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*, *pfheme1*, 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\(_{50}\) values. 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 (Wellem and Plowe, 2001). This also happened to the antifolates drug pyrimehtamine (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...
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 (Noord 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 (Wongrachanali 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 (Eklund and Fidock, 2007). The K76T mutation in the *P. falciparum* chloroquine resistance transporter, pfcr, is a major determinant of CQ resistance (Fidock et al., 2000). Point mutations in dihydroprotoether 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 (*mdr1*) 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 promoter 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 *plasmodin* 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 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.

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 Nabaung 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 PQ, 320 nM for PO, 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 *pfcr* fragments covering codons 72–76 and 220, two *pfmdr1* fragments including codons 86, 184, 1042 and 1246, a *pfhdfr* fragment containing codons 51, 59, 108 and 164, two *pfddhs* fragments containing codons 436, 437, 540, and 581, a *pfne1* 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 (IC50) 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. IC50 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 IC50s of drugs were determined using Spearman’s test in the R package and MATLAB R2013a. Associations between IC50s 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 IC50 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 test) (Table 1). For CQ, 14.0% and 86.0% parasite isolates were close to 100 nM for resistance (Table 1, Fig. 1). For the Mannich base drug PND, the median IC50 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 IC50 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 IC50 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 IC50s 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, IC50s 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 IC50s to AS and DHA.

Table 1

| Drugs         | Median IC50 (nM) | Range (Mean ± SD) | IC50 (Mean ± SD) | P* | Cutoff (nM) | % (IC50s) 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 IC50s from the field isolates. Note for mefloquine and pyronaridine, the second cutoff value was based on mean + 2 × SD of IC50s from the field isolates in this study.
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 sensitivities 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 \( pfcr \), 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
overall prevalence of 3.7%, whereas the latter was not detected in the study population (Table 2).

Major mutations mediating resistance to PY in \( \text{pf} \text{dfhfr} \) were all highly prevalent in the study parasites (Table 2). In particular, C59R and S437A between 2008 and 2010 (\( P = 0.0266 \)), whereas the latter was not detected in the ND, not done. The prevalence of mutations in genes associated with drug resistance in di\( \text{Y. Bai et al.} \)

| Gene   | Residue position | 2007 (n = 21) | 2008 (n = 41) | 2009 (n = 22) | 2010 (n = 10) | 2012 (n = 13) | Total (n = 107) |
|--------|------------------|--------------|--------------|--------------|--------------|--------------|---------------|
| PFMR1  |                  |              |              |              |              |              |               |
|        |                  |              |              |              |              |              |               |
|        |                  |              |              |              |              |              |               |
| PDHFR  |                  |              |              |              |              |              |               |
|        |                  |              |              |              |              |              |               |
| PDHPS  |                  |              |              |              |              |              |               |
|        |                  |              |              |              |              |              |               |
| PDK13  |                  |              |              |              |              |              |               |
|        |                  |              |              |              |              |              |               |
|        |                  |              |              |              |              |              |               |

ND, not done.

\( ^a \) Significant differences in mutation prevalence are highlighted in bold (\( \chi^2 \) test): \( \text{pdhps} \) S436A between 2009 and 2012 (\( P = 0.0358 \)) and between 2010 and 2012 (\( P = 0.0266 \)); \( \text{pdhfr} \) A581G 2007 and 2012 (\( P = 0.0089 \)); \( \text{pfmrp1} \) H191Y between 2008 and 2010 (\( P = 0.0061 \)) and between 2010 and 2012 (\( P = 0.0078 \)); \( \text{pfmrp1} \) S437A between 2008 and 2010 (\( P = 0.0169 \)) and between 2009 and 2010 (\( P = 0.0436 \)); \( \text{pfmrp1} \) H785N between 2008 and 2012 (\( P = 0.0155 \)) and between 2009 and 2012 (\( P = 0.0101 \)); \( \text{PK13} \) 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 \( \text{pfk13} \) gene.

3.5. Association of gene polymorphisms with in vitro drug sensitivities

We compared the IC\(_{50}\) 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 \( \text{pfdfhfr} \) gene, mutations in the codon N51I was significantly associated with increased in vitro resistance to PY, whereas parasites carrying I164 and I164L had similar PY sensitivities (Fig. S2A). Mutations in \( \text{pfmrp1} \) and \( \text{pfk13} \) may affect parasite's sensitivities to multiple drugs. In Africa, the N86Y mutation is associated decreased sensitivity to aminooquinolines 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 \( \text{pfmrp1} \) mutations, we identified that parasites with the mutation N104D showed significantly increased sensitivity to PND (Fig. S2B).
addition, parasites with MS-6 (with one type 1 repeat) expressed significantly increased sensitivity to MQ compared to those carrying the MS-7 allele (Fig. S2H, I).

### 3.6. Haplotype diversity of drug resistance genes

For pfcr *, 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 pfhve1 gene, 11 haplotypes were detected and MS-7 occurred in 58.9% of the parasites. This haplotype has been associated with reduced in vitro IC50 to quinine. The H719N and the P574L were associated with reduced in vitro sensitivity to CQ and increased sensitivity to PY, respectively (Fig. S2J, K). In addition, parasites with MS-6 (with one type 1 repeat) expressed significantly increased sensitivity to MQ compared to those carrying the MS-7 allele (Fig. S2H, I).

#### Table 3

| Gene | Hapelotype | 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 |
| 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 |
| Pfhdhr | 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 |
| Pfhps | 12 | SGEA | 9.5 | 7.3 | – | 20.0 | – | 6.5 |
| | | AGEA | 47.6 | 31.7 | 45.5 | 40.0 | 23.1 | 37.4 |
| | | SPEG | 19 | 31.7 | 22.7 | 30.0 | 38.5 | 28.0 |
| | | AGEG | – | 9.8 | 9.1 | – | – | 5.6 |
| Pfmp1 | 22 | HNSHITF | 28.6 | 17.1 | 22.7 | 50.0 | 15.4 | 23.4 |
| | | YNAAVTF | 33.3 | 24.4 | 18.2 | – | – | 19.6 |
| | | YSAHITF | – | 9.8 | 4.5 | – | 7.7 | 5.6 |
| | | YNAPVTI | 4.8 | 12.2 | 9.1 | – | 7.7 | 8.4 |
| | | YNAHVMF | 4.8 | 9.8 | 27.3 | 10.0 | – | 11.2 |
| | | YNAVNF | 4.8 | 9.8 | – | 10.0 | 30.8 | 9.3 |
| Pfhe1 | 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 (χ² test): Pfmdr1 NYN between 2008 and 2010 (P = 0.0468), 2008 and 2012 (P = 0.0172) and between 2009 and 2012 (P = 0.0201); pfdhfr NFN between 2008 and 2010 (P = 0.0468) and 2008 and 2012 (P = 0.0172); Pfmp1 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).

* Haplotypes were based on amino acids at the positions pfcr (72, 74, 75, 76, 220); pfmdr1 (86, 184, 1042); pfdhfr (51, 59, 108, 164); pfdhps (436, 437, 540, 581); and pfmp1 (191, 325, 437, 785, 876, 1007, 1390). Mutant residues are in bold. For the pfhve1 haplotypes, the number in parenthesis indicates the copy number of DNNND repeats.

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 antimalarial 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 IC50 values during longitudinal monitoring of the field parasite isolates. This is in contrast to reports from other malaria endemic areas where...
cessation of CQ use is accompanied with a gradual decline of parasites carrying the pfcrt K76T mutation and concomitant return of CQ sensitivities (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 *plasmeispin 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 pfdhfr 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 IC50 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, stringent monitoring is needed.

Gene amplification such as pfmdr1 and plasmodins 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 pfcr, dhfr, and dhps. The high prevalence in 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 IC50 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.jidppdr.2018.05.003.

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