Molecular analysis of chloroquine resistance in *Plasmodium falciparum* in Yunnan Province, China

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**Summary**

Resistance of *Plasmodium falciparum* to chloroquine (CQ) is determined by the mutation at K76T of the *P. falciparum* chloroquine resistance transporter (*pfcrt*) gene and modified by other mutations in this gene and in the *P. falciparum* multidrug resistance 1 (*pfmdr1*) gene. To determine the extent of polymorphisms in these genes in field *P. falciparum* isolates from Yunnan province of China, we genotyped the *pfcrt* codon 76, *pfmdr1* codons 86 and 1246. Our results showed that although CQ has been withdrawn from treating falciparum malaria for over two decades, 90.3% of the parasites still carried the *pfcrt* K76T mutation. In contrast, mutations at *pfmdr1* codons 86 and 1246 were rare. Sequencing analysis of the *pfcrt* gene in 34 parasite field isolates revealed CVIET at positions 72–76 as the major type, consistent with the theory of Southeast Asian origin of CQ resistance in the parasite. In addition, two novel *pfcrt* haplotypes (75D/144Y/220A and 75E/144Y/220A) were identified. Real-time polymerase chain reaction was used to determine *pfmdr1* gene amplification, which is associated with melfloquine resistance. Our result indicated that in agreement with that melfloquine has not been used in this area, most (>90%) of the parasites had one *pfmdr1* copy. Genotyping at two hypervariable loci showed relatively low levels of genetic diversity of the parasite population. Meanwhile, 28.4% of cases were found to contain mixed clones, which favour genetic recombination. Furthermore, despite a unique history of antimalarial drugs in Yunnan, its geographical connections with three malarious countries facilitate gene flow among parasite populations and evolution of novel drug-resistant genotypes. Therefore, continuous surveillance of drug resistance in this area is necessary for timely adjustment of local drug policies and more effective malaria control.

**Keywords** malaria parasite, chloroquine resistance, *Plasmodium falciparum* chloroquine resistance transporter gene, *Plasmodium falciparum* multidrug resistance 1, genetic diversity

**Introduction**

The emergence and spread of malaria parasites resistant to many antimalarials, particularly chloroquine (CQ) and pyrimethamine (PY)–sulphadoxine (SD), is largely responsible for the recent global resurgence of malaria, which severely hampers our capacity to roll back malaria (Snow *et al.* 2004; Olliaro 2005). In China, malaria is still a major public health problem with most of the cases concentrated in two subtropical provinces, Yunnan and Hainan. Yunnan is located in Southwest China, bordering three malaria-endemic countries, Myanmar, Laos and Vietnam. Malaria here is perennial with peak transmission occurring from June through October, coincident with the rainy season. Malaria cases in Yunnan are concentrated in several counties near the borders and about one-third are due to *Plasmodium falciparum* infections. Here, CQ resistance in *P. falciparum* appeared in the early 1970s and spread rapidly. By the early 1980s, it was replaced by PY–SD to treat falciparum malaria (Liu *et al.* 2005).

Since then, longitudinal surveys have been carried out in these two malarious provinces and a general decline of CQ resistance was noticed (Liu *et al.* 2005). In Hainan, *in vitro* microtests demonstrated a drop in the rate of CQ-resistant *P. falciparum* from 97.9% in 1981 to 60.9% in 1991 and
26.7% in 1997 (Liu et al. 1995, 2005). Correspondingly, 4-week in vivo observations detected a decrease of CQ-resistant parasites from 84.2% in 1981 to 40% in 1991 and 18.4% in 1997. Although a similar trend was observed in Yunnan, the decrease of CQ resistance was insignificant and over 90% of P. falciparum remained CQ resistant in 1992 (Yang et al. 1994). A more recent survey in 2002 indicated that CQ resistance rate in Yunnan was still above 70% (Liu et al. 2005). While this trend of declining CQ resistance as its cessation in many endemic areas suggests a possible rotation of CQ in the future (Kublin et al. 2003), such a decision requires close regional monitoring because evolution of CQ resistance after the removal of drug pressure seems to differ considerably among parasite populations.

Chloroquine resistance is manifested by impaired CQ uptake by the parasite vacuole, which is correlated with mutations in several genes. A single mutation K76T in the P. falciparum chloroquine resistance transporter gene (pfcr) has been demonstrated to be a major determinant of CQ resistance in P. falciparum (Fidock et al. 2000; Sidhu et al. 2002; Lakshmanan et al. 2005). All P. falciparum clinical samples that are resistant to CQ contain the K76T mutation (Djimdé et al. 2001). As at least five founder mutations of K76T have been detected worldwide (Wellem & Plowe 2001; Wootton et al. 2002; Chen et al. 2005), genetic backgrounds of the resistant strains differ greatly. Other epistatic markers such as P. falciparum multidrug-resistant 1 (pfmdr1) gene also affect the final outcome of CQ resistance (Reed et al. 2000; Babiker et al. 2001). Five single nucleotide polymorphisms (SNPs) of pfmdr1 N86Y, Y183F, S1034C, N1042D and D1246Y were identified in field isolates from different regions of the world. Some correlation studies showed that 86Y was associated with CQ resistance in isolates from the Old World, whereas the C-terminal mutations were found in isolates from South America (Hayton & Su 2004). A recent study demonstrated that N1042D of pfmdr1, a prevalent mutation in South America, contributes to quinine instead of CQ resistance, suggesting that pfmdr1 mutations can affect parasite susceptibility to a wide range of antimalarials depending on the parasite’s genetic background (Sidhu et al. 2005). While the effect of pfmdr1 point mutations might affect the parasite’s sensitivity to different drugs, recent correlation studies demonstrated that pfmdr1 amplification is responsible for resistance to mefloquine (Wilson et al. 1993; Price et al. 1999, 2004; Nelson et al. 2005).

Currently, the K76T mutation is widely used as a reliable marker for CQ-resistance in epidemiological studies (Wongsrisrichalai et al. 2002). In different continents, this marker has been shown to correlate with in vivo CQ resistance (e.g. Jelinek et al. 2002; Berens et al. 2003; Daily et al. 2003). A recent molecular analysis of P. falciparum samples from Hainan province collected from 1978 to 2001 showed a continuous decrease in the prevalence of K76T, consistent with the increased CQ sensitivity determined by in vitro and in vivo drug assays (Wang et al. 2005). In Yunnan, most malaria drug resistance surveillance relied on an in vitro microtest described by Rieckmann et al. (1978) and in vivo monitoring (Yang et al. 1994, 1995, 1997), whereas molecular epidemiological studies of drug resistance have not been carried out. Here, we report the population diversity of P. falciparum parasites in Yunnan and evaluated the prevalence of polymorphisms at pfcr and pfmdr1. Our results showed a relatively low level of genetic diversity of the parasites with 28.4% mixed-strain infections. Despite the withdrawal of CQ from treating falciparum malaria for over two decades, the pfcr K76T mutation still had over 90% prevalence. In contrast to the high prevalence of 86Y mutation in certain Southeast Asian P. falciparum samples, pfmdr1 mutations at codons 86 and 1246 were very rare in the Yunnan samples. Consistent with that mefloquine has never been used here; the level of pfmdr1 gene amplification was low.

Materials and methods

Samples

Plasmodium falciparum field isolates were collected in 2003–2004 in two counties of Yunnan Province. Twenty samples were collected from Gengma county, Lincang district bordering Myanmar and 69 samples were from Mengla county, Xishuangbanna district, bordering Laos and Myanmar (Figure 1). Both areas had a similar level of malaria endemicity, within the range of 14–44 malaria incidence per 10 000 people in 2000. The human subject protocol for this study as part of the ongoing malaria epidemiological surveys of the Yunnan Institute of Parasitology was approved by the provincial review board. Parasite samples were collected from patients with uncomplicated P. falciparum infections before drug treatment. Initial diagnosis was carried out by microscopic examination of Giemsa-stained thick blood films. For each sample, approximately 200 µl of finger-prick blood were spotted on a piece of Whatman 3M filter paper and air-dried. The dried filters were stored in individual plastic bags at −20 °C until DNA extraction. Parasite DNA was extracted from the blood filters using either the Chelex boiling method (Plowe et al. 1995) or the QIAamp DNA mini kit (Qiagen, Valencia, CA, USA).
Parasite genotyping at merozoite surface protein 1 (msp1) and glutamate-rich protein (glurp) loci

Nested polymerase chain reaction (PCR) was performed to differentiate the three molecular types of msp1 in the polymorphic block 2 as described (Kaneko et al. 1997). First PCR was performed with conserved outer primers followed by three individual nested PCR using primer pairs specific for allelic variant families of the msp1 block 2. The central repeat fragment of glurp was amplified similarly by nested PCR using primers described earlier (Roper et al. 1998). Sizes of the PCR products were estimated following electrophoresis on agarose gels. Alleles with similar sizes (e.g. 350 and 375 bp) were binned around an average size. The multiplicity of infection (MOI), or number of genotypes per infection, was calculated as the highest number of genotypes at any of the two genes.

Determination of prevalence of *Plasmodium falciparum* chloroquine resistance transporter K76T, *Plasmodium falciparum* multidrug-resistant 1 N86Y and D1246Y mutations

To identify other *pfcr* mutations in the Yunnan isolates, we selected 34 samples of the 76T genotype for sequencing of the *pfcr* fragments. These samples were pre-determined by genotyping msp1 and glurp to be of single-clone infections in order to avoid complications during direct sequencing of the PCR products. In brief, three fragments of the *pfcr* gene were amplified from each sample using primers described by Chen et al. (2003). PCR products were purified by filtration columns (Qiagen) and sequenced directly using the BigDye terminator chemistry on an automated ABI sequencer. The accuracy of sequences was confirmed by sequencing from both ends of the PCR products.

Determination of *Plasmodium falciparum* multidrug-resistant 1 copy number by real-time polymerase chain reaction analysis

An increased *pfmdr1* copy number has been associated with *in vitro* and *in vivo* mefloquine resistance (Wilson et al. 1993; Price et al. 1999, 2004; Nelson et al. 2005). To assess the *pfmdr1* copy number in the Yunnan *P. falciparum* isolates, we performed real-time PCR analysis for 60 single-clone infections on the basis of the genotyping results. Real-time PCR was performed as described (Ferreira et al. 2006) using FastStart SYBR Green mix (Roche) with primers for *pfmdr1* and a reference gene on chromosome 14 encoding fructose-bisphosphate aldolase (*fba*) (*fba*-F: GGGAATATTCTCGAAGTAA and *fba*-R: CGGTGATGGTGTTTTTAG). Data were analysed using the OpticonMonitor Analysis Software and the threshold cycle, \( C_t \), was defined as the cycle number at which the change of fluorescence in the reaction exceeds 10 SDs above the background. Background fluorescence was calculated as the mean fluorescence between cycle 3 and 7 (Miao et al. 2006). For each sample, the \( C_t \) value was determined for both *pfmdr1* and *fba* genes. All reactions were performed in triplicate and the result was rejected if the SD value was above 0.5. Reactions performed with serial dilutions of DNA from the 3D7 clone, known to harbour a single copy of *pfmdr1* and *fba*, were used to

**Figure 1** Map of Yunnan Province showing the locations (stars) where parasite samples were collected.

1246 were previously described (Djimde et al. 2001). Apo I, which cuts the K76 codon, was used to distinguish wild-type from the mutant. To determine the polymorphisms at the *pfmdr1* codons 86 and 1246, *Afl* III and *Eco* RV were used, which cut the 86Y and 1246Y mutants, respectively (Duraisingh et al. 1997; Syafruddin et al. 2005).
calibrate the $C_t$ values from the field samples. Copy number of pfmdr1 gene was calculated using the $2^{-\Delta \Delta C_t}$ method: $\Delta C_t = (C_{t-\text{mdr}1} - C_{t-\text{fa}1}) - (C_{t-\text{mdr}1} - C_{t-\text{fa}1})_{375}$, where X is the test sample (Ferreira et al. 2006) (Table 3).

Results

Parasite population diversity

Parasites were genotyped at the msp1 and glurp loci to evaluate the population diversity at the two study sites in Yunnan. Both genes were successfully amplified from 88/89 samples (Table 1). While the result showed the presence of all three msp1 variant types (K1, MAD20 and RO33), the predominant allele family was MAD20, which was originally defined in an isolate from Papua New Guinea (PNG) (Tanabe et al. 1987). This suggests that MAD20 may be the prevalent type circulating in certain malarious regions of Asia. While only one size of approximately 150 bp was detected for the MAD20 type, the K1 type was more variable with four sizes (200, 300, 350 and 375 bp) being detected. The RO33 type was the least abundant at both sites. Genotyping msp1 also detected samples containing different allele families indicative of mixed-strain infections, which accounted for 21.1% and 30.4% of the samples from Gengma and Mengla, respectively. Comparing with a total of six alleles found for msp1, parasites at the glurp locus were less diverse with only four alleles observed and their distributions were also biased. The frequencies for the 375, 475, 550 and 800 bp alleles were 18.3%, 52.1%, 21.1% and 8.55%, respectively. The MOI of Mengla samples (1.3) was slightly higher than that of Gengma samples (1.21) (Table 1).

**Plasmodium falciparum** chloroquine resistance transporter allele types and their prevalence in Yunnan province

*In vitro* microtests indicated that although CQ was abandoned for one and two decades, the prevalence of CQ resistance in *P. falciparum*-endemic regions of Yunnan province remained high (Yang et al. 1994; Liu et al. 2005). Consistent with this notion, the prevalence of pfcr K76T mutation was 78.3% and 93.3% in *P. falciparum* samples from Gengma and Mengla counties, respectively. Overall, the K76T mutation had a 90.3% prevalence in the Yunnan samples.

In addition to the major CQ-resistance determinant K76T, there are at least 10 other pfcr mutations associated with CQ-resistant isolates from various geographical regions. To assess the levels of polymorphisms in 10 of these codons, we employed a strategy used by Chen et al. (2003) to amplify short fragments of pfcr from 34 single clones for sequencing. Consistent with the result from PCR–RFLP analysis, sequencing confirmed the presence of K76T mutation in all 34 samples. A total of five haplotypes were observed with the haplotype a as the most prevalent, accounting for 70.6% of the sequenced samples (Table 2). This haplotype was also the major one observed in CQ-resistant parasites from Asia and Africa (Wootton et al. 2002). Compared with the wild-type CQ-sensitive sequence of the 3D7 clone, all the pfcr sequences from Yunnan had 5–6 mutant codons. Sequences at positions 72–76 were either CVIET or CVIDT. None of the predominant sequences found in the Philippines (CVMHT) or Papua New Guinea (SVMNT) was observed in Yunnan. Whereas the A220S mutation is associated with the K76T mutation in most CQ-resistant parasites, 9/34 sequenced Yunnan samples with K76T mutation had the wild-type 220A (Table 2). This haplotype was also the major one observed in CQ-resistant parasites from Asia and Africa (Wootton et al. 2002). Compared with the wild-type CQ-sensitive sequence of the 3D7 clone, all the pfcr sequences from Yunnan had 5–6 mutant codons. Sequences at positions 72–76 were either CVIET or CVIDT. None of the predominant sequences found in the Philippines (CVMHT) or Papua New Guinea (SVMNT) was observed in Yunnan. Whereas the A220S mutation is associated with the K76T mutation in most CQ-resistant parasites, 9/34 sequenced Yunnan samples with K76T mutation had the wild-type 220A (Table 2). A similar observation was made in a subset of samples from the Philippines (Chen et al. 2003). Furthermore, two abundant mutations A144T and L160Y identified in samples from the Philippines (Chen et al. 2003) and the A144F mutation observed in other Southeast Asian samples (Durrand et al. 2004) were not found in the Yunnan samples. Instead, a novel A144Y mutation was found in 9/34 sequences. Interestingly, all the A144Y mutants were linked with the wild-type A220. Collectively, this study identified two novel pfcr haplotypes 75D/144Y/220A and 75E/144Y/220A.

**Table 1**  Parasite diversity at the msp1 site

<table>
<thead>
<tr>
<th>Sites</th>
<th>K1 (%)</th>
<th>MAD20 (%)</th>
<th>RO33 (%)</th>
<th>Number of samples†</th>
<th>Number of isolates</th>
<th>MOI</th>
<th>Number of mixed infections (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gengma</td>
<td>21.7</td>
<td>69.6</td>
<td>8.7</td>
<td>19/20</td>
<td>23</td>
<td>1.21</td>
<td>4 (21.1)</td>
</tr>
<tr>
<td>Mengla</td>
<td>31.1</td>
<td>67.8</td>
<td>1.1</td>
<td>69/69</td>
<td>90</td>
<td>1.30</td>
<td>21 (30.4)</td>
</tr>
<tr>
<td>Total</td>
<td>29.2</td>
<td>68.1</td>
<td>2.7</td>
<td>88/89</td>
<td>113</td>
<td>1.28</td>
<td>25 (28.4)</td>
</tr>
</tbody>
</table>

MOI, multiplicity of infection.
†Number of samples successfully tested/total number of samples.
Plasmodium falciparum multidrug-resistant 1 single nucleotide polymorphisms and copy number

In sharp contrast to the prevalence of K76T mutation in the Yunnan P. falciparum samples, only one isolate (<1%) had the pfmdr1 N86Y mutation, while none was mutated at codon 1246 (Table 3). To rule out that the result obtained by PCR–RFLP analysis was due to incomplete enzyme digestion, we randomly selected 10 PCR products from each pfmdr1 fragment for sequencing, which further confirmed the presence of wild-type sequences at the two codons (data not shown).

From 60 single-clone infections, the pfmdr1 copy number was successfully determined in 53 samples, 44 of which were from Mengla county. When rounded to the closest integer, 90.6% isolates had a single copy pfmdr1, 7.5% had two copies, and only one sample (1.9%) had three copies (Table 3). Interestingly, all these five isolates with amplified pfmdr1 were from Mengla.

Table 2 Sequence polymorphisms in pfcr

<table>
<thead>
<tr>
<th>Origin</th>
<th>Isolate</th>
<th>Amino acid in pfcr at position</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yunnan, China</td>
<td>a (24)</td>
<td>C I E T H A L S E I</td>
</tr>
<tr>
<td>b (7)</td>
<td>C I D T H Y L A E R</td>
<td></td>
</tr>
<tr>
<td>c (1)</td>
<td>C I D T H Y L A E I</td>
<td></td>
</tr>
<tr>
<td>d (1)</td>
<td>C I E T H Y L A E R</td>
<td></td>
</tr>
<tr>
<td>e (1)</td>
<td>C I E T H A L S E R</td>
<td></td>
</tr>
<tr>
<td>Thailand</td>
<td>a</td>
<td>C I E T H A L S E I</td>
</tr>
<tr>
<td>b</td>
<td>C I E T L A L S E I</td>
<td></td>
</tr>
<tr>
<td>Cambodia</td>
<td>a</td>
<td>C I E T H A L S E I</td>
</tr>
<tr>
<td>b</td>
<td>C I D T H S E R</td>
<td></td>
</tr>
<tr>
<td>c</td>
<td>C M N T A</td>
<td></td>
</tr>
<tr>
<td>d</td>
<td>C T N S</td>
<td></td>
</tr>
<tr>
<td>PNG</td>
<td>a</td>
<td>S M N T H A L S Q R</td>
</tr>
<tr>
<td>b</td>
<td>S M N T H A L A Q R</td>
<td></td>
</tr>
<tr>
<td>Solomon</td>
<td>S M N T H A L S Q R</td>
<td></td>
</tr>
<tr>
<td>Philippines</td>
<td>a</td>
<td>C M N T H T Y A Q R</td>
</tr>
<tr>
<td>b</td>
<td>S M N T H T Y A Q R</td>
<td></td>
</tr>
<tr>
<td>c</td>
<td>C I E T H A L S E I</td>
<td></td>
</tr>
<tr>
<td>Brazil</td>
<td>S M N T H A L S Q R</td>
<td></td>
</tr>
<tr>
<td>Ecuador</td>
<td>C M N T H S Q R</td>
<td></td>
</tr>
<tr>
<td>Colombia</td>
<td>C M E T Q S T</td>
<td></td>
</tr>
</tbody>
</table>

Table 3 Copy number and frequencies of mutations in pfmdr1

<table>
<thead>
<tr>
<th>Polymorphisms</th>
<th>Copy number</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>N86Y</td>
<td>0.9%</td>
<td></td>
</tr>
<tr>
<td>D1246Y</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>1X</td>
<td>90.6%</td>
<td></td>
</tr>
<tr>
<td>2X</td>
<td>7.5%</td>
<td></td>
</tr>
<tr>
<td>3X</td>
<td>1.9%</td>
<td></td>
</tr>
</tbody>
</table>

Number in parenthesis indicates the number of isolates with that respective haplotype.

Discussion

The widespread resistance to CQ and other antimalarials occurring in Southeast Asia has fostered the deployment of artemisinin-based combination therapy for falciparum malaria. Beginning in 2005, artemisinin-based combination therapy (dihydroartemisinin–piperaquine, Artekin) began to be the choice of P. falciparum therapy in Yunnan. Since widespread resistance to piperaquine has been observed (Yang et al. 1995), suitability of this partner drug for artesunate in this area is questioned. Meanwhile, declining resistance to CQ observed in P. falciparum in many malarious regions after its withdrawal suggests a possibility of future reintroduction of the drug. This consider especially requires better understanding of the evolution of resistance in the absence of the drug pressure.

While drug resistance is expected to drop after the removal of selective pressure, this process can vary greatly between locations. More surprisingly, the two closely located provinces in China also experienced quite different paces in the evolution of CQ resistance although CQ was withdrawn almost concomitantly from the two provinces.
two decades ago. Whereas a significant decline of CQ resistance was observed in Hainan following the cessation of CQ use (Liu et al. 1995; Wang et al. 2005), CQ resistance in Yunnan province remained high (Yang et al. 1997). Our molecular analysis of the major genetic determinant of CQ, the K76T mutation in \textit{pfcrt}, also supports the results from the \textit{in vitro} microtests. Although the exact mechanism remains to be elucidated, we speculate that several factors may have contributed to the persistence of the K76T mutation in the Yunnan \textit{P. falciparum} populations.

First, vivax malaria accounts for approximately 2/3 of all malaria cases in Yunnan and CQ is still the recommended drug for treating vivax malaria. In such \textit{P. vivax}–\textit{P. falciparum} sympatric areas, mixed-species infections are common and can reach as high as 20% (Snounou et al. 1993). Moreover, some of these co-infections are often diagnosed as vivax malaria by conventional microscopic examination and treated with CQ, when 
cryptic

\textit{CQ-resistant \textit{P. falciparum}} infections emerge after treatment (Mayxay et al. 2001). Undoubtedly, CQ selective pressure on \textit{P. falciparum} is retained in areas of \textit{falciparum–vivax} co-existence. This factor may be responsible for the slow decline of \textit{P. falciparum} CQ resistance in countries and regions of the Mekong Delta (Berens et al. 2003; Lim et al. 2003; Durrand et al. 2004; Congpuong et al. 2005).

Second, unlike Hainan Island that is isolated from other malarious regions, Yunnan borders three Southeast Asian countries, where malaria is highly endemic and CQ resistance levels are high (Labbe et al. 2001; Ngo et al. 2003; Dittrich et al. 2005; Hatabu et al. 2005). Genetic exchange and spread of resistant genotypes likely play an important role in maintaining CQ resistance in this area.

Third, deployment of structurally similar drugs with cross-resistance will slow down the decline of resistance. A prominent example is cross-resistance between CQ and amodiaquine (Holmgren et al. 2006). Although amodiaquine has never been used for malaria control in Yunnan, \textit{P. falciparum} is highly resistant to this drug (Yang et al. 1997). In malaria-endemic areas of China, another quinoline drug, piperaquine has been used extensively in the light of CQ resistance, and widespread resistance to this drug has been observed (Yang et al. 1995). While cross-resistance between the two drugs has not been clearly established (Basco & Ringwald 2003), the fact that they both contain similar structural components suggests that extensive deployment of piperaquine may have imposed a positive selection pressure on \textit{pfcrt} mutations. Last, while development of drug resistance generally has fitness cost (Walliker et al. 2005), the evidence for reduced fitness in CQR parasites is circumstantial, which may vary from population to population. It is possible that the acquisition of CQR alleles in the studied parasite populations in Yunnan might not result in reduced fitness as observed elsewhere (Kublin et al. 2003; Wang et al. 2005); thus the mutations would persist even in the absence of drug pressure. Future detailed studies of the genetic backgrounds of parasites in this area are needed for the elucidation of resistance to quinoline drugs.

Molecular and population studies suggest that CQ resistance in \textit{P. falciparum} has evolved independently in at least four regions: Southeast Asia, PNG, and two in South America (Wellens & Plowe 2001; Wootton et al. 2002). The recent detection of other novel \textit{pfcrt} alleles in the Philippines suggests the possibility of additional founder events (Chen et al. 2003). A cluster of mutations in \textit{pfcrt} amino acids 72–76 distinguishes three major haplotypes in the global \textit{P. falciparum} populations: the wild-type CVMK, CVIET found in Asia/Africa, SVMNT associated with PNG and South America (Wootton et al. 2002). Besides these major haplotypes, others have been associated with different geographical populations of the parasite. For example, SVIET, CVIKT, CVIDT and CVTNT have been found in Southeast Asia (Nagesha et al. 2003; Lim et al. 2003). It is interesting that the SVMNT type, originally considered to be associated only with the PNG/ South America populations (Mehlotra et al. 2001), was recently found to be the predominant haplotype in the Philippines (Chen et al. 2003), India (Vathsala et al. 2004) and Iran (Ursing et al. 2006). Our analysis of 34 samples from Yunnan identified two haplotypes CVIET (76%) and CVIDT (24%). That the former is the major haplotype present in Asia is consistent with the theory of Southeast Asian origin of the CQ-resistant parasites. The observation of the east-to-west increase of CQ resistance in Cambodia provides stronger support of this theory (Lim et al. 2003).

As acquisition of CQ resistance is likely a stepwise process, the N75D and N75E mutations observed in the present and earlier studies might be different steps [\textit{AAT(N)} \rightarrow \textit{GAT(D)} \rightarrow \textit{GAA(E)}] in the evolution of \textit{pfcrt} (Lim et al. 2003; Mittra et al. 2006). It is noteworthy that the N75E mutants also exhibit much higher IC\textsubscript{50} values to CQ than the N75D mutants (Durrand et al. 2004). While the significance of other \textit{pfcrt} mutations is less well understood, the A220S mutation has been tightly linked with the K76T mutation in most of the global CQ-resistant lines. Our analysis of the Yunnan \textit{P. falciparum} isolates revealed that 25/34 of the isolates sequenced had such an association, whereas approximately 25% of the K76T isolates were wild-type at A220. The 76T/220A combination was also found as the main type in a subset of samples from the Philippines (Chen et al. 2003). The simultaneous finding of A144T and L160Y mutations in these samples led the authors to hypothesize that these might be compensatory
mutations that confer CQ resistance in the absence of the A220S mutation. Interestingly, we did not observe the A144T and L160Y mutations in the Yunnan samples. Instead, our analysis of limited samples found that the 76T/220A haplotype was always associated with the A144Y mutation. The role of these new pfcr1 haplotypes in CQ resistance awaits further investigations.

The history of antimalarial drug usage in Yunnan is distinctive from those of neighbouring countries and other parts of the world. As a result of extensive CQ resistance in many parts of the world, in late 1970s, this drug was withdrawn from treating falciparum malaria in 1983 in Yunnan. After replacement with PY-SP for a short while, artemisinin treated falciparum malaria in 1983 in Yunnan. After P. falciparum parts of the world. As a result of extensive CQ resistance in late 1970s, this drug was withdrawn from treating falciparum malaria in 1983 in Yunnan. After replacement with PY-SP for a short while, artemisinin drugs were introduced and gradually became the mainstay of malaria therapy. Other drugs that have been used widely in bordering countries such as mefloquine have not been used in this region. Such a unique history of antimalarial drug usage probably has shaped the current parasite populations with different genetic backgrounds.

Of particular interest is the pfmdr1 gene encoding an ABC transporter, which is a possible modulator of drug resistance in P. falciparum. Its effect in modulating resistance to CQ and other antimalarials probably depend on the genetic background of the parasite (Reed et al. 2000; Sidhu et al. 2005). Many field studies aiming to establish a statistical association of the widespread N86Y mutation in Asia and Africa with CQ resistance have yielded conflicting results (Wellems & Plowe 2001). Recent studies with Southeast Asian strains of P. falciparum also failed to demonstrate the N86Y mutation in CQ resistance, but established the role of pfmdr1 amplification in mefloquine resistance (Price et al. 1999, 2004; Pickard et al. 2003; Nelson et al. 2005). In contrast to the high prevalence of 86Y mutation in some parts of Southeast Asia such as Indonesia (Syfruddin et al. 2005), we found that mutations at codons 86 and 1246 were very rare in the Yunnan P. falciparum population. This result is congruent with similar findings of other P. falciparum populations from the Mekong Delta (Congpuong et al. 2005; Khim et al. 2005; Wang et al. 2005). It is likely that pfmdr1 mutations may not play an important role in CQ resistance in this large region. Furthermore, real-time PCR analysis showed that less than 10% of the parasites from Yunnan had the pfmdr1 gene amplification, which contrasted with 54% of isolates at the Thai–Myanmar border (Price et al. 2004). Again, this difference can be explained by the distinct mefloquine use histories in the two countries. Whereas mefloquine has never been used in Yunnan and parasites here are generally susceptible to this drug, it has been widely deployed in Thailand since 1985 (Yang et al. 1997; Sattabongkot et al. 2004). The lack of large geographical barriers between the two sites may facilitate the spread of resistant parasites, which may account for the low-level pfmdr1 amplification observed in the Yunnan parasite population.

Genetic diversity of the malaria parasite at a given region is correlated with the local transmission intensity. Drug selection, however, tends to diminish genetic diversity, especially near the resistance-determinant locus (Wootton et al. 2002). In agreement with Yunnan as a malaria hypoendemic region, genetic diversity at the hypervariable sites msp1 (six alleles) and glurp (four alleles) was lower than what was observed previously in a similarly hypoendemic region in western Thailand (nine msp1 and seven glurp alleles) (Paul et al. 1998) and in hyperendemic regions such as Tanzania (17 msp1 alleles) (Babiker et al. 1994). The low number of msp1 and glurp alleles and the predominance of a CQ haplotype may indicate that parasite population in this region is genetically homogeneous, which could be the result of the CQ-selective sweep. Nevertheless, despite low transmission intensity, the observed near 30% mixed-strain infections in Yunnan favour genetic recombination. In addition, its geographical connections with several malarious countries and the resultant frequent population migration across borders ensure large regional gene flows. Therefore, continuous surveillance of drug resistance is needed to extend the life span of individual antimalarial drugs and help local authorities develop more appropriate malaria control policies.

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References


Chloroquine resistance in Plasmodium falciparum


Nagesha HS, Casey GJ, Riekmann KH et al. (2003) New haplotypes of the Plasmodium falciparum chloroquine resistance


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Analyze moléculaire de la résistance à la chloroquine chez Plasmodium falciparum dans la province de Yunnan en Chine

La résistance du Plasmodium falciparum à la chloroquine (QC) est déterminée par la mutation K76T sur le gène transporteur de résistance à la chloroquine (pfcrt) et modifiée par d’autres mutations sur ce gène et sur le gène de la multi-résistance de P. falciparum (pfmdr1). Afin de déterminer sur le terrain l’ampleur du polymorphisme dans ces gènes chez des isolats de P. falciparum provenant de la province de Yunnan en Chine, nous avons génotypé le codon 76 de pfcrt et les codons 86 et 1246 de pfmdr1. Nos résultats démontrent que bien que la QC ait été retirée du traitement de la malaria falciparum depuis plus de deux décennies, 90,3% des parasites portaient toujours la mutation K76T de pfcrt. En revanche, les mutations au niveau des codons 86 et 1246 de pfmdr1 étaient rares. L’analyse des séquences du gène pfcrt chez 34 parasites isolés sur le terrain a indiqué un haplotype CVIET au niveau des positions 72–76 comme étant le type majeur, ce qui est consistant avec la théorie de l’origine sud-est asiatique de la résistance du parasite à la QC. En outre, deux nouveaux haplotypes de pfcrt (75D/144Y/220A et 75E/144Y/220A) ont été identifiés. La réaction en chaine de la polymérase en temps réel a été utilisée pour déterminer l’amplification du gène pfmdr1, associé à la résistance à la méfloquine. Nos résultats confirment cela en accord avec le fait que la méfloquine n’a pas été utilisée dans cet endroit. En effet, la plupart (>90%) des parasites avaient une seule copie de pfmdr1. Le génotypage de deux loci hypervariables a révélé des niveaux relativement faibles de la diversité génétique de la population de parasite. Toutefois, 28,4% de cas se sont avérés contenir des mélanges de clones, ce qui favorisent la recombinaison génétique. En plus, malgré l’historique unique des médicaments antimalariques à Yunnan, ses connections géographiques avec trois pays malariques facilitent le transfert de gènes au sein des populations de parasites et l’évolution de nouveaux génotypes résistants aux médicaments. Par conséquent, la surveillance continue de la résistance aux médicaments dans ce secteur est nécessaire pour l’ajustement ponctuel des politiques locales sur les médicaments et pour un contrôle plus efficace de la malaria.

mots clés parasite de malaria, résistance à la chloroquine, pfcrt, pfmdr1, diversité génétique

Análisis molecular de la resistencia a cloroquina en Plasmodium falciparum en la Provincia de Yunnan, China

La resistencia de Plasmodium falciparum a la cloroquina (CQ) está determinada por la mutación de K76T del gen del transportador de resistencia a cloroquina de P. falciparum (pfcrt), y modificada por otras mutaciones de este gen, así como en el gen de multiresistencia 1 de P. falciparum (pfmdr1). Con el fin de determinar la extensión del polimorfismo de estos genes en aislados silvestres de P. falciparum de la Provincia de Yunnan en China, hemos genotipado el codon 76 de pfcrt, los codones 86 y el 1246 de pfmdr1. Nuestros resultados muestran que, aunque hace más de dos décadas que se retiró la CQ como tratamiento para la malaria por falciparum, un 90,3% de los parásitos aún tiene la mutación K76T del pfcrt. En cambio, las mutaciones en los codones 86 y 1246 del gen pfmdr1 son raras. La secuenciación del gen pfcrt en 34 parásitos de campo, reveló que el CVIET en las posiciones 72–76 es el tipo predominante, de forma consistente con la teoría del origen sureste-asiaítico de la resistencia a CQ en el parásito. Adicionalmente, se identificaron dos nuevos haplotipos de pfcrt (75D/144Y/220A y 75E/144Y/220A). Se utilizó la PCR a tiempo real para determinar la amplificación del gen pfmdr1, la cual se asociaba con la resistencia a mefloquina. Nuestros resultados indican que, en línea con el hecho de que la mefloquina no se ha utilizado en esta área, la mayoría de los parásitos (>90%) tenían una copia del pfmdr1. El genotipado de dos loci hipervariables mostró niveles relativamente bajos de diversidad genética en la población parasitaria. Por otro lado, se encontró que un 28,4% de los casos tenían clones mixtos, lo cual favorece la recombinación genética. Más aún, a pesar de la historia única de antimaláricos en Yunnan, las conexiones geográficas con tres países endémicos para malaria facilita el flujo genético entre las poblaciones de parásitos y la evolución de genotipos resistentes novedosos. Por lo tanto, en esta área es necesario mantener una vigilancia continua de resistencia a antimaláricos, con el fin de ajustar a tiempo las políticas de medicamentos y ejercer un control de la malaria más efectivo.

palabras clave parásito de malaria, resistencia a cloroquina, pfcrt, pfmdr1, diversidad genética