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Longitudinal surveillance of drug resistance in *Plasmodium falciparum* isolates from the China-Myanmar border reveals persistent circulation of multidrug resistant parasites

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ABSTRACT

Multidrug-resistant *Plasmodium falciparum* in the Greater Mekong Subregion of Southeast Asia is a major threat to malaria elimination and requires close surveillance. In this study, we collected 107 longitudinal clinical samples of *P. falciparum* in 2007–2012 from the malaria hypoendemic region of the China-Myanmar border and measured their in vitro susceptibilities to 10 antimalarial drugs. Overall, parasites had significantly different IC_{50} values to all the drugs tested as compared to the reference 3D7 strain. Parasites were also genotyped in seven genes that were associated with drug resistance including *pfcrt, pfmdr1, pfmrp1, pfdhfr, pfdhps, pfnhe1*, and *PfK13* genes. Despite withdrawal of chloroquine and antifolates from treating *P. falciparum*, parasites remained highly resistant to these drugs and mutations in *pfcrt, pfdhfr*, and *pfdhps* genes were highly prevalent and almost reached fixation in the study parasite population. Except for pyronaridine, quinine and lumefantrine, all other tested drugs exhibited significant temporal variations at least between some years, but only chloroquine and piperaquine had a clear temporal trend of continuous increase of IC_{50}s. For the *pfmrp1* gene, several mutations were associated with altered sensitivity to a number of drugs tested including chloroquine, piperaquine, lumefantrine and dihydroartemisinin. The association of *PfK13* mutations with resistance to multiple drugs suggests potential evolution of *PfK13* mutations amid multidrug resistance genetic background. Furthermore, network analysis of drug resistance genes indicated that certain haplotypes associated multidrug resistance persisted in these years, albeit there were year-to-year fluctuations of the predominant haplotypes.

1. Introduction

Malaria, a life-threatening disease caused by the *Plasmodium* parasites, has claimed over 400 000 human lives globally in 2016 (WHO, 2017). In the tropical and subtropical areas of the Greater Mekong Subregion (GMS), recent achievements in malaria control have encouraged countries within this region to pursue malaria elimination, aiming to reach this goal by 2030. Chemotherapy is an essential tool for malaria management, but its effectiveness is compromised by the emergence and spread of drug-resistant *Plasmodium falciparum* strains. Chloroquine (CQ) was one of the most widely used antimalarial drugs. Only several years after its introduction, CQ-resistant cases emerged firstly in Southeast Asia, then appeared in Latin America, and spread to all other endemic areas (Wellems and Plowe, 2001). This also happened to the antifolates drug pyrimethamine (PY). The GMS is a breeding ground of antimalarial drug resistance, and *P. falciparum* has developed resistance to essentially all commonly used antimalarial drugs (Fairhurst and Dondorp, 2016). Multidrug-resistant (MDR) parasites

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have led to the deployment of artemisinin combination therapies (ACTs). However, artemisinin resistance in *P. falciparum* has also emerged in the same place of the GMS (Noedl et al., 2008; Dondorp et al., 2009), where CQ and PY resistance first emerged. Furthermore, resistance to the partner drug mefloquine (MQ) and recently piperaquine (PQ) have resulted in increased clinical failures of the artesunate (AS)-MQ and dihydroartemisinin (DHA)-PQ, respectively (Wongsrichanalai and Meshnick, 2008; Saunders et al., 2014; Spring et al., 2015). With the unfolding of malaria elimination campaign in the GMS, heightened surveillance of drug resistance in *P. falciparum* is required in order to monitor the situation, prevent the spread of resistant parasites, and make timely changes of the national drug treatment policies.

The identification of drug resistance mechanisms facilitates molecular surveillance of antimalarial drug resistance (Ekland and Fidock, 2007). The K76T mutation in the *P. falciparum* chloroquine resistance transporter, pfcrt, is a major determinant of CQ resistance (Fidock et al., 2000). Point mutations in dihydropteroate synthase (dhps) and dihydrofolate reductase (dhfr), two key enzymes in the folate biosynthesis pathway, mediate resistance to the antifolates sulfadoxine and PY, respectively (Gregson and Plowe, 2005). As its name indicates, point mutations in the multidrug resistance 1 (*mdr*1) gene confer resistance to a number of drugs, while *mdr1* gene amplification is responsible for clinical resistance to MQ (Price et al., 2004), and in vitro resistance to other amino alcohol drugs (Sidhu et al., 2006). In recent years, the advancement of genomic tools allowed accelerated identification of resistance mechanisms to artemisinin and PQ. Through a combination of in vitro selection, genomics and population biology, artemisinin resistance was found to be associated with point mutations in the propeller domain of the *PfK13* gene (Ariey et al., 2014), which were subsequently confirmed by genetic manipulations (Ghorbal et al., 2014; Straimer et al., 2015). Similarly, genome-wide association studies revealed that amplification of two protease genes *plasmepsin 2/3* was associated with clinical resistance to PQ in Cambodia (Amato et al., 2017; Witkowski et al., 2017). In areas of low transmission where host immunity against the malaria parasites is low, molecular markers serve as proxies for the prediction of efficacies of antimalarial drugs and provide convenient assessment of the epidemiology of drug resistance in malaria parasites.

“Border malaria” – concentrated malaria transmission along international borders – brings extreme difficulties for surveillance, and malaria re-introduction by cross-border migratory human populations could plunge people of malaria eliminating countries into malaria resurgence (Delacollette et al., 2009; Cui et al., 2012). Since drug policies in neighboring countries may differ considerably, parasite populations at the border may experience divergent drug selection pressures, favoring the emergence of MDR parasites (Zeng et al., 2017). The China-Myanmar border used to be a malaria hyperendemic region with a distinct antimalarial drug use history. Since 1979, PQ has been used extensively as a replacement drug of CQ in China, which has led to clinical resistance to PQ (Gao et al., 1993; Yang et al., 1999). Also, artemisinin drugs had been deployed mostly as monotherapies prior to 2005. After 2005, the national antimalarial drug policy has changed to ACT, mostly DHA-PQ, as the frontline treatment for uncomplicated *P. falciparum* cases in this region. Clinical follow-ups in recent years showed that DHA–PQ remained highly efficacious for treating uncomplicated falciparum malaria (Liu et al., 2015; Wang et al., 2015a). Further studies of parasites from this region also showed that day-3 parasite positivity as well as delayed parasite clearance were associated with *PfK13* mutations (Huang et al., 2015; Wang et al., 2015c). Moreover, consistent with extensive deployment of ACT, the proportions of parasites carrying the *PfK13* mutations have been increasing (Wang et al., 2015b). Possibly reflecting the divergent antimalarial drug histories, parasites from this region showed a *PfK13* mutation pattern that is distinct from that in Cambodia (Ariey et al., 2014; Tun et al., 2015; Wang et al., 2015b). Thus, continuous monitoring of antimalarial drug resistance in this region is warranted.

In the present study, we performed a longitudinal follow-up of in vitro sensitivities in *P. falciparum* clinical isolates collected during 2007–2012 to commonly-used antimalarial drugs and determined dynamic changes in drug sensitivities and polymorphisms of genes associated with drug resistance. These data combined allowed us to further detect and confirm associations between drug-resistant genes and in vitro sensitivities to several antimalarial drugs. Our results revealed the persistent circulation of MDR parasites and further highlighted the necessity of close drug-resistance monitoring in the GMS in order to use updated drug policy for a specific region and period.

2. Material and methods

2.1. Parasite sample collection

To longitudinally follow *P. falciparum* in vitro sensitivities to antimalarial drugs at the China-Myanmar border, we collected 107 clinical parasite samples from acute, uncomplicated *P. falciparum* infections from malaria clinics located near the Nabang township in west Yunnan Province, China, and the Laiza township, Kachin State, Myanmar, during 2007–2012. Malaria diagnosis was based on microscopy of Giemsa-stained blood smears, and 2–5 ml venous blood was drawn from patients with falciparum malaria. Blood samples were stored in liquid nitrogen and used for culture adaptation. All patients in this study signed informed consent forms voluntarily and the research project was approved by the institutional review board of Kunming Medical University.

2.2. Parasite culture and in vitro drug assay

Culture-adapted parasite isolates were assayed for their in vitro sensitivities to 10 antimalarial drugs. CQ, MQ, quinine (QN), and PY were purchased from Sigma (St. Louis, MO, USA). PQ was from Chongqing Kangle Pharmaceutical Co. (Chongqing, China), pyronaridine (PND) was obtained from the China Institute of Pharmaceutical and Biological Products (Beijing, China), while naphthoquine (NQ), lumefantrine (LMF), AS, and DHA were from Kunming Pharmaceutical Co. (Kunming, Yunnan, China). Stock solutions of CQ, NQ, PND, and PQ were prepared in distilled water, MQ, QN, LMF, AS and DHA in ethanol, and PY in 1% acetic acid. Only monoclonal isolates were used for drug assays (Meng et al., 2010; Yuan et al., 2013). Parasite culture, synchronization and drug assay using the SYBR Green I-based method were performed as described (Smilkstein et al., 2004; Wang et al., 2016). Drugs were added to each well of a 96-well microplate at an initial concentration of 3.75 μM for CQ and PY, 256 nM for NQ and MQ, 1.5 μM for AS and DHA, 160 nM for PND, 320 nM for PQ, 10.24 μM for QN, and 800 nM for LMF, which were serially diluted. Each parasite strain was assayed with three technical repeats and two biological replications, and the 3D7 strain was included in all assays as an internal reference.

2.3. Sequencing analysis of drug resistance genes

Parasite genomic DNA was extracted from cultured parasites using a QiaAmp DNA minikit (Qiagen). Polymorphisms in drug resistance genes were determined by PCR and sequencing as previously reported (Yang et al., 2011; Gupta et al., 2014; Wang et al., 2015b). These include two *pfcrt* fragments covering codons 72–76 and 220, two *pfmdr1* fragments including codons 86, 184, 1042 and 1246, a *pfdhfr* fragments containing codons 51, 59, 108 and 164, two *pfdhps* fragments containing codons 436, 437, 540, and 581, a *pfnhe1* fragment containing the ms4760 minisatellite, and two *pfmrp1* gene fragments containing codons 191, 325, 437, 785, 876, 1007, 1390 and the complete sequence of *PfK13* gene.

2.4. Statistical analyses

The geometric mean of the half-maximal inhibitory concentration (IC_{50}) was calculated by fitting the drug response data to a sigmoid curve. Median and interquartile range (IQR) were used since the data were not normally distributed. IQR, mean and standard deviation (SD) were determined using GraphPad Prism 6.0 for Windows. IC_{50} values of parasite isolates among the years as well as between the field isolates and 3D7 were compared by Mann-Whitney *U* test. Correlations between IC_{50}s of drugs were determined using Spearman's test in the R package and MATLAB R2013a. Associations between IC_{50}s and mutations were investigated by multiple *t*-tests. The relationship among the haplotypes was analyzed by using the neighbor-joining algorithm in MEGA version 7 (Kumar et al., 2016) and the haplotype network program pegas in the R package (https://cran.r-project.org/web/packages/pegas/index.html).

3. Results

3.1. In vitro susceptibilities of parasite isolates to antimalarial drugs

A total of 107 clinical *P. falciparum* samples from the China-Myanmar border in 2007 (22 isolates), 2008 (41 isolates), 2009 (22 isolates), 2010 (10 isolates), and 2012 (13 isolates) were culture-adapted and assayed for in vitro sensitivities to 10 antimalarial drugs (Table 1, Fig. 1). Overall, the field parasite isolates had significantly higher IC_{50} values to all drugs than 3D7 ($P < 0.0001$, Mann-Whitney *U* test) (Table 1). For CQ, 14.0% and 86.0% parasite isolates were considered moderately resistant (25 nM ≤ IC_{50} < 100 nM) and highly resistant (IC_{50} ≥ 100 nM) based on criterion described earlier (Table 1) (Ringwald et al., 1996; Nkhoma et al., 2007; Chaijaroenkul et al., 2010). The other two 4-aminoquinoline drugs PQ and NQ remained highly active against parasites in culture with median IC_{50}s being in the lower nanomolar range, at 11.0 and 10.8 nM, respectively. Since the in vitro resistance thresholds for PQ and NQ were not defined, we arbitrarily estimated the cutoff values for resistance using the tradition of calculating the mean plus 2 SDs of the IC_{50}s of the 107 field isolates (Pascual et al., 2015), which were both close to 30 nM (Table 1). If these cutoff values were used, 6.5% and 4.7% parasite isolates were above these cutoffs for PQ and NQ, respectively (Table 1, Fig. 1). For both drugs, the IC_{50}s had relatively wide ranges, and the IC_{50}s between the least and most sensitive parasite isolates differed by 25–30 folds, suggesting the presence of parasite strains with much decreased sensitivity to these drugs (Hao et al., 2013).

Among the aryl aminoalcohol drugs, LMF was highly active against the parasites with a median IC_{50} in the lower nanomolar concentration (5.1 nM) and a relatively narrow range (~13 folds between the least and most sensitive strains), but five (4.7%) parasite isolates had IC_{50}s of > 10.6 nM. Though MQ has not been used in here, the median IC_{50} (45.4 nM) was greater than the 30 nM threshold used to define resistance in an earlier study (Ringwald et al., 1996), and 83.2% (89/107) parasite isolates had IC_{50} values above this cutoff. The IC_{50}s of three parasite isolates were close to 100 nM, about 25 times of that in the most sensitive isolates. QN has been extensively used to treat falciparum malaria in the study area. Although the median IC_{50} of 464.8 nM was below 600 nM, the arbitrarily defined threshold for resistance (Ringwald et al., 1996), 25.2% (27/107) parasites displayed IC_{50} values above this threshold. Moreover, five samples had IC_{50}s exceeding 1000 nM and one isolate had IC_{50} of > 2000 nM, suggesting potential QN resistance in the study parasite population, a result that is consistent with our previous report (Meng et al., 2010).

For the two artemisinin derivatives tested, both AS and DHA were highly active against these isolates with median IC_{50}s being in the sub-nanomolar range (Table 1). Using mean + 2 SDs to arbitrarily define the cutoffs for reduced sensitivity, 2.8% and 3.7% of parasites had IC_{50}s above the cutoffs for AS and DHA, respectively (Table 1). For the Mannich base drug PND, the median IC_{50} was 10.3 nM, below the 15 nM threshold value for resistance defined earlier (Pradines et al., 1998). Despite this, 31.8% of the tested parasites had IC_{50}s above this cutoff value. If we arbitrarily defined the cutoff value by using the mean + 2 SDs, 6.5% parasites showed reduced sensitivity to this drug (Table 1).

Antifolates have been deployed extensively for the treatment of *P. falciparum* malaria as well as for malaria prophylaxis in the past. *In vitro* resistance to PY was very high, and only one parasite isolate from the 2007 samples was considered sensitive based on the threshold of 100 nM for resistance (Table 1, Fig. 1) (Ringwald et al., 1996; Aubouy et al., 2003). Moreover, 80.2% parasite isolates had PY IC_{50} values exceeding 2000 nM, a value to define high PY resistance.

3.2. Temporal trends of in vitro sensitivities

Except for PND, QN and LMF, all other tested drugs exhibited significant temporal variations at least between some years (Fig. S1, Table S1). Consistent with an earlier observation (Hao et al., 2013), in vitro IC_{50}s to CQ displayed a clear trend of continuous annual increase from a median value of 145.0 nM in 2007 to 692.3 nM in 2012 (Fig. S1). In addition, IC_{50}s for PQ and PY also increased annually during the study period except in 2012 for PY. Despite extensive use of artemisinin drugs in this region, there were no significant annual increases in IC_{50}s to AS and DHA.

Table 1
In vitro IC_{50} values (nM) of *P. falciparum* field isolates from the China-Myanmar border to 10 antimalarial drugs.

Drugs	Median (IQR)	Range	3D7 (Mean ± SD)	*P**	Cutoff (nM)	# (%) of isolates above cutoff
Chloroquine	273.4 (154.9–559.2)	38.7–2563.0	17.8 ± 8.1	< 0.0001	100[#]	92 (86.0%)
Piperaquine	11.0 (6.5–14.9)	1.7–43.0	5.1 ± 2.0	< 0.0001	29.0	7 (6.5%)
Naphthoquine	10.8 (6.4–14.3)	1.6–32.0	8.5 ± 5.0	< 0.0001	25.4	5 (4.7%)
Mefloquine	45.4 (34.5–57.7)	5.7–121.1	18.1 ± 7.6	< 0.0001	30[#]; 90.6	89 (83.2%); 4 (3.7%)
Lumefantrine	5.1 (3.8–6.7)	1.7–17.0	4.8 ± 2.9	< 0.0001	10.6	5 (4.7%)
Quinine	464.8 (298.7–605.5)	30.6–2123.0	83.3 ± 41.5	< 0.0001	600[#]	27 (25.2%)
Pyrimethamine	4129.0 (2698.0–5588.0)	8.4–10519.0	62.5 ± 44.9	< 0.0001	100[#]	106 (99.1%)
Pyronaridine	10.3 (6.6–17.3)	2.2–39.3	5.6 ± 6.2	< 0.0001	15[§]; 27.4	34 (31.8%); 7 (6.5%)
Artesunate	10.7 (8.1–14.2)	1.6–29.7	7.4 ± 4.4	< 0.0001	22.2	3 (2.8%)
Dihydroartemisinin	4.1 (2.7–6.0)	1.1–11.0	3.0 ± 2.5	< 0.0001	8.9	4 (3.7%)

IQR, interquartile range; SD, standard deviation; ND, not defined.
**P* values are from Mann-Whitney *U* test for comparison between field isolates and 3D7.
Cutoffs for resistance are based on earlier report in Ringwald et al. (1996)[#] and Pradines et al. (1998)[§]. The rest of the cutoff values were based on calculated based on mean + 2 × SD of IC_{50}s from the field isolates. Note for mefloquine and pyronaridine, the second cutoff value was based on mean + 2 × SD of IC_{50}s from the field isolates in this study.

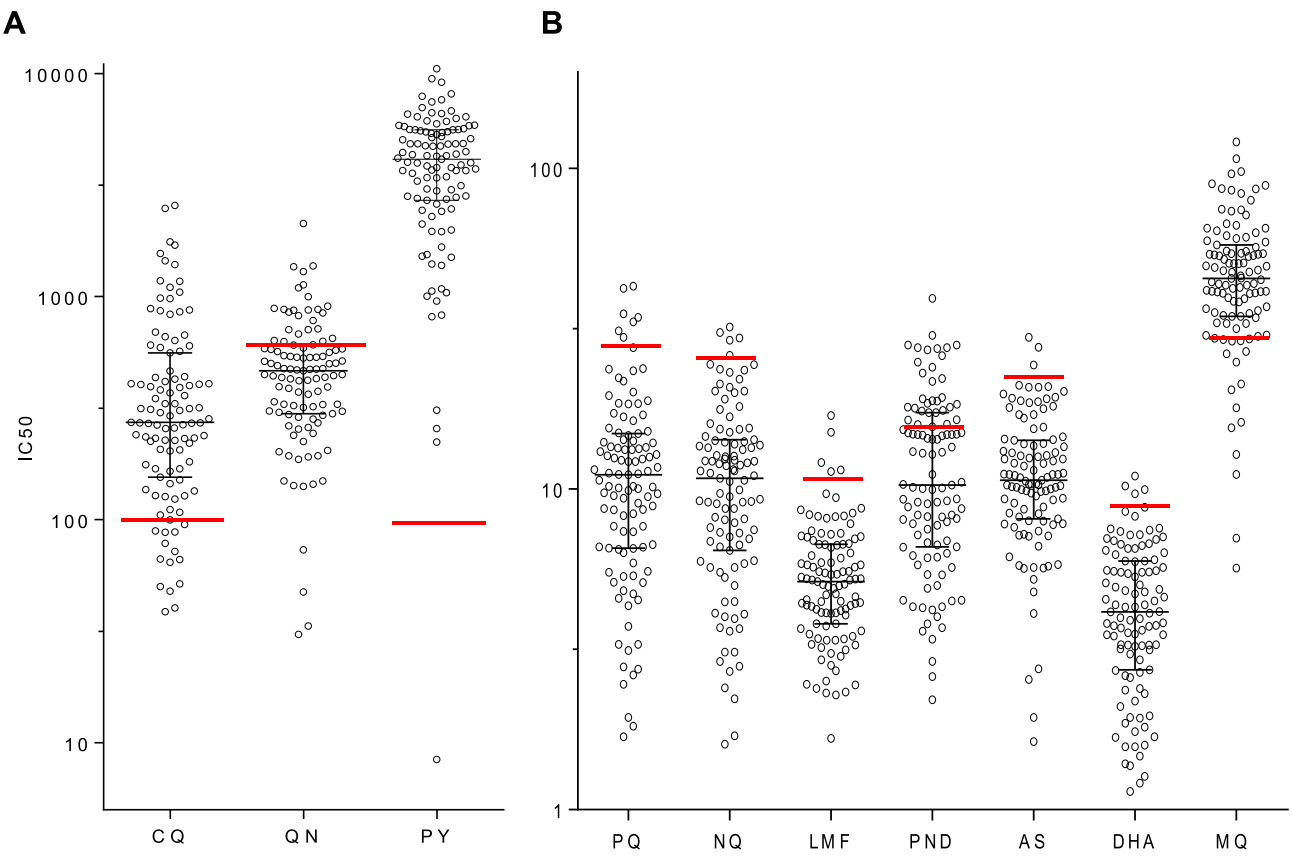

Fig. 1. *In vitro* susceptibilities of parasite isolates from the China-Myanmar border to 10 antimalarial drugs. Graph A shows the dot plot of IC_{50}s of the 107 parasite isolates to chloroquine (CQ), quinine (QN) and pyrimethamine (PY), while graph B shows their IC_{50}s to piperaquine (PQ), naphthoquine (NQ), lumefantrine (LMF), pyronaridine (PND), artesunate (AS), dihydroartemisinin (DHA) and mefloquine (MQ). Red bars indicate the cutoffs for resistance as defined in Table 1. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.3. Relationships between in vitro sensitivities to different drugs

Pairwise comparison showed that there were highly significant, positive correlations between sensitivities to AS and DHA (Fig. 2, $P < 0.0001$, Spearman's test). In addition, as we have reported earlier (Hao et al., 2013), susceptibilities to the two 4-aminoquinoline drugs CQ and PQ were significantly correlated ($P < 0.0001$). Also, sensitivities to aminoalcohol drugs and artemisinin derivatives were correlated. Specifically, the sensitivity to MQ was correlated with those to QN and DHA ($P < 0.05$). Similarly, QN showed significant correlations with DHA and AS ($P < 0.01$) as well as LMF ($P < 0.0001$). Sensitivities to NQ and PND were significantly correlated ($P < 0.01$). In addition, PY and LMF also showed significant correlation ($P < 0.01$).

To determine whether the CQ resistance background affects the in vitro susceptibilities to other antimalarial drugs, parasite isolates were divided into moderately CQ-resistant (15 parasites) and highly CQ-resistant (92 parasites) groups (Table S2). Among the drugs tested, only PQ and DHA were less active against highly CQ-resistant parasite strains, whereas other drugs were similarly active against parasites in these two groups.

3.4. Polymorphisms in drug resistance genes

We genotyped seven genes to determine the prevalence of mutations associated with drug resistance. For *pfcrt*, M74I, N75E, K76T and A220S all reached fixation in the study parasite population. For *pfmdr1*, the N86Y mutation was rare in the parasite population with a prevalence of 0.9%, whereas the Y184F mutation reached 30.8% and appeared to have been decreasing through the years. Of the other two mutations N1042D and D1246Y, the former was detected in some years with an

*, $P < 0.05$, **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$

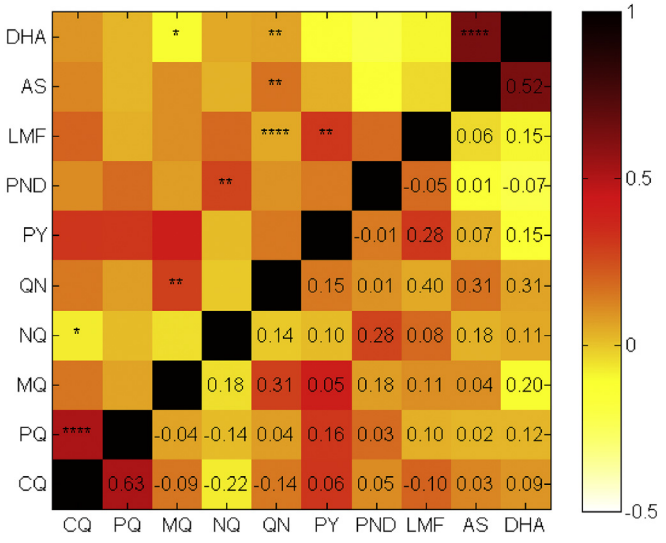

Fig. 2. Correlation between IC_{50} values of parasite isolates to 10 antimalarial drugs. Correlations between IC_{50} values were analyzed by Spearman's test and the degree of correlation between two drugs was colored coded. The coefficients are shown below the diagonal, while their levels of significance are shown as asterisks above the diagonal. Abbreviations of the drugs are the same as in Fig. 1.

Table 2
The prevalence of mutations in genes associated with drug resistance in different years.[a]

Gene	Residue position	2007 (n = 21)	2008 (n = 41)	2009 (n = 22)	2010 (n = 10)	2012 (n = 13)	Total (n = 107)
Pfcrt[c]	C72S	–	–	–	–	–	–
	M74I	100	100	100	100	100	100
	N75E	100	100	100	100	100	100
	K76T	100	100	100	100	100	100
	A220S	100	100	100	100	100	100
Pfmdr1	N86Y	4.8	–	–	–	–	0.9
	Y184F	28.6	43.9	31.8	10	7.7	30.8
	N1042D	4.8	–	13.6	–	–	3.7
Pfdhfr	N51I	61.9	65.9	63.6	70	76.9	66.4
	C59R	95.2	100	100	100	100	99.1
	S108N	95.2	100	100	100	100	99.1
	I164L	71.4	85.4	77.2	80	61.5	77.5
Pfdhps	S436A	61.9	51.2	**68.2**	40	**30.8**	53.3
	A437G	95.2	95.1	95.4	100	100	96.3
	K540E/N	90.5	85.4	**90.9**	**100**	**61.5**	86.0
	A581G	**23.8**	46.3	36.4	40	**69.2**	42.1
Pfmrp1	H191Y	66.7	**75.6**	63.6	**30**	**84.6**	68.2
	N325S	4.8	12.2	9.1	10	15.4	10.3
	S437A	66.7	**70.7**	**68.2**	**30**	69.2	65.4
	H785N	19.0	**9.8**	**4.8**	10	**38.5**	14.0
	I876V	57.1	61	68.2	50	53.8	59.8
	T1007M	19.0	22	31.8	20	46.2	26.2
	F1390I	9.5	14.6	10	–	7.7	10.3
PfK13[c]	NN[b]	42.9	61.5	68.2	80	69.2	60.7
	K189T	–	–	–	10	–	1.0
	E252Q	4.8	5.1	–	–	–	2.9
	P441L	4.8	–	–	–	–	1.0
	F446I	19.0	30.8	18.1	10	46.2	25.7
	R539T	–	7.7	–	–	7.7	3.8
	P574L	9.5	2.6	–	–	–	2.9
	C580Y	–	2.6	–	–	–	1.0
	A676D	–	5.1	–	–	–	2.9
	H719N	–	–	**18.1**	–	–	3.8

ND, not done.

[a] Significant differences in mutation prevalence are highlighted in bold (χ^2 test): *pfdhps* S436A between 2009 and 2012 ($P = 0.0358$) and between 2010 and 2012 ($P = 0.0266$); *pfdhps* A581G 2007 and 2012 ($P = 0.0089$); *pfmrp1* H191Y between 2008 and 2010 ($P = 0.0061$) and between 2010 and 2012 ($P = 0.0078$); *pfmrp1* S437A between 2008 and 2010 ($P = 0.0169$) and between 2009 and 2010 ($P = 0.0436$); *pfmrp1* H785N between 2008 and 2012 ($P = 0.0155$) and between 2009 and 2012 ($P = 0.0101$); *PfK13* H719N between 2008 and 2009 ($P = 0.0140$).

[b] NN insertion between amino acids 136 and 137.

[c] *39* samples were genotyped in 2008, giving a total of 105 samples genotyped for the *pfk13* gene.

overall prevalence of 3.7%, whereas the latter was not detected in the study population (Table 2).

Major mutations mediating resistance to PY in *pfdhfr* were all highly prevalent in the study parasites (Table 2). In particular, C59R and S108N almost reached fixation with only one parasite isolate from 2007 remaining as the wild type. The other two major mutations N51I and I164L also reached high prevalence (66.4% and 77.5%, respectively). Among the mutations in *pfdhps* that confer resistance to sulfadoxine, the A437G mutation was approaching fixation at 96.3%. The K540E/N mutation was also highly prevalent at 86.0%.

Pfmrp1 gene had seven mutations with 10.3–68.2% frequencies, among which two mutations H191Y and S437A exceeded 60% in the parasite population. The minisatellite in *pfnhe1* was associated with altered sensitivity to QN in some parasite populations (Ferdig et al., 2004; Bennett et al., 2007; Henry et al., 2009; Meng et al., 2010). In this study, a total of 11 *pfnhe1* ms4760 alleles were found; two (MS-6 and MS-7) reached substantial levels (13.1% and 58.9%, respectively) in the parasite population.

Genotyping *PfK13* gene detected the NN insertion between amino acids 136 and 137 in 60.7% parasites and nine point mutations in 45% of parasites. Among these point mutations, seven were located in the propeller domain (> 440 amino acids). Consistent with our earlier findings (Wang et al., 2015b, 2015c), F446I was the predominant mutation with a prevalence of 25.7%. The other six were rare mutations P441L (1.0%), R539T (3.8%), P574L (2.9%), C580Y (1.0%), A676D (2.9%) and H719N (3.8%) (Tables 2 and 3).

3.5. Association of gene polymorphisms with in vitro drug sensitivities

We compared the IC_{50}s between parasites carrying the wild-type alleles and those with the mutant alleles. Fixation of the major CQ-resistant alleles (K76T and A220S) was consistent with the in vitro assay result. For the *pfdhfr* gene, mutations in the codon N51I was significantly associated with increased in vitro resistance to PY, whereas parasites carrying I164 and 164L had similar PY sensitivities (Fig. S2A). Mutations in *pfmdr1* and *pfmrp1* may affect parasite's sensitivities to multiple drugs. In Africa, the N86Y mutation is associated decreased sensitivity to aminoquinolines but increased sensitivity to arylamino alcohol drugs such as MQ, LMF and halofantrine (Dokomajilar et al., 2006; Mwai et al., 2009). In our sample set, this mutation was found in only one parasite isolate. For the rest of *pfmdr1* mutations, we identified that parasites with the mutation N1042D showed significantly increased sensitivity to PND (Fig. S2B). For the *pfmrp1* gene, several mutations were associated with altered sensitivity to a number of drugs tested. The prevalent T1007M was correlated with elevated IC_{50} values to two 4-aminoquinoline drugs CQ and PQ (Fig. S2C, D). In addition, H785N was linked to decreased sensitivity to PQ and LMF, whereas F1390I was associated with increased sensitivity to PY and DHA (Fig. S2D-G).

Consistent with our previous finding, parasites with the *pfnhe1* ms4760 haplotype MS-7 (with three type 1 repeats) exhibited significantly reduced sensitivity to QN compared to those carrying MS-1 (with two type 1 repeats) and MS-5 allele (with four type 1 repeats). In

Table 3
Prevalence of major haplotypes of known drug resistance genes in parasites from different years.

Gene	H	Haplotype[a]	2007 (n = 21)	2008 (n = 41)	2009 (n = 22)	2010 (n = 10)	2012 (n = 13)	Total (n = 107)
Pfcrt	1	**CIETS**	100	100	100	100	100	100
Pfmdr1	5	NYN	66.7	**56.1**	**54.5**	**90.0**	**92.3**	65.4
		NFN	23.8	**43.9**	31.8	**10.0**	**7.7**	29.9
Pfdhfr	4	**IRNI**	19.0	12.2	22.7	20.0	23.1	17.8
		NRNL	28.6	31.7	36.4	30.0	7.7	29.0
		IRNL	42.9	53.7	40.9	50.0	53.8	48.6
Pfdhps	12	**SGEA**	9.5	7.3	–	20.0	–	6.5
		AGEA	47.6	31.7	45.5	40.0	23.1	37.4
		SGEG	19	31.7	22.7	30.0	38.5	28.0
		AGEG	–	9.8	9.1	–	–	5.6
Pfmrp1	22	HNSHITF	28.6	**17.1**	22.7	**50.0**	15.4	23.4
		YNAHVTF	33.3	24.4	18.2	–	–	19.6
		YSAHITF	–	9.8	4.5	–	7.7	5.6
		YNAHVTI	4.8	12.2	9.1	–	7.7	8.4
		YNAHVMF	4.8	9.8	27.3	10.0	–	11.2
		YNANVMF	4.8	9.8	–	10.0	30.8	9.3
Pfnhe1	11	MS-1 (2)	14.3	9.8	9.1	10.0	7.7	10.3
		MS-5 (4)	–	4.9	9.1	20.0	–	5.6
		MS-6 (1)	19.0	14.6	18.2	–	–	13.1
		MS-7 (3)	52.4	58.5	**50.0**	50.0	**84.6**	58.9

H, number of haplotypes. Only haplotypes with prevalence ≥5% were included.
Significant differences in the annual prevalence of haplotypes are highlighted in bold (χ^2 test): *Pfmdr1* NYN between 2008 and 2010 ($P = 0.0468$), 2008 and 2012 ($P = 0.0172$) and between 2009 and 2012 ($P = 0.0201$); *pfmdr1* NFN between 2008 and 2010 ($P = 0.0468$) and 2008 and 2012 ($P = 0.0172$); *pfmrp1* HNSHITF between 2008 and 2010 ($P = 0.0277$), and pfnhe1 MS-7 between 2008 and 2012 ($P = 0.0067$) and between 2009 and 2012 ($P = 0.0406$).

[a] Haplotypes were based on amino acids at the positions *pfcrt* (72, 74, 75, 76, 220); *pfmdr1*(86, 184,1042); *pfdhfr*(51, 59, 108, 164), *pfdhps*(436, 437, 540, 581); and*pfmrp1*(191, 325, 437, 785, 876, 1007, 1390). Mutant residues are in bold. For the *pfnhe1* haplotypes, the number in parenthesis indicates the copy number of DNNND repeats.

addition, parasites with MS-6 (with one type 1 repeat) expressed significantly increased sensitivity to MQ compared to those carrying the MS-1, MS-5 and MS-7 allele (Fig. S2H, I).

Artemisinin resistance shown as delayed clearance is associated with mutations in the propeller domain of the *PfK13* gene. Whereas none of the propeller mutations identified in this study were linked to increased IC_{50}s to AS and DHA, F446I was linked to increased resistance to both CQ and PQ (Fig. S2J, K). In addition, the N-terminal insertion was also found to be associated with altered in vitro sensitivity to CQ and MQ. The H719N and the P574L were associated with reduced sensitivity to QN and increased sensitivity to PY, respectively (Fig. S2J, L).

3.6. Haplotype diversity of drug resistance genes

For pfcrt, **CIETS** at positions 72–76 occurred at 100%, consistent with this being the most prevalent CQ-resistant haplotype in Southeast Asia. For antifolate resistance, 95% parasites carried triple and quadruple mutations in *pfdhfr*, while 77.5% parasites carried triple and quadruple mutations in *pfdhps,* confirming that the parasite population was highly resistant to sulfadoxine-PY. However, more than 65% parasites carried the wild-type *pfmdr1*, and it reached 90% or higher in samples after 2010, albeit the sample size was small. For *pfmrp1*, seven mutations were detected, resulting in 22 haplotypes with six exceeding 5%. For the *pfnhe1* gene, 11 haplotypes were detected and MS-7 occurred in 58.9% of the parasites. This haplotype has been associated with reduced in vitro IC_{50} to quinine. For the *PfK13* gene, nine point mutations and 11 haplotypes were identified. All parasites contained single mutations in the propeller domain, whereas only one parasite carried the E252Q and F446I double mutations.

When all sequenced drug resistance-related genes with the exclusion of *pfnhe1* were considered, 96 haplotypes were identified (Fig. 3). These haplotypes include those with mutations in all sequenced genes, indicating MDR phenotypes. Phylogenetic analysis revealed five major groups, with each of the haplotype groups containing isolates from different years (Fig. 3). Similarly, haplotype network analysis did not identify substantial clustering of the haplotypes by years (Fig. S3). Although there were haplotypes that persisted through these years, the predominant haplotypes showed changes in each year.

4. Discussion

Chemotherapy remains a cornerstone in malaria treatment, but the *P. falciparum* parasite is highly adept at developing resistance to antimalarial drugs. Especially, parasite strains from the GMS have developed resistance to essentially all antimalarials deployed so far. This arms race between the use of new drugs and evolution of resistance has forced malaria-endemic nations to frequently change their drug policies to maintain relatively high efficacies of the frontline antimalarials. This demands close surveillance of drug efficacy and resistance development and spread. In vitro assays of clinical parasite isolates for sensitivity to antimalarials and monitoring known molecular markers associated resistance offer complementary ways of resistance surveillance. In this study, we used these two approaches to monitor the longitudinal trends of in vitro susceptibilities of *P. falciparum* using an archived collection of parasite isolates from the China-Myanmar border region. All drugs except MQ have been used with varied extents in this region. Consistently, these clinical parasite isolates exhibited significant differences in their in vitro susceptibilities to all drugs compared to 3D7. However, except for CQ and PQ, sensitivities to other drugs did not show a consistent temporal trend of continuous decrease over the years.

CQ has been withdrawn from treating falciparum malaria in this region in the 1970s, but parasites remained highly resistant to CQ. Interestingly, we even observed continued escalation of in vitro CQ IC_{50} values during longitudinal monitoring of the field parasite isolates. This is in contrast to reports from other malaria endemic areas where

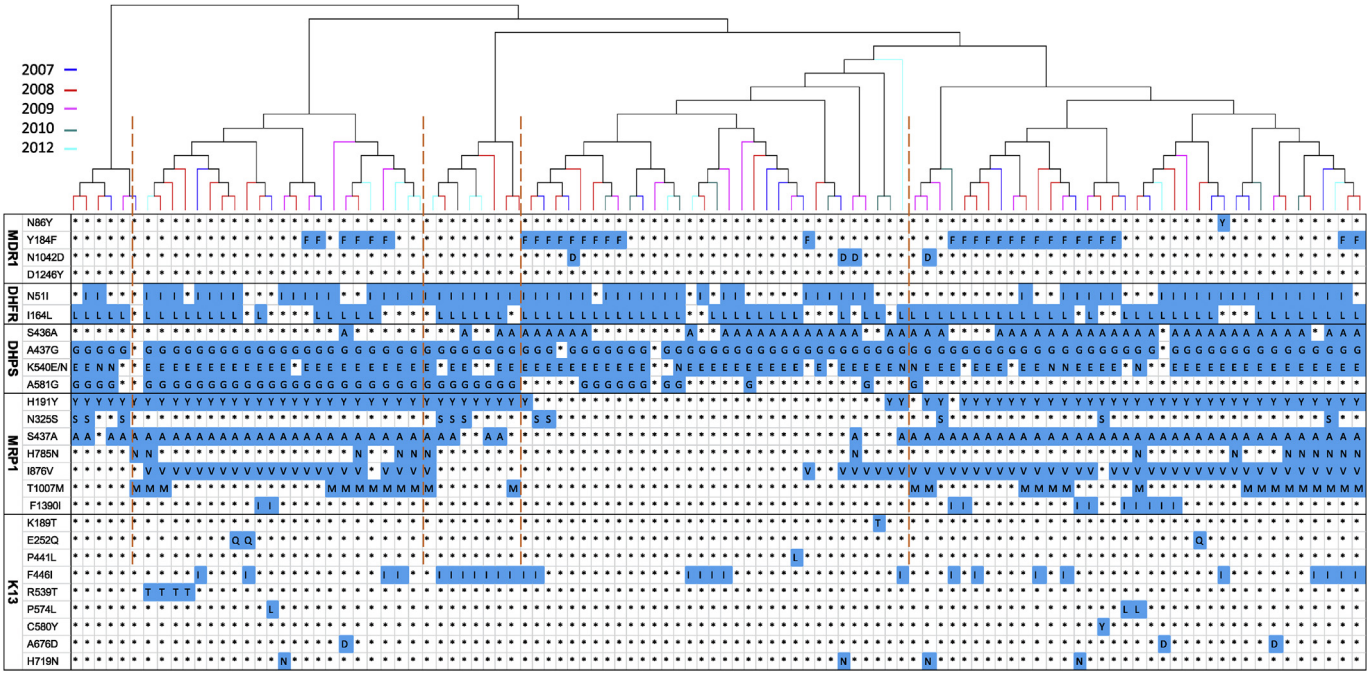

Fig. 3. Phylogenetic clustering of the haplotypes based on the mutations in five genes associated with drug resistance in *P. falciparum*. The tree was constructed using the neighbor-joining method implemented in the MEGA program. Parasites were color-coded by the years of collection. Haplotypes are shown with mutations in each gene highlighted in blue color and wild-type residues shown as asterisks. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

cessation of CQ use is accompanied with a gradual decline of parasites carrying the pfcrt K76T mutation and concomitant return of CQ sensitivity (Kublin et al., 2003; Laufer et al., 2010). The reasons for the persistent and continuous evolution of CQ resistance in our study site could include 1) continued selection pressure due to the use of CQ for treating sympatric *P. vivax* cases, and 2) an almost complete lack of competing wild type parasites as the major *pfcrt* mutant alleles are fixed. It is also possible that the extensive use of PQ as the ACT partner drug has exerted selective pressure on CQ resistance given their structural similarity, which is also reflected in the significant correlation in sensitivities to CQ and PQ. Since we only determined the sequence polymorphisms around the K76 and A220 position of the *pfcrt* gene, it is possible that the parasites, like those identified in Cambodia, may evolve new *pfcrt* mutations, which could confer CQ resistance without incurring fitness cost (Gabryszewski et al., 2016). Future studies will need to elucidate the evolution of the pfcrt locus and its pleotropic effects on resistance development to other antimalarial drugs.

As a replacement drug for CQ, extensive deployment of PQ as a monotherapy has led to the development of clinical resistance to PQ in China (Gao et al., 1993; Yang et al., 1999). Though clinical resistance to DHA-PQ has recently emerged in Cambodia (Amato et al., 2017; Witkowski et al., 2017), this ACT remained highly efficacious for treating falciparum cases in the China-Myanmar border region (Liu et al., 2015; Wang et al., 2015a). In Cambodia, the DHA-PQ failure involves resistance to both DHA and PQ; the latter apparently evolved in the background of artemisinin resistance and was associated with amplification of the aspartic protease genes *plasmepsin 2/3* (Amato et al., 2017; Witkowski et al., 2017). The decrease of in vitro PQ sensitivity observed in some parasite isolates used in this study may involve different mechanisms. Two mutations in the *pfmrp1* gene were associated with reduced susceptibility to PQ. In addition, the F446I mutation in PfK13 was also associated with reduced susceptibility to PQ and CQ. Though PfK13 mutations may not be mediating PQ resistance per se, it is possible that artemisinin resistance might have evolved in the background of PQ resistance or vice versa, like what was observed in Cambodia (Duru et al., 2015; Spring et al., 2015). The other 4-aminoquinoline drug NQ, an antimalarial drug developed in China, has not been applied widely in this region and parasites were relatively sensitive to this drug. Interestingly, sensitivities to NQ and CQ appear to be negatively correlated, suggesting *pfcrt* mutations do not confer cross resistance to NQ.

The antifolate drugs have also been discontinued for malaria treatment, but resistance to PY remained very high, and mutations in *dhfr* and *dhps* conferring resistance to PY and sulfadoxine, respectively, were highly prevalent in the parasite population. We did not notice a consistently declining trend of *dhfr* and *dhps* mutant alleles through the years of monitoring, which is different from what has been observed in other malaria regions such as Ethiopia after withdrawal of sulfadoxine -PY (Tessema et al., 2015). It is noteworthy that intermittent preventive treatment based on sulfadoxine-PY has not been carried out in the study area. It is curious whether selective pressure maintaining the *dhfr* and *dhps* mutations is due to the use of antifolate drugs for treating bacterial infections, a common practice in the study area.

Artemisinins have been used for more than three decades in the China-Myanmar border area, mostly as monotherapies prior to 2005. There are indications that artemisinin resistance also has emerged in this area, and clinical cases remaining parasitemic three days after treatment with ACT were associated with increased in vitro ring-stage survival and mutations in the propeller domain of PfK13 (Wang et al., 2015c). Unlike other parts of the GMS, parasites from this border area have high prevalence of the F446I mutation (Huang et al., 2015; Wang et al., 2015b; Ye et al., 2016). Yet, it is noteworthy that the conventional IC_{50} values of the parasite strains could not predict artemisinin resistance that is restricted to the early ring stage because this method subjects the parasites to continuous exposure to antimalarials through most of the developmental cycle (Dondorp et al., 2009; Witkowski et al., 2013). Nonetheless, our genotyping result was consistent with previous findings, showing F446I as the predominant mutation in this region. Importantly, we also identified the presence of some mutations such as R539T and C580Y, which were highly prevalent in the Cambodian parasite populations and were confirmed genetically to confer artemisinin resistance in vitro (Straimer et al., 2015). Thus, despite

excellent ACT efficacy at the China-Myanmar border, strenuous monitoring is needed.

Gene amplification such as *pfmdr1* and *plasmepsins 2/3* are associated with drug resistance in *P. falciparum*. Especially in some GMS countries where MQ has been extensively deployed, *pfmdr1* amplification is highly prevalent and is associated with increased risk of recrudescence in patients treated with MQ and LUM (Price et al., 2004; Alker et al., 2007; Lim et al., 2009). Whereas increased *pfmdr1* copies are associated with in vitro MQ resistance (Price et al., 1999), in vitro selection for PQ resistance found deamplification of the *pfmdr1* gene in PQ-resistant parasites (Eastman et al., 2011). Consistent with this in vitro finding, the recently emerged resistance in Cambodia to DHA-PQ was also associated with single-copy *pfmdr1* (Amato et al., 2017; Witkowski et al., 2017), suggesting that PQ selects against *pfmdr1* amplification. Our earlier studies of *pfmdr1* copy numbers in parasites from the China-Myanmar border region did not identify *pfmdr1* amplification (Meng et al., 2010; Wang et al., 2012), which is consistent with no MQ but extensive DHA-PQ use in this area. Nevertheless, analysis of amplification of these drug resistance-associated genes in future parasite isolates from this region needs to be vigorously followed up.

Molecular surveillance of the genetic markers associated with drug resistance agreed well with the in vitro drug assay results for well-characterized genes such as *pfcrt*, *dhfr* and *dhps*. The high prevalence of resistance-conferring mutations in these genes is consistent with MDR phenotypes of the parasites. However, given that the sample sizes in this study are relatively small, especially in year 2010, caution is needed to make conclusions on drug resistance. Furthermore, the lack of well-defined cutoffs for in vitro drug resistance for most of the drugs assayed also warns against dichotomous division of drug resistance based solely on in vitro assay data and arbitrary cutoffs. Whereas the in vitro data clearly confirmed drug resistance to CQ and PY, the IC_{50} data for other drugs can only serve as references to guide future work, which should certainly involve clinical efficacy studies. Network analysis in this study showed that certain haplotypes associated MDR have been collected in multiple years, indicating relative persistence of these MDR parasites in this region. In addition, there have been year-to-year fluctuations of the predominant haplotypes, which could be due to the introduction of parasite genotypes as the result of migration of internally displaced people to the border region (Lo et al., 2015).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ijpddr.2018.05.003.

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