Plasmodium falciparum multidrug resistance protein 1 (pfmrp1) gene and its association with in vitro drug susceptibility of parasite isolates from north-east Myanmar

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Objectives: Plasmodium falciparum multidrug resistance protein 1 (pfmrp1) has recently emerged as an important determinant of drug resistance and mutations in the gene have been associated with several drugs. The aim of this study was to understand the level of genetic diversity in pfmrp1 and to determine the association of different mutations with altered drug susceptibilities of P. falciparum.

Methods: We analysed 193 sequences of pfmrp1 from South-East Asia, west Asia, Africa, Oceania and South America. We measured the level of genetic diversity and determined signatures of selection on the gene. In vitro susceptibilities of 28 P. falciparum isolates from north-east Myanmar to a panel of seven commonly used antimalarials were determined. Statistical analysis was performed to determine the association of different mutations with in vitro drug susceptibilities.

Results: A total of 28 single nucleotide polymorphisms were identified in 193 sequences, of which 22 were non-synonymous. Whereas mutations in the pfmrp1 gene were conserved among different countries within a continent, they were different between continents. Seven non-synonymous mutations were identified in the north-east Myanmar isolates; all were relatively frequent in this region as well as in other neighbouring countries. Molecular evolutionary analysis detected signatures of positive selection on the gene. Moreover, some mutations in this gene were found to be associated with reduced susceptibilities to chloroquine, mefloquine, pyronaridine and lumefantrine.

Conclusions: Evidence of the positive selection of pfmrp1 and its association with the susceptibilities of parasites to multiple drugs signifies its potential as an important candidate for monitoring drug resistance.

Keywords: malaria parasite, drug resistance, ABC transporter

Introduction

Over the past decade, the total number of malaria cases and deaths due to malaria has progressively decreased in most of the countries where malaria is endemic. A recent World Malaria Report from the WHO confirmed 219 million cases and 660000 deaths due to malaria in 2010.1 Remarkably, the Greater Mekong Sub-region (GMS) has achieved an 81% reduction in the number of deaths due to malaria from 1998 to 2010.2 This significant gain has become possible with multi-sectoral efforts including the use of artemisinin-based combination therapies. Unfortunately, the recent rise of artemisinin-resistant Plasmodium falciparum in the GMS has posed a serious threat to the elimination of malaria. In recent history, chloroquine resistance has put a dent in treatment efforts. Adding to the woes, we do not have effective alternatives in hand to replace artemisinins. In these circumstances, it is imperative to prolong the life span of the drugs currently in use against malaria. For this purpose, strategic monitoring of drug resistance in endemic regions and deciphering the molecular mechanisms of drug resistance are critical measures for detecting the emergence of drug resistance and deterring its spread. In the GMS, Myanmar contributes the highest number of cases of malaria annually and most malaria cases come from the international border regions of the country.2 Moreover, reduced susceptibilities to most antimalarials currently in use have been continuously reported from Myanmar,3–5 which has clearly shown an alarming
served for Vacutainers and centrifuged, and the red blood cells (RBCs) were cryopreserved for in vitro drug assay. Written informed consent was obtained from the participants or their guardians. This study was approved by the Institutional Review Board of Kunming Medical University.

**PCR and sequencing of pfmrp1**

Parasite genomic DNA was extracted from the filter papers using a QiaAmp DNA Mini Kit (Qiagen, Germany). DNA was genotyped for three polymorphic genes—merozoite surface protein 1 (msp1; block 2), msp2 and glutathione rich protein (glurp)—by nested PCR as previously described. The pfmrp1 open reading frame was amplified using long-range PCR, and was further divided into seven fragments for amplification by nested PCR using primers and conditions specified in earlier publications. PCR products were first visualized by gel electrophoresis and purified using the High Pure PCR cleanup microkit (Roche) and sequenced using BigDye Terminator v3.1. Sequences were assembled using Lasergene software (DNASTAR) with manual editing.

**Molecular evolutionary analysis of pfmrp1**

We used 193 full-length pfmrp1 sequences for analysis, which included 38 samples from Africa, 13 from South America, 15 from Oceania, 5 from west Asia and 122 from South-East Asia (GenBank accession numbers FJ477732–477813 and FJ477817–477834), of which 93 samples were from the current study. The sequences were aligned using ClustalW15 implementing MEGA v5.05.16 Nucleotide and amino acid positions were numbered according to the 3D7 reference sequence (PF3D7_0112200). Since some sequences contain missing information, sequence analyses were performed after pairwise deletion of missing data using MEGA v5.05. Molecular evolutionary analysis was first done for the north-east Myanmar isolates and then the complete set of 193 sequences. As a measure of nucleotide diversity, the number of polymorphic sites, the proportion of each allele per SNP, and synonymous and non-synonymous substitutions were determined. Two summary statistics, π and θ, were also estimated, where π depicts the average pairwise nucleotide diversity and θ equals the average number of segregating sites.

In order to assess the potential selection of this gene, we estimated the rate of synonymous substitutions at silent sites (ds), which are presumed to be neutral, and the rate of substitution at non-synonymous sites (dn), which are usually under selection. ds exceeds dn only if natural selection promotes changes in the gene, whereas dn suppresses ds when natural selection does not favour changes in the gene. Based on this assumption, we performed a codon-based Z test, averaging overall sequences, to calculate the probability of departure from neutrality assuming dn=ds as a null model. Values of probability less than 0.05 were considered significant and the variance of the difference was computed with 500 replicates by the bootstrap method using the Nei and Gojobori model.19 All of the above test statistics were performed using MEGA v5.05 with pairwise deletion of missing data. To further support the evidence of positive selection of the gene, other statistical tests such as the Tajima’s D and McDonald–Krietman’ (MK) tests were also performed. The details of these tests are provided in the Supplementary data available at JAC Online.

Since multiple mutations in a gene might have differential roles in drug resistance, the degree of random association between different alleles was determined using pairwise linkage disequilibrium (LD) estimates. For this, we estimated the correlation coefficient R220 using DNASP v5.0 and assessed the significance of each association using the Chi-square test after Bonferroni correction. In addition, the SNP data were employed to generate haplotypes and to calculate haplotype frequencies. A haplotype network was constructed with Network4.611 software (http://www.fluxus-engineering.com) using the median joining algorithm11 with default parameters. Out-group probabilities were estimated from the haplotype network using TCS 1.21 in order to identify the ancestral haplotype.22

**Materials and methods**

**Parasite clinical isolates**

*P. falciparum* samples were collected from 18 May 2007 to 1 August 2010 from patients presenting with uncomplicated malaria infection at two malaria clinics in Laiza township, Kachin State, north-east Myanmar. Malaria infection was diagnosed by microscopy of Giemsa-stained thick and thin blood smears. If *P. falciparum* infection was confirmed, 0.2 mL of finger-prick blood was spotted onto a piece of Whatman 3 mm filter paper, air dried and stored individually for later analyses. An additional 2 mL of venous blood was drawn from each patient in EDTA-coated Vacutainers and centrifuged, and the red blood cells (RBCs) were cryopreserved for in vitro drug assay. Written informed consent was obtained from

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A knowledge of the molecular markers underlying antimalarial drug resistance is very important for monitoring the emergence and dissemination of drug-resistant parasites in endemic regions. Whereas the molecular mechanisms of resistance to certain antimalarials such as chloroquine and sulfadoxine/pyrimethamine are well known, the molecular markers for resistance to other antimalarial drugs remain poorly understood. Many *P. falciparum* drug transporters, including ATP-binding cassette (ABC) transporters, have been the focus of research because of their potential involvement in antimalarial drug resistance. Among the 16 members of *P. falciparum* ABC family, the multidrug resistance protein 1 (*pfmrp1*) is the best studied ABC transporter. Point mutations and amplification of this gene are linked to reduced susceptibility to a number of antimalarials such as chloroquine, mefloquine, quinine and artemisinin derivatives. The malaria parasite *P. falciparum* encodes two full transporter members of the ABC subfamily, which are also known as multidrug resistance-associated proteins. Both *pfmrp1* and *pfmrp2* are localized to the parasite plasma membrane. Single nucleotide polymorphisms (SNPs) of *pfmrp1* have previously been associated with a number of drugs, i.e. chloroquine, quinine, mefloquine and artemisinin derivatives. Consistent with this, our recent study from the China–Myanmar border region reported a significant association of this gene with chloroquine resistance. In particular, the K1466R mutation in *pfmrp1* was selected by sulfadoxine/pyrimethamine treatment, whereas artemether-lumefantrine selection led to an increased prevalence of 1876V in recrudescent infections. Furthermore, a *pfmrp1* knockout parasite displayed an increased intracellular accumulation of glutathione, chloroquine and quinine and became more susceptible to a number of antimalarials, including chloroquine, quinine, artemisinin, piperaquine and primaquine, suggesting that *pfmrp1* might be involved in drug efflux.

Following our initial analysis of the association of *pfmrp1* SNPs with altered susceptibility to chloroquine, we performed a thorough molecular evolution analysis of the *pfmrp1* gene from the north-east Myanmar area. We obtained complete sequences of this gene from 93 clinical parasite isolates from this region and aimed to identify signatures within this gene indicating drug selection. In addition, we used a small subset of these parasite isolates to determine whether this gene has SNPs that are associated with altered susceptibilities to a panel of commonly used antimalarial drugs in an in vitro study.
Table 1. Prevalence of point mutations in the pfmrp1 nucleotide and amino acid sequence of 93 P. falciparum isolates from north-east Myanmar during the years 2007–09

<table>
<thead>
<tr>
<th>Substitutions</th>
<th>Proportion of mutated allele</th>
<th>Mutant isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>nucleotidea</td>
<td>amino acida</td>
<td>2007 (n=26)</td>
</tr>
<tr>
<td>T571C</td>
<td>H191Y</td>
<td>0.58</td>
</tr>
<tr>
<td>G974A</td>
<td>N325S</td>
<td>0.08</td>
</tr>
<tr>
<td>G1309T</td>
<td>S437A</td>
<td>0.58</td>
</tr>
<tr>
<td>C2353A</td>
<td>H785N</td>
<td>0.15</td>
</tr>
<tr>
<td>G2626A</td>
<td>I876V</td>
<td>0.5</td>
</tr>
<tr>
<td>C3020T</td>
<td>T1007M</td>
<td>0.15</td>
</tr>
<tr>
<td>T4168A</td>
<td>F1390T</td>
<td>0.08</td>
</tr>
</tbody>
</table>

aUnderlined letters are the mutant alleles compared with 3D7.

In vitro drug assay

A total of 28 parasite isolates were adapted to continuous in vitro culture in type 0+ RBCs in complete medium supplemented with 25 mM HEPES, 50 mg/L hypoxanthine, 25 mM NaHCO3, 0.5% Albumax II and 40 mg/mL gentamicin sulphate as previously described. Cultures were maintained at 37°C in an atmosphere of 90% N2/5% O2/3% CO2. An SYBR Green I-based fluorescence assay was used to measure susceptibility of the parasites to seven antimalarial drugs: pyronaridine, lumefantrine, piperaquine, mefloquine, chloroquine, quinine, sulfadoxine/pyrimethamine (20:1, w/w). Pyronaridine, lumefantrine and piperaquine were obtained from Kunming Pharmaceutical Co. (Kunming, Yunnan, China), while the other drugs were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). Cultures were synchronized by two rounds of 5% D-sorbitol treatment, and ring-stage parasites were assayed for drug susceptibility in 96-well microtitre plates with a 1% haematocrit and 0.5% parasitaemia as described earlier. For each parasite isolate and drug concentration, the assay was performed in three biological replicates, and each biological replicate with two technical replicates. To reduce the variation between plates, the standard laboratory clone 3D7 was included as a reference.

Statistical analysis

All statistical analyses were performed using SPSS version 22 (SPSS IBM, New York, USA) and Graph Pad Prism (www.graphpad.com). The frequencies of mutations and haplotypes in different years were compared using the Student’s t-test. The mean of the half-maximal inhibitory concentration (IC50) and standard error for the seven drugs were determined using Graph Pad Prism. The reduction in susceptibility of each drug was tested by comparing the IC50 values for all the isolates with that for 3D7 and the significance of the difference was tested by a one-sample t-test. Spearman’s correlation coefficients were used to investigate the degree and significance of the relation between the IC50 values for all seven drugs. Differences in drug responses between different alleles of each mutation and haplotypes were compared using the Student’s t-test for each drug. Values of P ≤ 0.05 were considered statistically significant for all the tests. We also performed bootstrap analysis using 1000 replicates to increase the power of the analysis.

Results

Genetic diversity of pfmrp1 from the north-east Myanmar region

We obtained full-length pfmrp1 sequences (5469 bp) for 93 P. falciparum isolates collected from the north-east Myanmar area from 18 May 2007 to 1 August 2010 (GenBank accession numbers KJ713058–KJ713150). Compared with the 3D7 reference sequence, eight polymorphic sites were observed, seven of which resulted in non-synonymous mutations (Table 1). One synonymous SNP at nucleotide 4497 contained C in all 93 isolates compared with A in 3D7. Thus, only seven non-synonymous nucleotide variations were identified in the 93 sequences. All the mutations were bi-allelic and four were transitions. Moreover, these mutations were identified previously in P. falciparum from various geographical regions (Figure 1). Allele K1466 previously associated with sulfadoxine/pyrimethamine was found to be fixed in all 93 isolates. Not considering the synonymous mutation at nucleotide 4497, 26 isolates (28%) were the same as the 3D7 sequence, whereas 67 isolates showed mutations at one or more sites. Of the seven non-synonymous SNPs, Y191 was found to be the most abundant (66%), whereas N785 and I1390 occurred in 10% of the samples (Table 1). The proportions of most of these mutant alleles appeared to have increased in 2008 and 2009 as compared with 2007 (Table 1), although the differences were not statistically significant.

Consistent with the low prevalence of SNPs in the gene, very low values of π = 0.00043 and θ = 0.00025 were observed. The value of π is higher than θ (Table 2) because the proportions of all seven mutations within the samples were rather high, ranging from 10% to 66% (Table 1). This is also evident from the value of Tajima’s D (1.6625), which was found to be positively deviated from neutral equilibrium, although the value was not statistically significant (Table 2).

Worldwide genetic diversity in the pfmrp1 gene

To gain a comprehensive knowledge of global genetic diversity in the pfmrp1 gene, we analysed 193 pfmrp1 sequences including an additional 100 sequences from different countries where malaria was endemic (GenBank accession numbers FJ477732–FJ477813 and FJ477817–FJ477834). A total of 28 SNPs were observed, six of which were synonymous and 22 non-synonymous (Table S1, available as Supplementary data at JAC Online). These included 15 singletons and eight mutations with a prevalence of ≥8% (Table S1). Interestingly, all eight mutations were observed in the north–east Myanmar isolates. Compared with the previously reported SNPs, the present analysis revealed four new SNPs: three at positions 3981, 4337 and 4892 were
observed only in single isolates from Cambodia, Liberia and Guinea Bissau, respectively. The fourth SNP, an A to C transversion at position 4497, was seen in all 93 isolates from north-east Myanmar. The regional non-synonymous mutations observed in the \textit{pfmrp1} gene are shown in Figure 1. Similar to previous observations, SNPs were found to be heterogenetically distributed, and the seven mutations observed in north-east Myanmar were also present in two GMS countries—Thailand and Cambodia. Mutations at position 876 and 1390 were comparatively widely distributed around the globe, while the other five mutations were specific to Asia and Oceania (Figure 1 and Table S1).

The estimates of nucleotide diversity were very low, with values of $\theta=0.0009$ and $\pi=0.0006$ in the worldwide samples. The value of $\theta$ was found to be slightly higher than the estimate of $\pi$; but the normalized difference between the two values as estimated by Tajima’s D test ($-1.0069$) was not statistically significant. In terms of region (38 sequences from Africa, 5 from west Asia, 122 from South-East Asia, 15 from Oceania and 13 from South America), the number of SNPs ranged from 18 in South-East Asia to eight in isolates from either Africa and Oceania. As reported earlier,\textsuperscript{10} the South American isolates contained no mutations. The South-East Asian isolates contained

\begin{table}[h]
\centering
\begin{tabular}{|l|c|c|c|c|c|c|c|}
\hline
Region & No. of isolates & No. of SNPs & No. of singletons & $\theta$ & $\pi$ & Tajima’s D & $d_u-d_s$ \\
\hline
Myanmar & 93 & 8 & 0 & 0.00025 & 0.00043 & 1.6625 & 2.200\textsuperscript{a} \\
Africa & 38 & 8 & 5 & 0.00035 & 0.00016 & $-1.838$ & 1.378 \\
South-East Asia & 122 & 18 & 8 & 0.0006 & 0.0005 & $-0.3048$ & 0.628 \\
Oceania & 15 & 8 & 1 & 0.00045 & 0.00033 & $-0.9741$ & 2.480\textsuperscript{a} \\
West Asia & 5 & 5 & 1 & 0.00044 & 0.00037 & $-1.1239$ & 1.895 \\
South America & 13 & 0 & 0 & 0 & 0 & — & — \\
Total & 193 & 28 & 15 & 0.0009 & 0.0006 & $-1.0069$ & 0.363 \\
\hline
\end{tabular}
\caption{Nucleotide variations and summary statistics of the \textit{pfmrp1} gene from different geographical regions}
\end{table}

\textsuperscript{a}Statistically significant with $P$ values $\leq 0.05$.

\textsuperscript{b}Myanmar isolates are included in the South-East Asian isolates.
eight singletons, followed by five in African sequences. The level of genetic diversity was found to be the highest in the South-East Asian isolates, with values of $\pi = 0.0005$ and $\theta = 0.0006$, followed by west Asia, where the values for $\pi$ and $\theta$ were 0.00037 and 0.00044, respectively. The values of $\theta$ were found to be slightly higher than the estimates for $\pi$ in all five geographical regions excluding Myanmar (Table 2).

**Signature of purifying selection**

We performed several molecular evolutionary analyses ($d_N$-$d_S$, Tajima’s D and the MK test) to assess the role of natural selection on pfmrp1. The occurrence of positive selection of the pfmrp1 gene was evidenced by the positive $d_N$-$d_S$ estimations (Table 2). Moreover, $d_N$ was found to be significantly higher than $d_S$ in the isolates from Oceania and north-east Myanmar based on the codon-based Z-test (Table 2). Positive selection of the gene was further supported by the Tajima’s D and MK tests (see the Supplementary data available at JAC Online).

**Haplotype diversity**

For haplotype analysis, we used only seven mutations that were identified in the north-east Myanmar isolates (Table 1). Out of 193 sequences, 31 sequences that contained either missing information or mixed alleles at those seven nucleotide positions were excluded from further analysis. We analysed 162 sequences that included 112 from South-East Asia, 26 from Africa, 5 from west Asia, 10 from Oceania and 9 from South America. In total, 23 haplotypes were observed (Figure 2a), with a haplotype diversity of 0.812. Twelve of the haplotypes were singleton haplotypes, of which 10 were found in South-East Asian isolates and two in African isolates (Figure 2a). Highly frequent haplotype H_4 (HNSHITF) resembling 3D7 was found in 61 of the total isolates and the haplotype H_5 (YNAHVTI) resembling the Plasmodium reichenowi sequence was observed in 17 isolates (Figure 2a). Haplotype H_4 was observed in all the regions studied here, whereas H_5 was observed in isolates from Thailand, Myanmar and Oceania (Figure 2). The second most highly frequent haplotype H_2 (YNAHVTI) was observed in 23 isolates, followed by H_6 in 19 isolates and then H_5 in 17 isolates (Figure 2). Interestingly, these three haplotypes contained Y191, A437 and V876 mutations in common. Moreover, this triple mutation combination was observed in 73 out of the 162 isolates, where they were found in combination with mutations at other positions. Out of a total of 23 haplotypes, 15 were found only in north-east Myanmar region, of which H_2 showed a high frequency (Figure 2b).

Pairwise LD analysis revealed several significant associations after Bonferroni correction between different alleles (Table S2, available as Supplementary data at JAC Online). Notably, positions

<table>
<thead>
<tr>
<th>Name</th>
<th>Number</th>
<th>Haplotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hap_1</td>
<td>5 (3)</td>
<td>YNAHITF</td>
</tr>
<tr>
<td>Hap_2</td>
<td>23 (22)</td>
<td>YNAHVTI</td>
</tr>
<tr>
<td>Hap_3</td>
<td>3 (1)</td>
<td>YNAHVTI</td>
</tr>
<tr>
<td>Hap_4</td>
<td>61 (29)</td>
<td>HNSHITF</td>
</tr>
<tr>
<td>Hap_5</td>
<td>17 (9)</td>
<td>YNAHVTI</td>
</tr>
<tr>
<td>Hap_6</td>
<td>19 (19)</td>
<td>YNAHVTI</td>
</tr>
<tr>
<td>Hap_7</td>
<td>4 (4)</td>
<td>YNAHVTI</td>
</tr>
<tr>
<td>Hap_8</td>
<td>7 (7)</td>
<td>YSAHTF</td>
</tr>
<tr>
<td>Hap_9</td>
<td>1 (0)</td>
<td>HNSHITF</td>
</tr>
<tr>
<td>Hap_10</td>
<td>5 (2)</td>
<td>HNSHITF</td>
</tr>
<tr>
<td>Hap_11</td>
<td>1 (0)</td>
<td>HNSHITF</td>
</tr>
<tr>
<td>Hap_12</td>
<td>4 (4)</td>
<td>YSAHTF</td>
</tr>
<tr>
<td>Hap_13</td>
<td>1 (1)</td>
<td>YNAHVTI</td>
</tr>
<tr>
<td>Hap_14</td>
<td>1 (1)</td>
<td>YNAHVTI</td>
</tr>
<tr>
<td>Hap_15</td>
<td>2 (2)</td>
<td>HNSHITF</td>
</tr>
<tr>
<td>Hap_16</td>
<td>1 (1)</td>
<td>YNAHVTI</td>
</tr>
<tr>
<td>Hap_17</td>
<td>1 (1)</td>
<td>YNAHVTI</td>
</tr>
<tr>
<td>Hap_18</td>
<td>1 (1)</td>
<td>YSAHTF</td>
</tr>
<tr>
<td>Hap_19</td>
<td>1 (1)</td>
<td>YNAHVTI</td>
</tr>
<tr>
<td>Hap_20</td>
<td>1 (1)</td>
<td>HNSHITF</td>
</tr>
<tr>
<td>Hap_21</td>
<td>1 (1)</td>
<td>HNSHITF</td>
</tr>
<tr>
<td>Hap_22</td>
<td>1 (1)</td>
<td>YSAHTF</td>
</tr>
<tr>
<td>Hap_23</td>
<td>1 (1)</td>
<td>YSAHTF</td>
</tr>
</tbody>
</table>

Numbers in brackets are South-East Asian isolates in Fig (b)

**Figure 2.** Haplotype network for (a) 23 haplotypes in 162 *P. falciparum* isolates and (b) 21 haplotypes in 112 South-East Asian *P. falciparum* isolates. The size of the circle indicates the number of haplotypes and the lines between them are the interconnections based on type and number of mutations between two haplotypes. Each colour codes for different geographical regions: yellow, South-East Asia; red, Africa; green, South America; dark blue Oceania; black, west Asia; pink, Myanmar; light blue, Cambodia; purple, Thailand. Hap_4 (wild-type) is present in all the populations, whereas H_5 (resembling *P. reichenowi*) is reported only from north-east Myanmar, Thailand and Oceania. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.
191, 437 and 876 showed significant association with each other and also with positions 1007 and 1390 (Table S2). In haplotype network analysis, haplotype H, which contains the triple mutations at V_{191}^{Y}, A_{437}^{A} and V_{876}^{Y} was found to be the most interconnected with other haplotypes (Figure 2a) and revealed the highest out-group probability value ($P=0.11$) estimated from nested clade analysis using the TCS software. This indicates that alleles Y_{191}, A_{437} and V_{876} are ancestral and also resemble the alleles present in *P. reichenowi*.

### In vitro drug susceptibility assay

The IC_{50} values of seven drugs were estimated for 28 isolates using 3D7 as a reference. The IC_{50} values of each drug were found to be significantly higher than that of 3D7; however, the range of each drug was quite large. The means and ranges of the IC_{50} values for the seven drugs are shown in Table 3. The mean IC_{50} values of the isolates collected in different years from 2007 to 2010 were also determined. The IC_{50} values were not significantly different for the isolates from different years for any drug. Spearman’s correlation coefficient for the in vitro susceptibility of the drugs showed an association between different drugs (Table S3, available as Supplementary data at JAC Online). Mefloquine showed a significant positive correlation with pyronaridine, quinine and lumefantrine, whereas the in vitro response to chloroquine was negatively correlated with those to mefloquine and lumefantrine, although the correlations were not statistically significant.

### Association of in vitro drug susceptibilities and pfmrp1 variants

We performed genotype–phenotype association analysis for the seven drugs. On analysing the association between each allele and the in vitro response to the drugs, we identified significant associations between allele I_{1390} and response to chloroquine ($P=0.009$; Table S4, available as Supplementary data at JAC Online). We also observed a significant association between the haplotype Y_{191}^{A}A_{437}^{A}V_{876}^{Y}I_{1390} and the response to chloroquine ($P=0.048$), in contrast to the isolates bearing alleles V_{191}^{Y}A_{437}^{A}V_{876}^{Y}I_{1390}. With bootstrap analysis, we identified an association between the allele at T_{1007} and the response to mefloquine ($P=0.025$), and the H_{785} allele was found to be associated with the response to pyronaridine ($P=0.044$).

### Table 3. In vitro IC_{50} values (nM) of seven antimalarial drugs for 28 culture-adapted field isolates from north-east Myanmar

<table>
<thead>
<tr>
<th>Drug</th>
<th>Mean (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyronaridine</td>
<td>6.7 (2.5 – 14.4)</td>
</tr>
<tr>
<td>Lumefantrine</td>
<td>7.9 (2.0 – 16.2)</td>
</tr>
<tr>
<td>Piperaquine</td>
<td>20.2 (8.1 – 41.2)</td>
</tr>
<tr>
<td>Mefloquine</td>
<td>23.5 (6.3 – 44.6)</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>1066.6 (29.9 – 4110.0)</td>
</tr>
<tr>
<td>Quinine</td>
<td>62.3 (16.1 – 229.4)</td>
</tr>
<tr>
<td>Fansidar^{a}</td>
<td>146.2 (0.1 – 532.8)</td>
</tr>
</tbody>
</table>

^{a}IC_{50} of fansidar is given in µg/mL.

### Discussion

Assessing the signatures of positive selection of a candidate gene involved in drug resistance is a good indicator of its importance in natural populations. Common signatures of positive selection such as low levels of genetic diversity, an excess of rare alleles and deviation from neutrality with a higher rate of non-synonymous substitutions can be identified by molecular evolutionary analysis. This approach has been widely exploited in a genome-wide analysis of *P. falciparum* to identify drug resistance markers.^{25–28} In particular, transporter genes belonging to the ABC family have been extensively explored as potential determinants of resistance to antimalarial drugs. *pfmrp1* from the ABC family of transporters has recently emerged as a potential genetic target for multiple drugs. The mutations in this gene have been significantly associated with responses to chloroquine, quinine, mefloquine, sulfadoxine/pyrimethamine and artemisinin derivatives.^{9–12} Knockout of this gene in the *P. falciparum* W2 strain results in an increased susceptibility of the parasite to chloroquine, quinine, piperaquine, primaquine and artemisinin,^{13} indicating a potential role of this gene in the response to multiple antimalarials. Moreover, recent genome-wide association analysis revealed that an F1390I mutation in this gene was associated with the artemisinin clearance rate in the Cambodian *P. falciparum* population.^{29} A previous analysis of this gene in isolates from the China–Myanmar border also showed an association of the I876V mutation with the in vitro susceptibility of *P. falciparum* isolates to chloroquine.^{3} All these findings favour *pfmrp1* as an important candidate gene for drug resistance and prompted us to investigate the nature of drug selection of the gene. Here, we analysed full-length *pfmrp1* sequences from global parasite populations to infer signatures of drug selection and tried to identify the association of this gene in *P. falciparum* isolates from the north-east Myanmar region with the response to multiple drugs.

Eight polymorphic sites were observed in the 93 *P. falciparum* isolates studied, resulting in seven codons displaying a single point mutation. These observed mutations were previously known in *P. falciparum*.^{3,9–13} It is interesting to note that all the non-synonymous mutations were frequent, with a frequency ranging from 10% to 66%, and only one synonymous mutation was observed. The rate of non-synonymous substitutions per site (d_{s}/d_{n}) was found to be statistically significant. In addition, the MK test revealed a significant difference between synonymous and non-synonymous polymorphisms within and between species. The evidence of positive selection was further supported by the low level of nucleotide diversity measured by \( \theta \) and \( \alpha \). All analyses except for Tajima’s D revealed that the *pfmrp1* gene is under positive selection—the values of Tajima’s D were found to be positive due to the presence of frequent mutations in the gene.

In the worldwide isolates, the *pfmrp1* gene was found to be similarly less diverse in all geographical regions. In contrast to 22 non-synonymous mutations, only six synonymous mutations were observed. Moreover, five of the six synonymous mutations were singletons, the exception being one that was observed in 51% of the total isolates. In particular, this mutation at nucleotide 4497 had the same allele C in all the north-east Myanmar isolates, in contrast to A in all the other isolates from different regions. However, any direct biological relevance of the synonymous mutation could not be inferred, although synonymous mutations are known to influence protein expression, conformation and
Moreover, a geographically specific distribution of nucleotide diversity in pfmrp1 has previously been observed and was also observed in the distribution map of non-synonymous mutations of this gene (Figure 1). The reasons for these differences are unclear; one possible reason is the spatial and temporal variations that arise due to the different patterns and times of drug usage and the history of the emergence of drug resistance. This gene was found to be less diverse in all the regions showing low values of $\pi$ and $\theta$ estimates. Moreover, we observed positive values of $d_{N} - d_{S}$ and negative values of Tajima’s $D$ in all regions, which further suggests a positive selection of this gene.

Haplotype analysis of the pfmrp1 gene also revealed an interesting pattern. A total of 23 haplotypes were observed, of which 12 were found only once. The wild haplotype resembling 3D7-type alleles (H_4) was the most frequent and widely distributed, being detected in 61 isolates (~38%) from all geographical regions (Figure 2a). The rest of the 22 mutant haplotypes in 101 isolates showed an interesting pattern, as 73 out of 101 isolates (~72%) contained mutations Y191A, A437 and Y876 either alone or in combination with mutations at four other positions. Moreover, LD analysis revealed significant associations among these three mutations (Table S2). This observation corroborates earlier findings that reported a significant association between H191Y and S437A in P. falciparum. Haplotype network analysis also revealed that the haplotype containing these three mutations was the most ancestral of all and that all other haplotypes might have originated from this haplotype. The fact that the P. reichenowi mrp1 sequence contains the same alleles at these nucleotide positions lends further support to this assumption. This might be attributed to a common origin, selection pressure or a functional relevance of these mutations. Notably, the haplotype H_2 that bears these three mutations was found only in Myanmar (22 isolates) and Oceania (one isolate) (Figure 2b). Moreover, all the nine haplotypes that share these mutations were found to be specific to South-East Asia and the Oceania region (Figure 2). In particular, the mutations found in north-east Myanmar were all identified earlier in the GMS countries Thailand and Cambodia, suggesting that the parasite populations in the GMS might have had the same origin. Whereas the new mutations found in Thailand and Cambodia might have been due to divergent drug selection pressures in these countries, the uniqueness of the north-east Myanmar parasite population might reflect limited gene flow or isolation of this population from other GMS populations. Such population isolation could be attributed to the restricted human movements in north-east Myanmar as a result of military conflicts between Kachin and the ruling government.

Whereas the functional relevance of these mutations has not yet been clearly defined, Mu et al. has established a significant association of these mutations with the responses to chloroquine and quinine in parasite isolates from Thailand. However, this association was not evident in a later study conducted on parasite isolates from the same region. A subsequent study reported an association of mefloquine with alleles H191 and S437 in isolates from Angola and Y191 and A437 in isolates from Thailand, respectively. The roles of these mutations have yet to be confirmed, but a geographically specific association between mutations is interesting. The third mutation at position 876 is present in the Walker sequence of the nucleotide binding domain of pfmrp1 and has been shown to play a significant role in changing the functionality of the protein. Moreover, recent studies have revealed its association with in vitro susceptibility to chloroquine in isolates from the China–Myanmar border area and with artesether-lumefantrine in African isolates. Conversely, none of these three mutations individually showed an association with any of the drugs analysed here. Interestingly, the isolates with the Y191A, A437, Y876F haplotype were found to be associated with a response to chloroquine ($P=0.009$); however, this association was not observed with the Y191A, A437, Y876F haplotype. This outcome may therefore have resulted from the influence of the F1390I mutation. Earlier reports also suggest that F1390I is associated with responses to artemisinin, mefloquine and lumefantrine. Like other multiple drug resistance genes (e.g. pfmdr1), pfmrp1 has shown an association between multiple drugs with a particular mutation (as discussed above for the F1390I mutation) as well as a single drug with multiple mutations (e.g. chloroquine with mutations at positions 191, 437, 876 and 1390). The latter highlights the importance of haplotypes (a combination of different mutations) in determining a particular phenotype.

Another important position, 1466 in pfmrp1, contained only the K allele, which in an earlier study has been shown to be associated with reduced susceptibility to sulfadoxine/pyrimethamine. The fixation of K1466 might be due to the high drug pressure of sulfadoxine/pyrimethamine in the north-east Myanmar region, which is also evident from the wide range of IC_{50} values for sulfadoxine/pyrimethamine (mean IC_{50}=146.2 µg/mL; range 0.1–532.8 µg/mL). In addition, the $\text{IC}_{50}$ in the $\text{IC}_{50}$ assay identified significant positive correlations of mefloquine with pyronaridine, lumefantrine and quinine, whereas the response to chloroquine was negatively correlated with the responses to mefloquine and lumefantrine. This is consistent with the different selection pressures of these drugs on the different parasite genotypes. For example, chloroquine has been shown to select the pfmdr1 186Y mutation, whereas lumefantrine tends to select the wild-type N86 at this position. Therefore, the prevalence of pfmrp1 mutations in different geographical regions and the associations of different mutations with multiple drugs indicate selection pressure by multiple drugs on the pfmrp1 gene, which signifies its potential as an important candidate gene for monitoring drug resistance.

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**Transparency declarations**

None to declare.
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Supplementary data
Supplementary data, including Tables S1 to S4, are available at JAC Online (http://jac.oxfordjournals.org/).

References