6.8% of the isolates exceeding this value. For PND, 5.1% isolates had higher IC₅₀ values than the cutoff value of 15 nM as proposed earlier.³¹ Given that there were no threshold values defined for resistance to LMF and NQ, we used the mean + 2 SDs as arbitrary cutoff values for resistance.³² Three isolates had higher IC₅₀ values above the 8.6 nM threshold level for LMF, and 5.1% of the isolates had IC₅₀ values above the cutoff value of 19 nM for NQ (Figure 1). For the antifolate drug PY, IC₅₀ $\leq$ 100 nM, 100 < IC₅₀ $\leq$ 2000 nM, and IC₅₀ $\geq$ 2000 nM were classified as sensitive, moderately resistant, and highly resistant.^{30,33} According to this classification scheme, all isolates tested were considered highly resistant to PY (IC₅₀s $\geq$ 2000 nM) (Figure 1).

Temporal trend of in vitro drug sensitivities. There appeared to be three types of temporal trends of parasites' sensitivities to the seven antimalarials tested (Figure 2). For CQ, there was a continuous decrease in mean IC₅₀ values in 2012, 2014, and 2016. The annual mean IC₅₀s for another 4-aminoquinoline drug NQ and PND also showed a similar decreasing trend during 2010–2016. Interestingly, in vitro IC₅₀s of the parasites to the three aminoalcohol drugs MFQ, QN, and LMF showed a similar trend of fluctuation with a significant decrease in 2014 and an increase in 2016. Among them, parasites showed a significant higher IC₅₀ to LMF and QN in 2016 than 2014 ($P < 0.01$, Mann–Whitney U test) (Figure 2). By contrast, only the IC₅₀ values to PY showed a significant upward annual trend, with the IC₅₀ values in 2014 and 2016 being significantly higher than the values in 2010 and 2012 ($P < 0.001$).

Polymorphisms of drug resistance genes. We genotyped *pfprt*, *pfmdr1*, *pfdhps*, and *pfdhfr* genes in the isolates collected in 2014 and 2016. For *pfprt*, mutations at the M74I,

FIGURE 1. In vitro IC_{50} values of culture-adapted parasite isolates collected from the China–Myanmar border to seven antimalarial drugs chloroquine (CQ), quinine (QN), pyrimethamine (PY), mefloquine (MFQ), lumefantrine (LMF), naphthoquine (NQ), and pyronaridine (PND). The dot plots show the median and interquartile range. Red bars indicate the cutoff values used to define resistance: CQ, 100 nM; QN, 600 nM; PY, 100 nM; MFQ, 30 nM; LMF, 9.6 nM; NQ, 19 nM; and PND, 15 nM. Note that except for CQ and PY, the cutoff values for other drugs are arbitrary and do not necessarily correspond to in vivo clinical resistance. This figure appears in color at www.ajtmh.org.

N75E, K76T, A220S, Q271E, N326S, and R371I positions were all fixed in these isolates. Only one of the 15 parasites genotyped in 2014 was wild type at the I356T position (Table 2). For the *pfmdr1* gene, the N86Y mutation was rare and found only in one isolate in 2014. Interestingly, the Y184F mutation was highly abundant in 2014 (86.7%), whereas it was relatively infrequent in other years (7.7–15%) ($P < 0.0001$, χ^2 test). Whereas the *pfmdr1* 1042 and 1246 positions all remained wild type, the F1226Y mutation was identified in 2016 in 80% (16/20) parasite isolates. For the *pfdhps* gene, the K540E/N mutations were fixed in samples from 2010, 2014, and 2016. Whereas the A581G mutation showed an increasing trend from 40% in 2010 to 95% in 2016, the S436A mutation showed an opposite trend, decreasing from 40% in 2010 to disappearance in 2016 ($P < 0.001$). For the *pfdhfr* gene, both the N511 and I164L mutations were increased in frequency from 2010 and reached fixation in 2014 and 2016 ($P < 0.05$), whereas the C59R and S108N mutations were fixed in all samples.

Association of mutations with in vitro drug susceptibilities.

With the identification of the *pfmdr1* F1226Y mutation in parasite samples from 2016, we wanted to determine whether the *pfmdr1* mutations were associated with altered drug sensitivities. Neither Y184F nor F1226Y was associated with in vitro sensitivities of the

parasites to CQ, NQ, or PY (data not shown). However, the Y184F mutation was associated with significantly increased sensitivities to the aminoalcoholic drugs QN ($P < 0.001$), LMF ($P < 0.01$), and MFQ ($P < 0.05$) as well as PND ($P < 0.05$) (Figure 3). The F1226Y mutation was associated with significantly reduced sensitivities to QN ($P < 0.001$) and LMF ($P < 0.05$).

Haplotypes of drug resistance genes. For *pfprt*, 98.2% of the parasite isolates carry the octuple mutations at the 74/75/76/220/271/326/356/371 amino acid positions, a CQ-resistant genotype similar to that of Dd2 (Table 3). Three haplotypes were identified for the *pfmdr1* gene at the 86/184/1226 positions: the wild type (NYY) was predominant in 2010 and 2012, whereas two haplotypes with a single mutation NFF and NYY were the predominant haplotypes in 2014 and 2016, respectively (Table 3). For the antifolate resistance genes, 96.7% of the parasite isolates carried triple or quadruple mutations at the 521/59/108/164 positions in *pfdhfr*, although 88.2% of the parasite isolates carried a single or double mutation at 436/540/581 positions in *pfdhps*. The quadruple mutant haplotype **IRNL** of *pfdhfr* was present in 50% of the isolates in 2010, as compared with complete fixation in 2014 and 2016 ($P < 0.0001$). By comparison, the double-mutation haplotypes (**AE/NA** and **SE/NG**) in *pfdhps*, which were prevalent in 2010 and 2012, were

TABLE 1

In vitro IC_{50} s (nM) of culture-adapted *Plasmodium falciparum* isolates collected in 2014 and 2016 from the China–Myanmar border to seven antimalarial drugs

Drug	2014 (N = 16)		2016 (N = 20)		3D7 control (mean $\pm$ SD)
	Median (IQR)	Range	Median (IQR)	Range	
Chloroquine*	386.1 (236.3–632.9)	72.7–826.9	254.0 (235.7–408.9)	222.6–727.0	17.8 $\pm$ 8.1
Mefloquine	16.9 (12.6–19.9)	7.2–29.8	17.6 (14.6–30.2)	10.0–51.6	19.9 $\pm$ 1.2
Lumefantrine	1.9 (1.6–2.3)	1.2–3.0	3.5 (2.5–4.4)	1.3–12.5	1.5 $\pm$ 0.2
Quinine	93.3 (59.1–128.6)	43.3–197.7	149.4 (108.5–198.1)	58.4–314.1	34.6 $\pm$ 1.7
Naphthoquine	5.6 (1.9–7.8)	1.0–14.8	4.1 (1.8–10.7)	1.0–17.0	7.5 $\pm$ 0.5
Pyrimethamine	9705.0 (6137.0–14256.0)	4839.0–16679.0	11021.0 (9229.0–12592.0)	6737.0–18522.0	67.5 $\pm$ 12.8
Pyronaridine	7.5 (5.1–9.6)	4.6–11.6	7.9 (5.4–10.3)	3.3–13.4	5.0 $\pm$ 0.5

IQR = interquartile range (Mann–Whitney U test).

* For chloroquine, 15 isolates were tested in 2014 and 17 in 2016.

FIGURE 2. In vitro IC_{50} values of culture-adapted parasite isolates from 2010, 2012, 2014 and 2016 to seven antimalarial drugs. The dot plot shows that the median and interquartile range. *, **, ***, and **** indicate significant difference between the years at $P < 0.05$, 0.01, 0.001, and 0.0001, respectively. Data for the 2010 and 2012 parasites were generated in a previous study.²⁶

replaced by the single-mutation haplotype (SE/NA) in 2014 and 2016.

DISCUSSION

Plasmodium falciparum accounts for the vast majority of malaria-associated morbidity and mortality worldwide. The

spread of multidrug-resistant *P. falciparum* is one of the most important factors hindering the global elimination of malaria. As the GMS is moving toward malaria elimination, the heavy malaria burden in Myanmar needs particular attention. Furthermore, with Myanmar's geographical connection to South Asia, drug resistance could be amplified here and spilled over to the Indian subcontinent. In this study, we further profiled

TABLE 2
Prevalence of non-synonymous mutations in drug resistance-associated genes in 2010–2016

Gene	Amino acid position	Prevalence of isolates (n[%])				Total (n = 59)	P-value‡
		2010 (n = 10)	2012 (n = 13)	2014 (n = 16)	2016 (n = 20)		
Pfcr ^t *	M74I	10 (100)	13 (100)	15 (100)	17 (100)	55 (100)	ND
	N75E	10 (100)	13 (100)	15 (100)	17 (100)	55 (100)	ND
	K76T	10 (100)	13 (100)	15 (100)	17 (100)	55 (100)	ND
	A220S	10 (100)	13 (100)	15 (100)	17 (100)	55 (100)	ND
	Q271E	10 (100)	13 (100)	15 (100)	17 (100)	55 (100)	ND
	N326S	10 (100)	13 (100)	15 (100)	17 (100)	55 (100)	ND
	I356T	10 (100)	13 (100)	14 (93.3)	17 (100)	54 (98.2)	0.438
	R371I	10 (100)	13 (100)	15 (100)	17 (100)	55 (100)	ND
Pfmdr1†	N86Y	0	0	1 (6.7)	0	1 (1.7)	0.405
	Y184F	1 (10)	1 (7.7)	13 (86.7)	3 (15.0)	18 (31)	< 0.0001
	F1226Y	0	0	0	16 (80.0)	16 (27.6)	< 0.0001
<i>P. falciparum</i> dihydrofolate reductase	N51I	7 (70)	10 (76.9)	16 (100)	20 (100)	53 (89.8)	0.013
	C59R	10 (100)	13 (100)	16 (100)	20 (100)	59 (100)	ND
	S108N	10 (100)	13 (100)	16 (100)	20 (100)	59 (100)	ND
	I164L	8 (80)	8 (61.5)	16 (100)	20 (100)	52 (88.1)	0.003
<i>P. falciparum</i> dihydropteroate synthase	S436A	4 (40)	4 (30.8)	1 (6.3)	0	9 (15.3)	0.008
	K540E/N	10 (100)	8 (61.5)	16 (100)	20 (100)	54 (91.5)	< 0.0001
	A581G	4 (40)	9 (69.2)	15 (93.8)	19 (95)	47 (79.7)	0.001

The 2010 and 2012 data from Bai et al.²⁶ were included for comparison. Mutations are shown in bold. ND = not done.

* *Plasmodium falciparum* chloroquine-resistant transporter (Pfcr^t) gene was genotyped in 15 isolates in 2014 and 17 isolates in 2016.

† *Plasmodium falciparum* multidrug resistance 1 (Pfmdr1) gene was genotyped in 15 isolates in 2014.

‡ χ^2 test comparison for each mutation among the 4 years.

FIGURE 3. Association of the *pfmdr1* Y184F and F1226Y mutations with in vitro sensitivities to quinine, lumefantrine, mefloquine, and pyronaridine in parasites collected from 2010 to 2016. *, **, and *** indicate significance at $P < 0.05$, 0.01, and 0.001, respectively.

in vitro drug sensitivities of parasite isolates obtained in recent years from the China–Myanmar border and genotyped molecular markers of antimalarial drug resistance in these isolates. Together with the drug sensitivity and molecular epidemiological data from earlier years,^{26,34} we provide detailed trends of drug resistance of the parasites from this region.

The drug sensitivity profile of the *P. falciparum* parasites may well reflect the local antimalarial drug policies. In Myanmar, ACTs (artemether–lumefantrine, DHA–PPQ, and AS–MFQ) were introduced in 2008 as the first-line treatment for uncomplicated *P. falciparum* malaria.³⁵ In the China–Myanmar border area, the predominant ACT used is DHA–PPQ, and earlier studies indicated that DHA–PPQ retained excellent efficacy for uncomplicated *P. falciparum* malaria.^{36,37} It is noteworthy that despite recommendations from the national malaria treatment guidelines, various antimalarial drugs including artemisinin monotherapies could be found in the private sectors. Therefore, although all parasites evaluated in this study were collected after the introduction of ACTs, the extensive uses of other antimalarials may underline the dynamic changes of drug sensitivities of the parasites observed in our study area over the years.

Chloroquine resistance was discovered on the Cambodia–Thailand border as early as in the late 1950s, and resistant parasite strains spread to countries in Southeast Asia in the late

1960s.³⁸ As we have found previously,²⁶ parasite isolates in recent years remained highly resistant to CQ, a phenomenon that is often attributed to the wide use of CQ for treating symptomatic *Plasmodium vivax* infections. This in vitro drug susceptibility result is further supported by the molecular study of the *pfcr1* gene, which showed that 98.2% of the parasites carried the octuple mutations in the *pfcr1* gene. This particular Dd2-like *pfcr1* genotype is the most predominant genotype present in the GMS, and from this genetic background, the artemisinin and PPQ resistance has evolved.^{39,40} Despite the persistence of the Dd2-like *pfcr1* genotype, compared with 2012, there was a significant reduction in IC_{50} to CQ among parasites collected in 2014 and 2016, although all except two parasite isolates still had in vitro IC_{50} values above the 100 nM cutoff value for high-level resistance (Figure 2). We speculate that this change may be mediated by changes in other molecular markers such as *pfmdr1* that modify CQ resistance. Despite that another 4-aminoquinoline drug NQ showed a similar trend of reduction in IC_{50} after the year 2010, parasites remained sensitive to NQ with most of the parasites in 2014–2016 having IC_{50} values below 15 nM. In support of this, single-dose NQ in combination with azithromycin has been shown to be highly effective in preventing malaria infections as a monthly malaria prophylaxis drug in the China–Myanmar border area.⁴¹ This suggests that the major resistance mechanisms of CQ and NQ might be

TABLE 3
Prevalence of major haplotypes of drug resistance-associated genes in *P. falciparum* isolates from different years

Gene	Haplotype‡	Prevalence of isolates (n[%])				Total (n = 59)	P-value§
		2010 (n = 10)†	2012 (n = 13)†	2014 (n = 16)	2016 (n = 20)		
Pfcr1*	IETSESTI	10 (100)	13 (100)	14 (93.3)	17 (100)	54 (98.2)	0.438
Pfmdr1*	NYF	9 (90)	12 (92.3)	1 (6.7)	1 (5)	23 (39.7)	< 0.0001
	NFF	1 (10)	1 (7.7)	13 (86.7)	3 (15.0)	18 (31)	< 0.0001
	NYF	—	—	—	16 (80.0)	16 (27.6)	< 0.0001
Pfdhfr	IRNI	2 (20)	3 (23.1)	—	—	5 (8.5)	0.035
	NRNL	3 (30)	1 (7.7)	—	—	4 (6.8)	0.011
	IRNL	5 (50)	7 (53.8)	16 (100)	20 (100)	48 (81.4)	< 0.0001
Pfdhps	SE/NA	2 (20)	—	15 (93.8)	19 (95)	36 (61.0)	< 0.0001
	AE/NA	4 (40)	3 (23.1)	—	—	7 (11.9)	0.003
	SE/NG	3 (30)	5 (38.5)	—	1 (5)	9 (15.3)	0.009

Only haplotypes with prevalence $\geq 5\%$ were included. Mutations are shown in bold.

* The *P. falciparum* chloroquine-resistant transporter (*pfcr1*) gene was tested in 15 strains in 2014 and 17 strains in 2016, and 55 strains in total. The *P. falciparum* multidrug resistance 1 (*pfmdr1*) genes were tested in 15 strains in 2014, and 58 strains in total.

† Data for 2010 and 2012 were from Yao Bai et al.²⁶

‡ Haplotypes were based on amino acids at the positions *pfcr1* (74, 75, 76, 220, 271, 326, 356, and 371); *pfmdr1* (86, 184, and 1226); *P. falciparum* dihydropteroate synthase (*pfdhps*) (436, 540, 581), and *P. falciparum* dihydrofolate reductase (*pfdhfr*) (51, 59, 108, and 164).

§ The *P*-value analyzes the overall trend between four years (χ^2 test, SPSS 20).

different.⁴² Another drug that showed a similar trend to the two aminoquinoline drugs is PND, a drug that is used as an injection at the China–Myanmar border. It is not widely used, and parasites remained highly sensitive to this drug. Resistance mechanism to PND is not clear, but cross-resistance to aminoquinolines was generally not observed.⁴³

Interestingly, in vitro sensitivities of longitudinally collected parasite isolates to the three aminoalcohol drugs QN, LMF, and MFQ showed a similar trend with a significant decline in IC₅₀s in 2014, but a subsequent increase in 2016. Overall, the mean IC₅₀ levels of these drugs in 2014–2016 remained much lower than in 2010–2012. *Plasmodium falciparum* multidrug resistance 1 amplification has been associated with resistance to this family of drugs, and extensive use of MFQ has selected parasites with high copy numbers of *pfmdr1*.^{20,28,44} The very low level of *pfmdr1* amplification in this region as shown in our earlier studies^{27,45} could be due to the lack of MFQ use and deployment of PPQ either as monotherapy or in ACT with DHA, which selects against *pfmdr1* gene amplification.^{24,25} Although such a temporal fluctuation of in vitro sensitivities to these three drugs was not clear, it might be related to the drug use changes. Artemether–lumefantrine has been recently introduced to the study area in addition to DHA–PPQ as the main ACT used in the study area. Correlations in in vitro sensitivities to these three drugs suggest similar resistance mechanisms, and cross-resistance need to be monitored.

Results from the in vitro drug assay of PY and genotyping the *pfdhfr* and *pfdhps* genes all suggest that parasites from this region maintained a high degree of resistance to antifolate drugs. All parasites tested showed IC₅₀s to PY above 2000 nM (highly resistant), and moreover, the in vitro susceptibilities of the parasite isolates to PY kept decreasing during 2014–2016, although antifolate drugs have been withdrawn from treating malaria a long time ago. Accompanying this increasing trend in in vitro IC₅₀, several resistance-conferring mutations in *pfdhfr* (N51I) and *pfdhps* (K540E/N and A581G) had drastically increased in frequency and were fixed or approached fixation in 2014 and 2016. We do not know the sources for the continuous selection on these antifolate resistance genes but speculate that it could be due to collateral selection from extensive use of antifolate drugs for bacterial infections.

In addition to the observed temporal increase in *pfdhfr* and *pfdhps* mutations, changes in two other genes are also worth noting. For *pfmdr1*, we detected the F1226Y mutation in 80% parasite isolates in 2016, all of them being the N₈₆Y₁₈₄Y₁₂₂₆ haplotype. A study from the Thailand–Myanmar border showed that F1226Y was associated with reduced in vitro artemisinin sensitivity,⁴⁶ suggesting that this change may be due to the widespread use of ACTs in recent years. It is also noteworthy that our earlier study found that all parasites collected in 2014–2016 contained the NN insertion at amino acids 136–137, the F446I mutation, or the new mutation G533S at the *PfK13* gene.³⁴ Thus, it is also possible that these parasites from 2014 to 2016 may represent newly introduced parasites with the migrant human populations escaping the Kachin civil war.⁴⁷

The combination of in vitro drug sensitivity assay and molecular surveillance provides a comprehensive picture of antimalarial drug resistance. Unfortunately, the cutoff values for resistance of some drugs such as NQ and PND are not defined, and molecular markers for resistance to some drugs are not known. One limitation of this study is the relatively small

sample size. In recent years, as malaria incidence has gone down drastically, collection of samples for laboratory culture has become difficult. Thus, future studies may need to include clinical efficacy studies, on-site ex vivo drug assays, and molecular epidemiology studies so that the drug resistance information can be used to inform timely changes of drug policy.

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