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Molecular characterisation of drug-resistant *Plasmodium falciparum* from Thailand

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Abstract

Background: The increasing levels of *Plasmodium falciparum* resistance to chloroquine (CQ) in Thailand have led to the use of alternative antimalarials, which are at present also becoming ineffective. In this context, any strategies that help improve the surveillance of drug resistance, become crucial in overcoming the problem.

Methods: In the present study, we have established the *in vitro* sensitivity to CQ, mefloquine (MF), quinine (QUIN) and amodiaquine (AMQ) of 52 *P. falciparum* isolates collected in Thailand, and assessed the prevalence of four putative genetic polymorphisms of drug resistance, *pfcrt* K76T, *pfmdr1* N86Y, *pfmdr1* D1042N and *pfmdr1* Y1246D, by PCR-RFLP.

Results: The percentage of isolates resistant to CQ, MF, and AMQ was 96% (50/52), 62% (32/52), and 58% (18/31), respectively, while all parasites were found to be sensitive to QUIN. In addition, 41 (79%) of the isolates assayed were resistant simultaneously to more than one drug; 25 to CQ and MF, 9 to CQ and AMQ, and 7 to all three drugs, CQ, MF and AMQ. There were two significant associations between drug sensitivity and presence of particular molecular markers, i) CQ resistance / *pfcrt* 76T ($P = 0.001$), and ii) MF resistance / *pfmdr1* 86N ($P < 0.001$)

Conclusions: i) In Thailand, the high levels of CQ pressure have led to strong selection of the *pfcrt* 76T polymorphism and ii) *pfmdr1* 86N appears to be a good predictor of *in vitro* MF resistance.

Background

Malaria due to *Plasmodium falciparum* affects 300 million people and claims an estimated 1.5 million lives every year. Our present inability to synthesise a fully protective vaccine means that chemotherapy stands as the only effective measure in the control of the disease. However, in many parts of the world the parasite *P. falciparum* has become resistant to most drugs presently used [1], seriously undermining efforts for controlling malaria.

Chloroquine (CQ) has long been the drug of choice for the treatment of malaria; however, CQ-resistant parasites

are now present in most areas where malaria is endemic [2]. Chloroquine resistance is especially well established in Thailand, after having been first described in that country in the late 1950's [3]. The decline in the efficacy of chloroquine has led to the use of alternative antimalarials, such as antifolates, mefloquine and artemisinin derivatives, but parasite resistance to these drugs is also becoming a real problem [2]. In this context, understanding the genetic basis of drug resistance is essential for implementing rational measures to overcome the problem.

Although significant progress has been made in trying to understand how resistance to CQ may occur, many aspects of it remain unclear, and the genetic mechanisms responsible for mefloquine and quinine resistance are largely unknown. Nevertheless, two main genes have been implicated in quinoline resistance; the *pfmdr1* (*P. falciparum multi-drug resistance1*) and the *Pfcrt* (*P. falciparum chloroquine resistance transporter*). There is evidence from the analysis of a genetic cross which indicates that point polymorphisms in the *pfmdr1* gene may modulate sensitivity to both mefloquine (MF) and artemisinin in *P. falciparum*[4]. Furthermore, recent genetic transfection work has suggested that single nucleotide polymorphisms in the *pfmdr1* gene encoding changes in aminoacids 1034, 1042 and 1246 can influence parasite responses to mefloquine, quinine and halofantrine as well as to the structurally unrelated drug artemisinin, and modulate sensitivity to chloroquine depending on the genetic background of the parasites strains [5]. However, chloroquine resistance was shown to segregate independently of the *pfmdr1* gene, following a genetic cross between a CQ-sensitive parasite, *P. falciparum* HB3, and a CQ-resistant one, Dd2 [6], and the absence of a clear association between *pfmdr1* and chloroquine responses in natural parasite populations [7–15], strongly suggests the involvement of other gene(s). Recently, detailed linkage analysis and fine chromosome mapping of progeny clones of the HB3 × Dd2 cross has allowed the identification of another gene, *pfcrt*, in which a mutation at aminoacid 76 (*pfcrt* K76T) is highly correlated with increased CQ tolerance among field parasite isolates of *P. falciparum*[16–24]. In addition, a causal relationship between *pfcrt* 76T and chloroquine resistance has been confirmed by genetic transfection experiments [16].

The study of the correlation between drug resistance in natural parasite populations and genetic polymorphisms may allow the development of molecular tools to help predict responses to drugs and, as mentioned above, the *pfcrt* and *pfmdr1* genes have been identified as putative markers of quinoline resistance. In the present work we have investigated possible associations between four molecular markers in these genes and sensitivity to chloroquine, mefloquine, quinine (QUIN) and amodiaquine (AMQ) of *P. falciparum* parasites collected in Thailand.

Methods

Study site and method of parasite collection

After confirmation of *P. falciparum* infection by microscopical observation of thin and thick Giemsa-stained blood films, approximately 20 μl of blood were collected by finger-prick from consenting subjects living in 4 different areas of Thailand where malaria is highly endemic; the Tak province (North-western Thailand), Kanchanaburi (Western Thailand: Thai-Myanmar border), Chonburi (Eastern Thailand), Chantaburi and Trat (Eastern Thailand: Thai-Cambodia border). Samples were placed in sterile 1.5 ml micro-centrifuge tubes, together with 0.5 ml of transport medium (10 ml RPMI 1640 complete medium without serum, plus 20 μl of heparin 5000 i.u./ml), carried to Chulalongkorn University of Bangkok on the day of collection, at ambient temperature and placed in *in vitro* culture, following established procedures [25].

Parasite phenotyping (micro-tests)

Assessment of *P. falciparum* susceptibility to CQ, MF, QUIN and AMQ was performed according to the M.I.C. test [26,27], whereby samples were exposed to a range of concentrations of the four drugs, in microtitre culture plates, for 72 hours, with daily changes of medium (in the presence or absence of drugs). After this period, thin film Giemsa-stained preparations from each culture plate well were observed under light microscopy for the presence of parasites. The results were expressed in "minimum inhibiting concentration" (M.I.C.) units, i.e., the lowest drug concentration required to kill all or nearly all parasites (I.C.99) after 72 hours [26,27].

Parasite reference strains of known sensitivity to the drugs concerned were tested in parallel to all field-collected isolates. In this manner, the drug response of each isolate was determined by comparison of the M.I.C. between each sample and the relevant reference strains. Accordingly, samples tested for CQ, MF, QUIN and AMQ responses were clustered into two groups; sensitive (S) or resistant (R) based on sensitivity thresholds established in previous studies [26–29].

Genotyping by PCR-RFLP

Genomic DNA was extracted prior to drug testing, by a standard phenol:chloroform DNA extraction method, precipitated with absolute ethanol and stored in TE Buffer (Tris-EDTA), following established protocols [30]. The resulting DNA was used as template in 50 μl PCR reactions, containing 1 μM of each oligonucleotide primer, 1 × PCR buffer (Promega™), 2.5 mM $MgCl_2$, 0.2 mM dNTP's and 0.025 U/μl of Promega™ *Taq* DNA polymerase. Accordingly, a fragment of the *pfcrt* gene containing codon 76 was amplified by PCR using a Nested-PCR approach. For amplification of DNA fragments containing *pfmdr1* polymorphisms we used oligonucleotide primers published

Table 1: Polymerase Chain Reaction for amplification of fragments containing *pfcrt* and *pfmdr1* gene polymorphisms

Primer	Sequence (5' → 3')	PCR
***Pfcrt* 76**		
1st round sense	CAAGAAGGAAGTAAGTATCCAAAAATGG	94°C, 30"; 56°C, 30"; 60°C, 60"; 45 cycles
Antisense	GTAGTTCTTGTAAGACCTATGAAGGC	
Nested sense	GCAAAAATGACGAGCGTTATAGAG	94°C, 30"; 59°C, 30"; 60°C, 60"; 45 cycles
Antisense	CTGAACAGGCATCTAACATGGATATAGC	
***Pfmdr1* 86**		
Sense	ATGGGTAAAGAGCAGAAAGAG	94°C, 30"; 53°C, 30"; 68°C, 60"; 10 cycles, followed by 94°C, 30"; 50°C, 30"; 68°C, 60", 35 cycles
Antisense	CGTACCAATTCCTGAACTCAC	
***Pfmdr1* 1042**		
1st round sense	TATGTCAAGCGGAGTTTTTGC	94°C, 30"; 50°C, 30"; 68°C, 60"; 45 cycles
Antisense	TCTGAATCTCCTTTTAAGGAC	
Semi-nested sense	GTAAATGCAGCTTTATGGG	94°C, 30"; 50°C, 30"; 68°C, 60"; 45 cycles
Antisense	TCTGAATCTCCTTTTAAGGAC	
***Pfmdr1* 1246**		
Sense	CTACAGCAATCGTTGGAGAAA	94°C, 30"; 53°C, 30"; 68°C, 60"; 10 cycles, followed by 94°C, 30"; 50°C, 30"; 68°C, 60", 35 cycles
Antisense	GCTCTAGCTATAGCTATTCTC	

elsewhere [31], as well as newly designed ones. In this manner, the fragments of the *pfmdr1* gene containing codons 86 and 1246 were amplified in a single-step PCR, whereas the sequence of codon *pfmdr1* 1042 was determined following amplification by semi-nested PCR. All primer sequences and respective PCR conditions are presented in Table 1.

Restriction enzymes generating RFLPs

Following amplification of the fragments concerned, polymorphisms in the *pfcrt* and *pfmdr1* genes were assessed as follows: *pfcrt* 76K and *pfmdr1* 86N were detected by incubation of the corresponding PCR fragments with *Apo*I (r/aatty), *pfmdr1* 1042N was detected using *Asn*I (at/taat), and *pfmdr1* 1246Y was determined by incubation with *Eco*RV (gat/atc). Endonucleases *Apo*I, *Asn*I and *Eco*RV had been obtained from New England BioLabs™, Roche Molecular Biochemicals™ and Stratagene™ respectively, and incubations were setup following the manufacturers instructions. Appropriate control DNA of samples with known *pfcrt* and *pfmdr1* sequences was used in parallel with field-collected parasite isolates in every PCR-RFLP protocol; these were 3D7 (genotype *pfcrt* 76K, *pfmdr1* 86N, *pfmdr1* 1042N, *pfmdr1* 1246D), HB3 (genotype *pfmdr1* 1042D), Dd2 (genotype *pfcrt* 76T, *pfmdr1* 86Y) and 180/92 (genotype *pfmdr1* 1246Y). The products resulting from restrictions of *pfmdr1* 1042 were resolved in 8% acrylamide gels, whereas *pfmdr1* 86, *pfmdr1* 1246 and *pfcrt* 76 digests were run on 2% agarose gels, with both types of gels made in 1 × TBE buffer. All gels were stained with ethidium bromide and visualised under UV (ultraviolet) transillumination.

Statistical analysis of the association between pfcrt and pfmdr1 markers and drug responses

We searched for statistically significant associations between sensitivity to each of the drugs among all isolates and the presence of each of the particular markers included in our study by using Fisher's Exact Test (2-tailed) after having arranged the data in 2 × 2 contingency tables (mixed infections were excluded from this analysis). An association between a particular marker and resistance to a given drug was considered to be significant if the *P* value was found to be lower than 0.05 ($P < 0.05$).

Results

Responses to drugs

In total, 52 samples were analysed *in vitro* for their susceptibility to chloroquine, mefloquine and quinine, and 31 for amodiaquine. The results of these tests are presented in Table 2.

Pfcrt and pfmdr1 genotypes

The *pfcrt* 76 PCR primers amplified a sub-fragment of the gene comprising 479 nucleotides, containing both a monomorphic and a polymorphic *Apo*I restriction site. In this manner, incubation of this fragment with endonuclease *Apo*I generated either 3 fragments of 122, 124 and 233 base-pairs (b.p.), or 2 fragments of 233 and 246 b.p., if K or T were present respectively (Figure 1). The PCR of *pfmdr1* 86 amplified a fragment of 504 b.p., within which

Table 2: Results of *in vitro* drug tests (CQ-chloroquine; MF-mefloquine; QUIN-quinine; AMQ-amodiaquine; S-sensitive; R-resistant)

	CQ	MF	QUIN	AMQ	CQ+MF only	CQ+AMQ only	MF+AMQ only	CQ+MF+AMQ
S	4% (2/52)	38% (20/52)	100% (52/52)	42% (13/31)	-	-	-	-
R	96% (50/52)	62% (32/52)	-	58% (18/31)	48% (25/52)	29% (9/31)	-(0/31)	23% (7/31)

Table 3: Frequencies of allelic polymorphisms in the *pfcrt* and *pfmdr1* genes

	Pfcrt						*Pfmdr1*					
Allele	76K	76T	Mixed	86N	86Y	Mixed	1042N	1042D	Mixed	1246D	1246Y	Mixed
Frequency	.04	.83	.13	.60	.23	.17	.92	.08	0	.92	.02	.06

the presence of asparagine (N) was determined by incubation with *Apo*I, originating segments of 255 and 249 b.p., which resolved as a single band in 2% agarose gels (Figure 1). For detection of 1042 polymorphisms (N or D) the 188 b.p. fragment obtained by PCR could be restricted with endonuclease *Asn*I, after which 3 segments were obtained of 116, 46 and 26 b.p. if 1042N were present in the sample, and two fragments of 162 and 26 b.p. in the presence of the alternative aminoacid (as with *pfcrt* 76, one of the restriction sites for *Asn*I within this segment is also monomorphic, therefore always resulting in at least one cut) (Figure 1). Codon 1246 polymorphisms (D or Y) were assayed following incubation of the corresponding 508 b.p. PCR product with *Eco*RV, which produced 2 fragments of 268 and 240 b.p. if the target DNA contained tyrosine (Y) (Figure 1). All data resulting from this analysis is compiled in Table 3, where allele frequencies for each of the markers are shown.

Association between pfcrt and pfmdr1 markers and responses to drugs

Two significant correlations were detected between the presence of a particular marker and in vitro outcomes (Tables 4 and 5); one between CQ resistance and the presence of pfcrt 76T (P = 0.001) and the other, between MF resistance and the presence of pfmdr1 86N (P < 0.001). There was an evident lack of an association between AMQ responses and all of the markers studied (Table 6), a fact that was corroborated after statistical analysis (data not shown). In addition, since all isolates proved to be quinine sensitive, any correlations between response to this drug and corresponding genotypes could not be established. The data of the in vitro tests for the 4 drugs, and corresponding genotypes is compiled in Table 7.

Discussion

The increasing failure rates of several antimalarial drugs in the majority of malaria-affected areas means that close monitoring of the epidemiology and dynamics of drug resistance are necessary if we are to implement measures to circumvent the problem. The identification and validation of easy, rapid molecular markers of drug resistance would greatly facilitate this process, and would allow us to overcome difficulties in the use of traditional methods for assaying drug sensitivity.

In Thailand, CQ resistance was first reported more than forty years ago [3], and after ten years, resistance to chloroquine had become so widespread that use of the drug against *P. falciparum* was abandoned. At present, even though the drug is used only against *P. vivax*, it is perhaps not surprising to find that most *P. falciparum* are largely unaffected by CQ. Our *in vitro* observations show a near total prevalence of CQR (96%) in the present study area, confirming what has been widely reported [1]. Among all CQR isolates tested in our study, the presence of *pfcrt* 76T was universal ($P = 0.001$), indicating complete selection of this polymorphism by the drug, a result that is in agreement with recently published work not only from Thai-originated parasites [22], but also from parasites of different areas of the globe [32].

The correlation between *pfmdr1* genotypes and quinoline resistance has often generated conflicting results; although it has been suggested that *pfmdr1* 86Y can be correlated with increased CQ resistance in parasites which originated from different areas of the world [33–36], other field studies have not corroborated these findings [7–15], and the results of a *P. falciparum* genetic cross indicat-

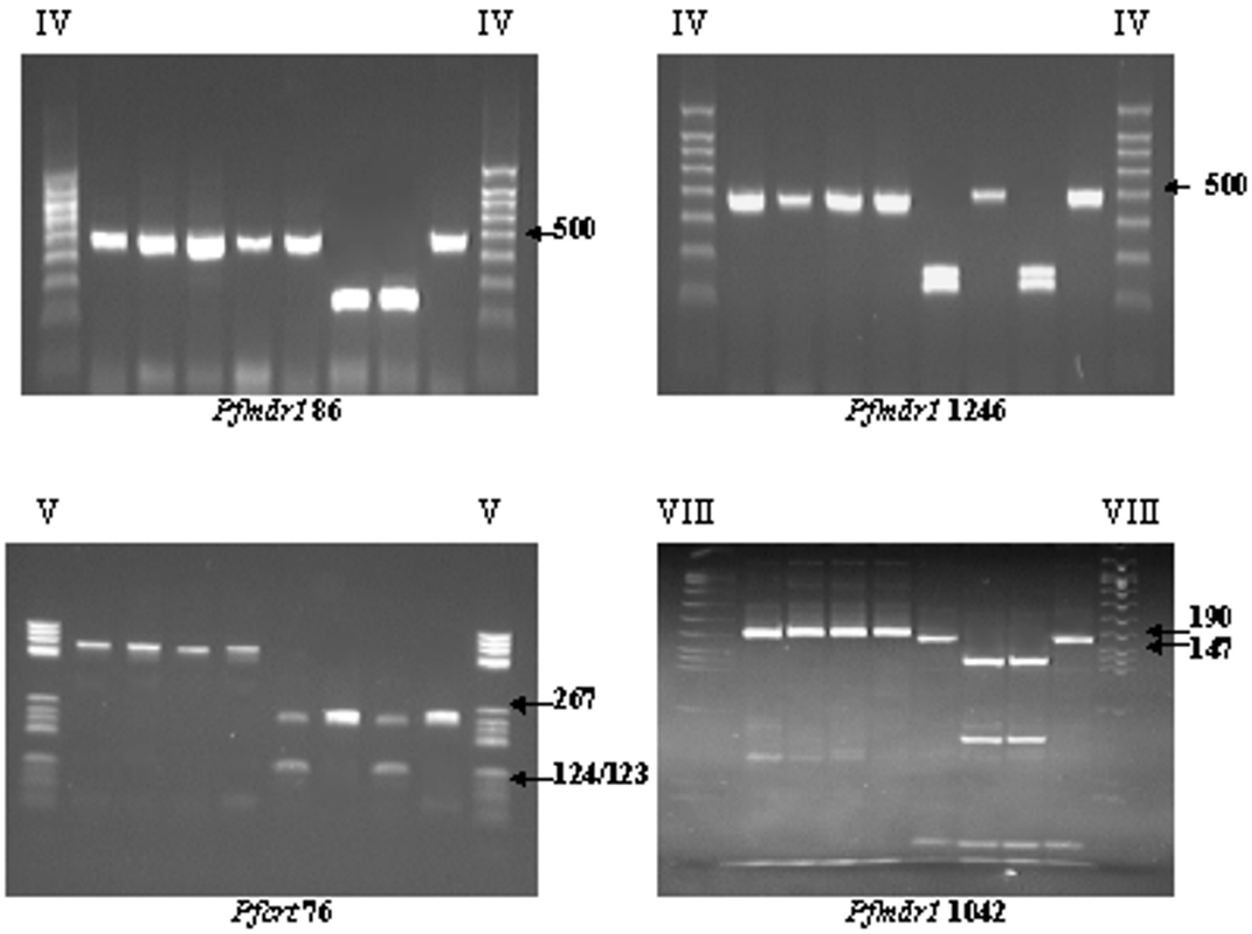

Figure 1
Agarose (*pfmdr1* 86, *pfmdr1* 1246, *pfcrt* 76), and acrylamide (*pfmdr1* 1042) gels showing PCR products and corresponding restriction digests, of control and field-collected samples of *P. falciparum*. (PCR-RFLP controls – 3D7 (genotype *pfcrt* 76K, *pfmdr1* 86N, *pfmdr1* 1042N, *pfmdr1* 1246D), HB3 (genotype *pfmdr1* 1042D), Dd2 (genotype *pfcrt* 76T, *pfmdr1* 86Y) and 180/92 (genotype *pfmdr1* 1246Y); IV, V and VIII – DNA Molecular Weight Markers IV, Bioline; V and VIII, Roche)

ed that CQR did not depend on the *pfmdr1* gene [6]. Our present findings do not implicate *pfmdr1* 86 in CQ resistance in Thailand, since the presence of both N and Y in our samples was largely independent of their CQ response, indicating that chloroquine does not appear to exert selective pressure on this area of the gene. Interestingly though, *pfmdr1* 1042D and *pfmdr1* 1246N, previously associated with increased CQ sensitivity following genetic transfection experiments [5], were largely absent in our samples, possibly suggesting a mechanism of chloroquine resistance that may in part depend on the presence of the alternative polymorphic alleles, *pfmdr1* 1042N and *pfmdr1* 1246D, respectively.

Mefloquine was introduced in Thailand in the form of Fansimef (mefloquine-sulfadoxine-pyrimethamine) during 1984 with an initial cure rate of 95%, but MF-resistant *P. falciparum* parasites have arisen and present a real threat to the control of malaria, especially in the Thai/Cambodia and Thai/Myanmar border areas [37]. In the present work the prevalence of MF resistance was 62%, indicating a worrying trend. A correlation between *pfcrt* 76T and mefloquine sensitivity would always be difficult to establish, since the near complete presence of this polymorphism is likely to have been selected by chloroquine pressure, whose mechanisms of action and resistance are probably distinct from those of MF. Earlier work from Thailand and other areas of the world has indicated that an increase in the level of mefloquine sensitivity among field isolates of

Table 4: *Plasmodium falciparum* isolates listed according to sensitivity to chloroquine (CQ), with corresponding *pfcrt* and *pfmdr1* markers

Isolate	CQ	*pfcrt* 76	*pfmdr1* 86	1042	1246
T9/94b3	S	K	Y	N	D
TM408	S	K	N	N	D
T101	R	T	N	N	D
T108	R	T	N	N	D
T113	R	T	N	N	D
T115	R	T	N	N	D
T116	R	T	N	N	D
T120	R	T	Y	N	D
T123	R	T	N	D	D
T130	R	T	N	N	D
T131	R	T	N	N	D
T132	R	T	N	N	D
T133	R	T	N	N	D
S3	R	T	Y	N	D
S64	R	T	Y	N	D
S71	R	T	Y	N	D
S90	R	T	Y	D	D
S118	R	T	Y	N	D
S149	R	T	NY	N	DY
S151	R	KT	NY	N	D
S152	R	T	Y	N	D
S153	R	T	Y	N	D
S157	R	KT	NY	N	D
S160	R	KT	NY	N	DY
CH1	R	T	Y	D	D
CH3	R	T	Y	D	D
CH7	R	T	N	N	D
TD2	R	T	N	N	D
TD3	R	T	N	N	D
TD8	R	T	N	N	D
TD14	R	T	N	N	D
TD21	R	T	N	N	D
TD27	R	T	N	N	D
TD49	R	T	N	N	D
TD56	R	T	N	N	D
TD61	R	KT	N	N	D
TD62	R	T	N	N	D
TD64	R	T	N	N	DY
TD79	R	KT	N	N	D
TD134	R	T	N	N	D
TD328	R	KT	N	N	D
TP4	R	T	Y	N	D
TP7	R	T	NY	N	D
TP13	R	T	NY	nd	D
TP17	R	T	NY	N	D
TP18	R	T	N	N	D
TP20	R	T	NY	N	D
TP21	R	KT	N	nd	D
TP26	R	T	N	N	D
TP34	R	T	N	N	D
TP40	R	T	N	nd	Y
RC17	R	T	NY	N	D

(S – sensitive; R – resistant; KT, NY and DY – mixed infections; nd – not determined; $P = 0.001$ for the association between CQR and presence of *pfcrt* 76T, mixed alleles excluded)

Table 5: *Plasmodium falciparum* isolates listed according to sensitivity to mefloquine (MF), with corresponding *pfcrt* and *pfmdr1* markers

Isolate	MF	*pfcrt* 76	86	*pfmdr1* 1042	1246
T9/94b3	S	K	Y	N	D
S3	S	T	Y	N	D
S64	S	T	Y	N	D
S71	S	T	Y	N	D
S90	S	T	Y	D	D
S118	S	T	Y	N	D
S149	S	T	NY	N	DY
S151	S	KT	NY	N	D
S152	S	T	Y	N	D
S153	S	T	Y	N	D
S157	S	KT	NY	N	D
S160	S	KT	NY	N	DY
CH1	S	T	Y	D	D
CH3	S	T	Y	D	D
TP4	S	T	Y	N	D
TP7	S	T	NY	N	D
TP13	S	T	NY	nd	D
TP17	S	T	NY	N	D
TP18	S	T	N	N	D
TP40	S	T	N	nd	Y
TM408	R	K	N	N	D
TP20	R	T	NY	N	D
TP21	R	KT	N	nd	D
RC17	R	T	NY	N	D
T108	R	T	N	N	D
T113	R	T	N	N	D
T115	R	T	N	N	D
T130	R	T	N	N	D
T131	R	T	N	N	D
T132	R	T	N	N	D
TD61	R	KT	N	N	D
TD14	R	T	N	N	D
T101	R	T	N	N	D
T116	R	T	N	N	D
T120	R	T	Y	N	D
T123	R	T	N	D	D
T133	R	T	N	N	D
CH7	R	T	N	N	D
TD2	R	T	N	N	D
TD3	R	T	N	N	D
TD8	R	T	N	N	D
TD21	R	T	N	N	D
TD27	R	T	N	N	D
TD49	R	T	N	N	D

Table 5: *Plasmodium falciparum* isolates listed according to sensitivity to mefloquine (MF), with corresponding *pfcrt* and *pfmdr1* markers

TD62	R	T	N	N	D
TD134	R	T	N	N	D
TD328	R	KT	N	N	D
TD56	R	T	N	N	D
TD64	R	T	N	N	DY
TD79	R	KT	N	N	D
TP26	R	T	N	N	D
TP34	R	T	N	N	D

(S – sensitive; R – resistant; KT, NY and DY – mixed infections; nd – not determined; $P < 0.001$ for the association between MFR and presence of *pfmdr1* 86N, mixed alleles excluded)

Table 6: *Plasmodium falciparum* isolates listed according to sensitivity to amodiaquine (AQ), with corresponding *pfcrt* and *pfmdr1* markers

				Pfmdr1	
Isolate	AQ	*pfcrt* 76	86	1042	1246
S153	S	T	Y	N	D
S157	S	KT	NY	N	D
S160	S	KT	NY	N	DY
TD3	S	T	N	N	D
TD49	S	T	N	N	D
TD56	S	T	N	N	D
TD62	S	T	N	N	D
TD79	S	KT	N	N	D
TD134	S	T	N	N	D
TD328	S	KT	N	N	D
TP4	S	T	Y	N	D
TP18	S	T	N	N	D
TP26	S	T	N	N	D
T9/94b3	R	K	Y	N	D
TM408	R	K	N	N	D
S3	R	T	Y	N	D
S118	R	T	Y	N	D
S149	R	T	NY	N	DY
S151	R	KT	NY	N	D
S152	R	T	Y	N	D
TD14	R	T	N	N	D
TD61	R	KT	N	N	D
TD64	R	T	N	N	DY
TP7	R	T	NY	N	D
TP13	R	T	NY	nd	D
TP17	R	T	NY	N	D
TP20	R	T	NY	N	D
TP21	R	KT	N	nd	D
TP34	R	T	N	N	D
TP40	R	T	N	nd	Y
RC17	R	T	NY	N	D

(S – sensitive; R – resistant; KT, NY and DY – mixed infections; nd – not determined)

Table 7: Number of isolates clustered according to sensitivity to each drug and corresponding *pfcrt* and *pfmdr1* genotypes

Drug		*Pfcrt*						*Pfmdr1*					
		76K	76T	76KT	86N	86Y	86NY	1042N	1042D	1042ND	1246D	1246Y	1246DY
CQ	***Total***												
S	**2**	2	0	0	1	1	0	2	0	0	2	0	0
R	**50**	0	43	7	30	11	9	43	4	0	46	1	3
MF													
S	**35**	2	27	6	2	11	7	29	3	0	32	1	2
R	**17**	0	16	1	29	1	2	16	1	0	16	0	1
QUIN													
S	**52**	2	43	7	31	12	9	45	4	0	48	1	3
R	**0**	0	0	0	0	0	0	0	0	0	0	0	0
AMQ													
S	**13**	0	9	4	9	2	2	13	0	0	12	0	1
R	**18**	2	13	3	7	4	7	15	0	0	15	1	2

(CQ – chloroquine; MF – mefloquine; QUIN – quinine; AMQ – amodiaquine; S – sensitive; R – resistant; 76KT, 86NY, 1042ND and 1246DY – mixed infections; shaded boxes indicate the two cases where there was a significant association between the presence of a particular marker and drug sensitivity: *pfcrt* 76T/CQR, $P = 0.001$ and *pfmdr1* 86N/MFR, $P < 0.001$)

P. falciparum may be correlated with a mutation in codon 86 of the *pfmdr1* gene (N86Y) [38–40]. In the present work, the occurrence of *pfmdr1* 86N was significantly associated with MF resistance as 31/32 resistant isolates carried this polymorphism ($P < 0.001$), strongly suggesting that 86N is an important event in the generation of MF resistance and may be a useful marker to monitor MF resistance in this country.

Interestingly, although the prevalence of AMQ resistance was high (58%), we did not detect a significant correlation between AMQ responses and any of the markers studied, contrary to what could be expected considering that AMQ is chemically very similar to CQ. These observations indicate that the mechanism of action and/or resistance differ between the two drugs, which may raise interesting questions about the design of new CQ-derivative compounds.

Quinine is one of the most effective drugs for the treatment of malaria in Thailand. Although the efficacy of this drug has been reduced, it has not yet become a serious problem, since the combination with tetracycline increases its cure rate. In fact, *P. falciparum* quinine sensitivity was total in all regions covered by our study, showing that despite its adverse side effects, quinine can still be used as a reliable resource of malaria therapy in Thailand. The reasons for the long-lasting efficacy of quinine may be manyfold, but its explanations lie outside the scope of this article. Most importantly, quinine may prove especially valuable in the treatment of multi-drug resistant Falciparum malaria, which we found to be largely present in our study as 41 out of 52 isolates were found to be resistant to more than one compound and 7 were unaffected by all drugs (CQ, MF and AMQ) except quinine.

Conclusions

Taken together, our results seem to be suggesting that CQ and MF are the major selective forces on the *pfcrt* and *pfmdr1* genes, whereby the presence of *pfcrt* 76T, and possibly *pfmdr1* 1042N and *pfmdr1* 1246D in Thai-originated parasites has been selected by chloroquine pressure. The *pfmdr1* 86N mutation seems to be important only for mefloquine resistance, and may represent a useful marker for monitoring resistance in this country, although its validation may require *in vivo* correlates and the analysis of a larger number of samples.

Authors' contributions

DL carried out a proportion of the parasite phenotyping, performed the molecular analysis of the majority of the samples and contributed for the elaboration of the manuscript. KR performed the molecular typing of a number of the isolates. FN participated in the parasite's phenotyping and genotyping. AS performed parasite collection and did a proportion of the micro-tests. JPG, VR and PC, conceived the study, participated in its design and co-ordination and were involved in phases of the experimental work.

Competing interests

None declared.

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