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Role of *Plasmodium falciparum* Chloroquine Resistance Transporter and Multidrug Resistance 1 Genes on *In Vitro* Chloroquine Resistance in Isolates of *Plasmodium falciparum* from Thailand

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Abstract. Resistance to chloroquine is a public health problem worldwide. Polymorphisms of the *Plasmodium falciparum* chloroquine resistance transporter (*pfcrt*) and *P. falciparum* multidrug resistance 1 (*pfmdr1*) genes have been linked to chloroquine resistance. Although the K76T mutation in the *pfcrt* gene has been shown to be a key determinant in chloroquine resistance, evidence suggests that the *pfmdr1* gene could modulate the level of chloroquine resistance. However, few studies of field isolates could identify the interactive role of these two genes in chloroquine resistance. Thus, we evaluated the influence of *pfcrt* and *pfmdr1* polymorphisms on *in vitro* chloroquine sensitivity in 89 adapted isolates of *P. falciparum* from Thailand. We found that 87 of 89 isolates contained the CVIET haplotype of the *pfcrt* gene. Two additional mutations in the *pfcrt* gene were identified, i.e., K6Q and H97L. For the *pfmdr1* polymorphisms, the 184F allele was common in the parasites isolated along the Thailand-Cambodia border, and those isolated along the Thailand-Myanmar border contained higher copy numbers. Our results indicate that the additional mutations, in particular H97L in the *pfcrt* gene and Y184F in the *pfmdr1* gene and its copy number, influence the level of chloroquine resistance.

INTRODUCTION

The development and spread of *Plasmodium falciparum* resistant to chloroquine has been a major factor in the resurgence of life-threatening *P. falciparum* malaria worldwide.[1] Chloroquine resistance is associated with reduced cellular drug accumulation at a high-affinity target site within the parasite food vacuole.[2] A genetic cross experiment identified a gene on chromosome 7, *Plasmodium falciparum* chloroquine resistance transporter (*pfcrt*), as the main determinant of chloroquine resistance.[3–5] A point mutation on the *pfcrt* gene resulting in replacement of lysine by threonine in the PfCRT at codon 76 has been proven to be critical for chloroquine resistance by transfection experiment.[6,7] The K76T mutation has been linked to chloroquine resistance in parasite isolates collected worldwide.[1,8–12] In addition, more than 15 mutations in the *pfcrt* gene resulting in amino acid changes have been reported.[13]

To date, a number of *pfcrt* haplotypes have been identified based on amino acids 72–76. These haplotypes have been linked to the geographic origin of the isolates and their chloroquine susceptibility status. Two common haplotypes, CVIET and SVMNT, were identified in chloroquine-resistant isolates from Asia/Africa and South America, respectively, and a CVMNK haplotype is universally identified in chloroquine-sensitive parasites.[6] Recent studies indicate the important role of amino acid polymorphisms in the PfCRT on the pattern and level of antimalarial drug resistance. A genetic cross experiment showed that parasites with CVIET and SVMNT haplotypes exhibited different patterns of chloroquine and monodesethylamodiaquine resistance.[14] Moreover, additional amino acid substitutes in the PfCRT altered the level of chloroquine resistance.[15–17]

There is compelling evidence that *P. falciparum* multidrug resistance 1 (*pfmdr1*), a gene on chromosome 5 encoding a P-glycoprotein homolog 1 (Pgh1), also contributes to chloroquine resistance.[18] A study by Reed and others using an allelic exchange experiment has definitively confirmed the involvement of *pfmdr1* polymorphisms in high-level chloroquine resistance.[19] In addition, a few studies of resistant parasites from *in vitro* drug selection indicate that alteration of the *pfmdr1* gene copy number contributes to changes in the level of chloroquine resistance.[20–22]

Thus, it has been postulated that the *pfmdr1* gene is working in concert with the *pfcrt* gene to control the level of chloroquine resistance.[23] However, some but not all studied of field isolates could identify this association.[24–26] Because different patterns of *pfcrt* and *pfmdr1* polymorphisms respond to different patterns of drug resistance, it would be useful to identify the distribution of these polymorphisms. In this study, *pfcrt* and *pfmdr1* polymorphisms in *P. falciparum* isolated along the Thailand-Myanmar and Thailand-Cambodia borders were determined. In addition, the role of these polymorphisms in controlling the level of chloroquine resistance was examined.

MATERIALS AND METHODS

***P. falciparum* strains and cultivation.** Eighty-nine isolates of *P. falciparum* used in this study were collected from malaria-endemic areas along the Thailand-Myanmar and Thailand-Cambodia borders during 2003–2005. Parasites were maintained in continuous cultures by using a modification of the method of Trager and Jensen.[27]

***In vitro* sensitivity assays.** Chloroquine sensitivity of *P. falciparum* isolates was determined by measurement of [^{3}H] hypoxanthine incorporation into parasite nucleic acids as described.[28] Drug IC_{50} (i.e., concentration of a drug that inhibits parasite growth by 50%) was determined from the log dose/response relationship as fitted by GRAFIT (Erithacus Software, Kent, United Kingdom).

Genotypic characterization of *pfcrt* and *pfmdr1* genes. Parasite DNA was extracted by using the Chelex-resin method.[29] Five microliters of DNA preparation was used for

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a 25-μL polymerase chain reaction (PCR). Nested PCR and allele-restricted PCR and/or restriction endonuclease digestion as described by Fidock and others[6] and Djimde and others[30] were used for detection of *pfcrt* mutations encoded amino acids at positions 76, 220, 271, 326, 356 and 371.

A primer pair, i.e., E1-2F (5′-CgACATTCCgATATATT TTAgAC-3′) and E1-2R (5′-TATATgTgTAATgTTTTATAT Tgg-3′) was used to amplify exon1 and 2 and produced the expected amplicon of 732 basepairs. The reaction mixture was composed of 1× colorless Go Taq® Flexi buffer, 1.5 mM $MgCl_2$, 0.2 mM each of dNTP, 2 units of Taq DNA polymerase, and 20 pmol of each primer. The mixture was processed in a programmable DNA thermal cycler (PTC-200 Peltier Thermal Cycler; MJ Research Inc., Waltham, MA). The program consisted of one cycle at 94°C for 3 minutes to denature the genomic DNA; then 35 cycles of denaturation at 92°C for 30 secons, annealing at 55°C for 30 seconds, and extension at 62°C for 1.5 minutes. For the last cycle, the extension step at 62°C was performed for 5 minutes to complete partial polymerization. The PCR amplicons were then stored at –20°C until used.

DNA purification and DNA sequencing were conducted by Bioservice Unit (Bangkok, Thailand). DNA sequences of the *pfcrt* gene and amino acid sequences of the PfCRT from 89 *P. falciparum* isolates were aligned against available sequences in GenBank (Dd2 and HB3) by using the Lasergene MegAlign Program (DNASTAR Inc., Madison, WI) with the method of ClustalV. The nucleotide sequences were translated to amino acid sequences. Each polymorphism position was identified as to whether located inside or outside the transmembrane protein of PfCRT by comparing with the predicted peptides from the TMMHM Program (CBS, Lyngby, Denmark).

Mutations in the *pfmdr1* gene were determined by nested PCR and the restriction endonuclease digestion method developed by Duraisingh and others[31] for detection of the mutations at codons 86, 184, 1034, 1042, and 1246. Strains K1 and 7G8 were used as positive controls. The *pfmdr1* gene copy number was determined by TaqMan real-time PCR (ABI sequence detector 7000; Applied Biosystems, Foster City, CA) as developed by Price and others.[32] The K1 and DD2 clone containing 1 and 4 *pfmdr1* copies, respectively was used as the reference DNA sample. The *pfmdr1* and β-tubulin amplification reactions were run in duplicate. Relative *pfmdr1* copy number was assessed as described.[32]

Statistical analysis. Data were analyzed by using SPSS for Windows (SPSS Inc., Chicago, IL). The chloroquine IC_{50} of each isolate was the mean IC_{50} of three independent experiments. Differences in mean IC_{50} and copy number of the *pfmdr1* gene among parasites from different areas were analyzed by using the independent *t*-test. Difference in median and interquartile range of chloroquine IC_{50} for parasites with different genotypes was analyzed by using the Mann-Whitney U test or the Kruskal-Wallis test. A chloroquine IC_{50} value > 25 nM was considered as *in vitro* chloroquine resistance, and an IC_{50} value > 100 nM was the cut-off point for *in vivo* chloroquine resistance.[33] Association between genotypes and *in vitro* chloroquine resistance of *P. falciparum* was analyzed by using the chi-square test and Fisher's exact test.

RESULTS

***In vitro* chloroquine sensitivities.** Characteristics of parasite isolates are shown in Table 1. The mean ± SD IC_{50} for chloroquine was 87.0 ± 43.8 nM (range = 13.9–190.5 nM). The parasites isolated along the Thailand-Cambodia border exhibited significantly more resistance to chloroquine than those from along the Thailand-Myanmar border. Of 89 isolates, 2 (2.2%), 55 (61.8%), and 32 (36.0%) were had IC_{50} values ≤ 25 nM, 26–100 nM, and > 100 nM, respectively.

Characterization of *pfcrt* and *pfmdr1* genes. Detection of the mutations in the *pfcrt* gene using nested PCR and allele-restricted PCR and/or restriction endonuclease digestion showed that all isolates contained the 220S, 271E, 326S, and 371I mutations. Two (2.2%) and four (4.5%) isolates contained the 76K and 356I mutations, respectively. Sequences of *pfcrt* codons 72–76 confirmed that two chloroquine-sensitive isolates contained the CVIEK haplotype and 87 chloroquine-resistant isolates contained the CVIET haplotype. In addition, sequencing of exons 1 and 2 of the *pfcrt* gene showed four additional points of nucleotide polymorphisms, i.e., 12, 16, 198, and 290. All additional mutations were found only in parasites containing the CVIET haplotype. Only nucleotide polymorphisms at positions 16 and 290 resulted in amino acid changes. A substitution of C for A at position 16 resulting in amino acid change from lysine to glutamine at position 6 (K6Q) was found in three chloroquine-resistant isolates. An amino acid change from histidine to leucine at position 97, caused by a replacement of T for A at position 290, was found in four chloroquine-resistant isolates. The replacement at positions 6 and 97 occurred in cytosol peptide 1 and transmembrane domain 2, respectively.

Of these 89 parasite isolates, 7 (7.9%), 36 (40.4%), 3 (3.4%), 3 (3.4%), and 0 (0%) isolates contained *pfmdr1* 86Y, 184F, 1034C, 1042D, and 1246Y mutations, respectively. These isolates had mean *pfmdr1* gene copy numbers of 2.6 (range = 0.8–5.6). The *pfmdr1* 184F allele was more common in parasites isolated along the Thailand-Cambodia than in those isolated along the Thailand-Myanmar border. In contrast, parasites isolated along the Thailand-Myanmar border had significantly higher copy numbers.

TABLE 1

In vitro sensitivities to chloroquine and distribution of *pfcrt* and *pfmdr*1 polymorphisms of 89 recently adapted *Plasmodium falciparum* isolates from Thailand-Myanmar and Thailand-Cambodia areas*

Area	No. of isolates	Mean ± SD chloroquine IC_{50} (nM)	*pfcrt* mutation	Mean ± SD *pfmdr1* copy number	*pfmdr1* mutations				
			76T		86Y	184F	1034C	1042D	1246Y
Thailand-Myanmar	68	76.9 ± 39.6	67 (98.5%)	3.0 ± 1.4	5 (7.4%)	19 (27.9%)	2 (2.9%)	2 (2.9%)	–
Thailand-Cambodia	21	119.7 ± 40.9†	20 (95.2%)	1.6 ± 0.9†	2 (9.5%)	17 (81.0%)‡	1 (4.8%)	1 (4.8%)	–
Total	89	87.0 ± 43.8	87 (97.8%)	2.6 ± 1.4	7 (7.9%)	36 (40.4%)	3 (3.4%)	3 (3.4%)	–

* *pfcrt* = *Plasmodium falciparum* chloroquine resistance transporter; *pfmdr1* = *P. falciparum* multidrug resistance 1; IC_{50} = 50% inhibitory concentration.
† Significant difference between 2 areas determined by independent *t* test ($P < 0.001$).
‡ Significant difference between 2 areas determined by Fisher's exact test ($P < 0.001$).

Association between *in vitro* chloroquine sensitivity and *pfcrt* and *pfmdr1* genes. Two parasite isolates containing *pfcrt* 76K exhibited absolute chloroquine sensitivity, and those harboring *pfcrt* 76T had a chloroquine IC_{50} > 25 nM. The *in vitro* chloroquine sensitivities of 87 isolates containing chloroquine-resistant haplotype CVIET with different additional *pfcrt* mutations and different *pfmdr1* genotypes are shown in Table 2. Of these 87 chloroquine-resistant isolates, 4 isolates containing additional PfCRT 97L exhibited significantly higher chloroquine IC_{50}, and isolates with PfCRT 6Q showed slightly lower chloroquine IC_{50}. Parasite isolates with *pfmdr1* 184F showed a higher level of chloroquine resistance, and those with a *pfmdr1* copy number ≥ 4 exhibited significantly lower chloroquine IC_{50}. The association between parasite genotypes and level of chloroquine resistance in 87 parasite isolates are shown in Table 3. Parasites with a chloroquine IC_{50} > 100 nM were significantly associated with PfCRT 97L and *pfmdr1* 184F, and a *pfmdr1* copy number ≥ 4 was more common in those with a chloroquine IC_{50} ≤ 100 nM.

According to their *pfcrt* and *pfmdr1* haplotypes, chloroquine-resistant parasites were classified into four groups (Table 4): i.e., isolates containing *pfcrt* 97H and *pfmdr1* 184Y with a copy number < 4; *pfcrt* 97L; *pfcrt* 97H and *pfmdr1* 184F with a copy number < 4; and *pfcrt* 97H and *pfmdr1* 184Y with a copy number ≥ 4. A total of 84 isolates were included in these four groups, and the other three isolates contained *pfmdr1*184Y with a copy number ≥ 4. To clearly determine the effect of the Y184F mutation and the copy number of the *pfmdr1* gene, we excluded these three isolates from the analysis. There were significant differences in chloroquine IC_{50}s among these groups (P < 0.001, by Kruskal-Wallis test). The parasites containing *pfmdr1* 184Y with a copy number ≥ 4 were significantly less resistant to chloroquine than the parasites containing *pfmdr1* 184Y with a copy number < 4 (P = 0.023) and *pfmdr1* 184F with a copy number < 4 (P = 0.001). In contrast, parasites containing *pfcrt* 97L were significantly more resistant than parasites containing *pfmdr1* 184Y with a copy number < 4 (P = 0.004), *pfmdr1* 184F with a copy number < 4 (P = 0.016), and *pfmdr1* 184Y with a copy number ≥ 4 (P = 0.003).

TABLE 2

Comparison of *in vitro* chloroquine sensitivity among *Plasmodium falciparum* isolates with different *pfcrt* and *pfmdr1* genotypes*

Parasite genotypes		No. (%)	Median CQ IC_{50} (IQR), nM	*P*
pfcrt				
K6Q	6K	84 (96.6)	76.8 (57.8–119.8)	0.043†
	6Q	3 (3.4)	47.2 (ND)	
H97L	97H	83 (95.4)	74.9 (55.4–115.8)	0.006†
	97L	4 (4.6)	158.6 (124.7–184.8)	
I356T	356I	4 (4.6)	99.3 (58.9–173.8)	0.325
	356T	83 (95.4)	75.8 (55.5–116.2)	
pfmdr1				
86	86N	81 (93.1)	75.0 (54.6–117.9)	0.873
	86Y	6 (6.9)	89.7 (69.9–125.1)	
184	184Y	51 (58.6)	67.5 (51.2–113.9)	0.008†
	184F	36 (41.4)	104.7 (63.9–120.8)	
1034	1034S	84 (96.6)	75.4 (55.8–116.2)	0.237
	1034C	3 (3.4)	170.7 (ND)	
1042	1042N	84 (96.6)	75.4 (55.8–116.2)	0.237
	1042D	3 (3.4)	170.7 (ND)	
Copy no.	< 4	71 (81.6)	80.4 (60.4–122.3)	0.002†
	≥ 4	16 (18.4)	56.1 (42.1–66.3)	
Total		87 (100)	75.8 (55.5–119.6)	

**pfcrt* = *Plasmodium falciparum* chloroquine resistance transporter; *pfmdr1* = *P. falciparum* multidrug resistance 1; CQ = chloroquine; IC_{50} = 50% inhibitory concentration; IQR = interquartile range; ND = not determined.
† Significant difference determined by Mann-Whitney U test.

TABLE 3

Association of parasite genotypes and the level of *in vitro* chloroquine sensitivity in *Plasmodium falciparum* isolates containing the *pfcrt* 76T allele*

Parasite genotypes		Chloroquine IC_{50}, no. (%) ≤ 100 nM	Chloroquine IC_{50}, no. (%) > 100 nM	*P*
pfcrt K6Q	K6	51 (60.7)	33 (39.3)	0.285
	6Q	3 (100)	0 (0)	
pfcrt H97L	H97	54 (65.1)	29 (34.9)	0.018†
	97L	0 (0)	4 (100)	
pfmdr1 Y184F	Y184	37 (72.5)	14 (27.5)	0.016‡
	184F	17 (47.2)	19 (52.8)	
pfmdr1 copy number	< 4	39 (54.9)	32 (45.1)	0.004†
	≥ 4	15 (93.8)	1 (6.3)	
Total		54 (62.1)	33 (37.9)	

**pfcrt* = *Plasmodium falciparum* chloroquine resistance transporter; *pfmdr1* = *P. falciparum* multidrug resistance 1; IC_{50} = 50% inhibitory concentration.
† Significant difference determined by Fisher's exact test.
‡ Significant difference determined by chi-square test.

DISCUSSION

In the present study, nearly all (87/89) isolates exhibited chloroquine-resistant haplotypes. The CVIET haplotype was identified in all 87 chloroquine-resistant isolates. This finding confirms the results of Hatabu and others, who showed that chloroquine-resistant isolates from Thailand contained CVIET.[34] In contrast, chloroquine-resistant isolates from other counties in Southeast Asia showed a different distribution of the *pfcrt* haplotypes. Three additional haplotypes, CVIDT, CVMNT, and CVTNT, were identified in *P. falciparum* isolates from Cambodia.[12,35] The SVMNT haplotype, was also detected in approximately 30% of parasites isolated from northern Lao PDRs.[36] In this study, only two chloroquine-sensitive haplotypes were identified. Both isolates contained CVIEK, a less common chloroquine-sensitive haplotype. This haplotype has only been reported in a few isolates from Sudan, India, and The Philippines.[6,26,37]

Sequencing of exons 1 and 2 of the *pfcrt* gene identified two other nucleotide polymorphisms that caused the changing of amino acid at positions 6 and 97. The K6Q mutation located in cytosol peptide 1 is reported in this study. Only two previous studies have reported polymorphisms at position 97 of the *pfcrt* gene. Interestingly, different patterns of amino acid changes at this position were identified according to the

TABLE 4

Comparison of *in vitro* chloroquine sensitivity among *Plasmodium falciparum* isolates with different *pfcrt* and *pfmdr1* haplotypes*

Group	Parasite haplotypes *pfcrt* K76T	*pfcrt* H97L	*Pfmdr1* Y184F	*Pfmdr1* Copy no.	No. (%)	Median CQ IC_{50} (nM)	IQR
1	76T	H97	Y184	< 4	36 (42.9)	72.1	54.8–121.3
2	76T	97L	Y184/184F	< 4	4 (4.8)	158.6	124.7–184.8
3	76T	H97	184F	< 4	31 (36.9)	105.9	34.4–119.9
4	76T	H97	Y184	≥ 4	13 (15.5)	56.7	42.4–65.1
Total					84 (100)	76.3	57.0–119.8

**pfcrt* = *Plasmodium falciparum* chloroquine resistance transporter; *pfmdr1* = *P. falciparum* multidrug resistance 1; CQ = chloroquine; IC_{50} = 50% inhibitory concentration; IQR = interquartile range. Significant differences (P < 0.001) observed among groups were determined by Kruskal-Wallis test.

geographic areas where the parasite isolates were found. Two parasites isolated from Colombia contained 97Q, and the 97L allele was identified in an isolate from Thailand.[6,10] Our study identified one and three isolates from the Thailand-Myanmar and Thailand-Cambodia borders, respectively, that contained the 97L allele.

Although an IC_{50} value > 100 nM is generally used for defining *in vivo* chloroquine resistance,[33] the chloroquine sensitive/resistant status might not be correlated with the *pfcrt* haplotypes in some studies.[8,35] Because this cut-off value was estimated from the correlation between *in vivo* and *in vitro* test results, this value reflects not only parasite's susceptibility but also host factors such as immune status. Thus, some studies, particularly those using laboratory isolates used a cut-off value of 25 nM.[38,39] Using this value, we found that *in vitro* parasite susceptibility was clearly correlated with *pfcrt* haplotypes in the present study. Two chloroquine-sensitive isolates containing the CVIEK haplotype showed an IC_{50} < 25 nM, and the chloroquine IC_{50} of those with the CVIET haplotype ranged from 25.2 to 190.5 nM. However, the parasite isolates containing the *pfcrt* 76T allele displayed such a broad range of chloroquine IC_{50}s, indicating that the level of chloroquine resistance must be under multilocus/multigenic control.

A few studies using field and laboratory isolates selected for drug resistance showed the influence of amino acid changes in PfCRT transmembrane domains on the chloroquine susceptibility of the parasite.[15–17] These amino acid substitutions, which resulted in changes in charge, are thought to affect the interaction of PfCRT and positively charged chloroquine, thus altering chloroquine accumulation in the food vacuole of the parasite and modulating chloroquine susceptibility. PfCRT is a member of the drug/metabolite transporter superfamily, which contains 10 transmembrane domains. On the basis of information from other members of the drug/metabolite transporter superfamily, each transmembrane domain has its specific function.[40] In the present study, chloroquine-resistant isolates with an amino acid substitution at position 97 in transmembrane domain 2 but not at position 356 in transmembrane domain 9 of the PfCRT exhibited significantly higher chloroquine resistance. Functions of transmembrane domain 2 are similar to those of transmembrane domain 1, where the K76T mutation is located, i.e., recognition and discrimination of substrate. Transmembrane domain 9 is also important for binding and translocation of the substrate. Our finding might be explained by replacement of positively charged histidine by neutral leucine at amino acid position 97; no alteration of charge for the I356T substitution was found. Alteration of charge in the transmembrane domain might affect the drug susceptibility of the parasite more than that occurring in the cytosol peptide. A slight difference in the chloroquine IC_{50} was noted between the parasite isolates harboring a positively charged lysine and a neutral glutamine at the PfCRT position 6.

Our study identified some known point mutations by PCR–restriction fragment length polymorphism and additional mutations in exons 1 and 2 by sequencing. To determine the influence of *pfcrt* polymorphisms on the level of chloroquine resistance, all mutations should be identified by full-length sequencing of the *pfcrt* gene. In addition, because all available evidence indicates the involvement of other gene(s) in *in vitro* chloroquine susceptibility, alteration of chloroquine IC_{50} in these parasites may not be completely modulated by *pfcrt* polymorphisms. The effect of these charge-loss mutations should be further investigated by suitable techniques such as the transfection technique.

A few studies of field isolates have reported significantly interactive roles of two genes, usually *pfcrt* 76T and *pfmdr1* 86Y, in chloroquine resistance.[24,41] In the present study, among the parasite isolates containing the *pfcrt* 76T allele, both copy number of the *pfmdr1* gene and the changed amino acid at position 184 in the Pgh1 influenced the level of chloroquine resistance. Different predominant *pfmdr1* haplotypes, that might confer different levels of chloroquine resistance were found in parasites isolated along the Thailand-Myanmar and Thailand-Cambodia borders. Parasites from the Thailand-Myanmar areas contained higher copy numbers of the *pfmdr1* gene, and most parasites from the Thailand-Cambodia border contained the *pfmdr1* 184F allele. Selection for different *pfmdr1* haplotypes in these two areas may have been caused by different drug policies in the past. The combination of mefloquine and artesunate has been strictly used for the treatment of uncomplicated *P. falciparum* malaria in the Thailand-Cambodia area for more than 15 years. In contrast, mefloquine with or without artesunate was used, depending on the level of mefloquine resistance, along the Thailand-Myanmar border. Until 2003, the combination of mefloquine and artesunate had been used as the first-line drug in this area.

In conclusion, our results indicated that an additional charge-loss mutation in the *pfcrt* gene and copy number and mutation of the *pfmdr1* gene influenced the level of chloroquine resistance. In addition, molecular markers for chloroquine resistance could be different in each geographic area.

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