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In Vitro Sensitivity of *Plasmodium falciparum* Clinical Isolates from the China-Myanmar Border Area to Quinine and Association with Polymorphism in the Na^+/H^+ Exchanger▽

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Quinine resistance (QNR) in *Plasmodium falciparum* has been detected in many regions of the world where malaria is endemic. Genetic polymorphisms in at least four genes are implicated in QN susceptibility, and their significance often depends on the genetic background of the parasites. In this study, we have culture-adapted 60 *P. falciparum* clinical isolates from the China-Myanmar border and assessed their *in vitro* responses to QN. Our results showed that >50% of the parasite isolates displayed reduced sensitivity to QN, with a half-maximal inhibitory concentration (IC_{50}) above 500 nM. Genotyping of *pfcrt* found that an overwhelming proportion of the parasite population had the chloroquine-resistant genotype, whereas *pfmdr1* mutation genotypes and gene amplification were rare. Genotyping of the *P. falciparum* Na^+/H^+ exchanger gene (*pfnhe1*) at the minisatellite ms4760 locus identified 10 haplotypes. Haplotype 7, which harbors three copies of the DNNND repeat, was the most predominant, accounting for nearly half of the parasite isolates. Correlation studies did not reveal significant associations of the polymorphisms in *pfcrt* and *pfmdr1* genes with QN response. However, the ms4760 haplotypes were highly associated with *in vitro* QN responses. In particular, parasite isolates with an increased DNNND copy number tended to have significantly reduced QN susceptibility, whereas parasite isolates with a higher NHNDNHNNDDD copy number had increased QN susceptibility. This study provided further support for the importance of *pfnhe1* polymorphisms in influencing QNR in *P. falciparum*.

According to the *World Malaria Report 2009*, malaria caused an estimated 243 million clinical cases, resulting in nearly 0.9 million deaths, in 2008 (43). While most of the malaria burden is in Africa, it has been estimated that Southeast Asia accounts for 30 and 8% of the global malaria morbidity and mortality, respectively. In the Greater Mekong subregion, malaria epidemiology is characterized by immense geographical heterogeneity in disease distribution with many areas of high endemicity (38). Effective chemotherapy is essential for malaria control, but the emergence and spread of drug resistance in malaria parasites have led to a sharp rise in malaria-related morbidity and mortality (20, 41). This situation is particularly grave in Southeast Asia, where multidrug-resistant (MDR) *Plasmodium falciparum* poses a major challenge to the control of malaria (39). Therefore, for effective and sustainable malaria management, resistance monitoring and mechanism studies are of high priority, particularly in the era of artemisinin-based combination therapy (42).

Quinine (QN) has been a critical antimalarial drug because of its efficacy against chloroquine (CQ)-resistant parasites. In many regions where malaria is endemic, QN is still a primary drug of choice for the treatment of complicated malaria (45). Through its long history in malaria treatment, QN has remained largely effective, and the evolution of QN resistance (QNR) in *P. falciparum* appears to be slow. However, the observation of reduced sensitivity of *P. falciparum* to QN in Southeast Asia, South America, and Africa has raised considerable concern (14, 21, 26, 33, 51). *In vitro* drug assays have found complex patterns of cross-resistance with other quinoline drugs, such as CQ and mefloquine (MQ), suggesting shared resistance mechanisms. Recent genetic and molecular studies indicate that QNR is multifactorial and involves at least four genes: *P. falciparum multidrug resistance 1* (*pfmdr1*), *P. falciparum CQ resistance transporter* (*pfcrt*), *P. falciparum multidrug resistance-associated protein* (*pfmrp*), and *P. falciparum Na^+/H^+ exchanger 1* gene (*pfnhe1*). As its name implies, *pfmdr1* is involved in resistance to a number of antimalarials. Global isolates of the parasite show that PfMDR1 harbors a large number of point mutations (11). Genetic studies have found that some PfMDR1 mutations, particularly those that are highly prevalent in South America (S1034C/N1042D/D1246Y), where QN has the longest history of use, are asso-

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ciated with increased QNR (30, 35). In addition, increased copy numbers of *pfmdr1* increase resistance not only to MQ (8, 22, 24, 25, 44) but also to other arylamino alcohol drugs, such as QN, halofantrine, and lumefantrine (34). Some mutations in PfCRT, the major CQ resistance (CQR) determinant, are found to be associated with stereo-specific changes in responses to QN and quinidine (6, 10). Recently, a genetic study identified *PfMRP* as playing a role in the efflux of glutathione, CQ, and QN and contributing to parasite responses to multiple antimalarial drugs (27). In addition to these genes, quantitative trait loci analysis of the Dd2 × HB3 cross further mapped QNR to the *pfnhe1* gene, which harbors the minisatellite ms4760 (9). Sequence analysis of laboratory-adapted parasite isolates found that increased copy numbers of the minisatellite repeat (DNNND) are associated with reduced susceptibility to QN (9, 13). Consistent with the role of *pfnhe1* in QN response, the Dd2 × HB3 progeny clones with higher levels of QNR also exhibited significantly elevated PfNHE activity (4). Direct evidence of the *pfnhe1* involvement in QNR came from transfection studies, where reduced *pfnhe1* expression was associated with a significant decrease in QN sensitivity (19). It is noteworthy that the effect of *pfnhe1* knockdown is strain specific, providing further support for the complex, multifactorial nature of QNR in *P. falciparum*. Yet, none of these genes studied so far has accounted for high-level QNR.

Southeast Asia has been an epicenter for MDR *P. falciparum.* Parasites in this region are notorious for their propensity to develop resistance to multiple antimalarial drugs (29). To counter the rapid emergence and spread of drug resistance, malaria drug policies of the countries in Southeast Asia where malaria is endemic have undergone constant changes. CQ and antifolate drugs were abandoned a long time ago (46); resistance to other antimalarial drugs such as MQ has emerged soon after deployment, and QN can no longer be used for malaria monotherapy in this region (17, 26). Although four genes are implicated in reduced QN sensitivity in parasite field isolates, their validity as molecular markers for predicting QNR has been evaluated in only a small number of parasite isolates from diverse regions of the world (13). Therefore, in this study we further investigated the potential association between *in vitro* QN susceptibility of *P. falciparum* isolates collected from the China-Myanmar border with genetic polymorphisms in the *pfnhe1*, *pfcrt*, and *pfmdr1* genes.

MATERIALS AND METHODS

Collection of parasite clinical samples. A total of 260 *P. falciparum* field isolates were collected in 2007 to 2009 from symptomatic patients presenting with uncomplicated *P. falciparum* infections at a malaria clinic in Laiza Township near the China-Myanmar border. The human subject protocol for this study was approved by the Institutional Review Board of Kunming Medical University. Malaria infections were diagnosed by microscopic examination of Giemsa-stained thick and thin blood films. If *P. falciparum* infection was confirmed, 0.2 ml of blood was spotted on a piece of Whatman 3MM filter paper and used for molecular studies. An additional 0.5 ml of whole blood was collected in heparinized tubes, stored in liquid nitrogen, and transported to the laboratory for culturing.

Establishment of parasite cultures. Since our earlier work found that up to 30% of patient samples from this region contained mixed strain infections (50), we first wanted to identify samples with single *P. falciparum* infections. For genotyping, parasite genomic DNA was extracted from the filter papers by using a QIAamp DNA microkit (Qiagen, Germany) following the manufacturer's instructions. DNA was eluted in 80 μl of elution buffer. Parasite samples were then genotyped at three polymorphic genes, *merozoite surface protein 1* (*msp1*), *msp2*, and *glutamate-rich protein* (*glurp*), by previously described methods (15, 31, 37). Parasite cultures were initiated only from samples containing monoclonal infections. To adapt parasite samples to continuous cultures, frozen parasite stocks were thawed, washed twice with RPMI 1640 medium at 37°C, and mixed with fresh type O^+ human erythrocytes suspended at a 5% hematocrit in complete medium containing HEPES (5.94 g/liter), hypoxanthine (50 mg/liter), Albumax II (5 g/liter), RPMI 1640 (10.4 g/liter), gentamicin (5 mg/liter), $NaHCO_3$ (2.1 g/liter), and 6% AB^+ human serum. Parasite cultures were maintained routinely at 37°C in 75-cm^2 flasks (Costar) under a gas environment of 92% N_2, 5% CO_2, and 3% O_2.

***In vitro* QN sensitivity assay.** Parasite sensitivity to QN was determined using a SYBR green I-based fluorescence assay (36). The QN stock solution (25.6 μM) was prepared in ethanol and diluted in complete medium to the desired final concentrations, ranging from 10 to 2,560 nM. Each synchronized parasite culture at the late ring or early trophozoite stage was diluted with complete medium and fresh human erythrocytes to a starting 4% hematocrit and 0.3% parasitemia. For the drug assays, 90 μl of the parasite suspension was dispensed into the test wells of a 96-well microtiter plate, to which 10 μl of QN solution was added to each well to obtain a final concentration of 0, 10, 40, 160, 320, 640, 1,280, or 2,560 nM. The microtiter plates were incubated at 37°C for 72 h as described previously (2). Afterwards, the plates were frozen and thawed, and 100 μl of lysis buffer was added (36). The contents were mixed thoroughly until erythrocyte sediment was not visible. After incubation in the dark at room temperature for 1 h, fluorescence was measured using the Fluoroskan Ascent FL microplate fluorometer (Thermo Scientific, Waltham, MA) with excitation and emission wavelengths centered at 485 and 538 nm, respectively. For each QN concentration, three replicates were performed. To ensure consistency and reproducibility of the results, two biological replicates of the drug assay were performed on separate days. To further enhance comparability of our study results, six laboratory strains that display a wide range of QN sensitivity (3D7, K1, HB3, Dd2, W2, and 7G8) obtained from the Malaria Research and Reference Reagent Resource Center (MR4) and two Chinese laboratory isolates (SM and FCC1/HN) were similarly cultured and assayed.

Analysis of genetic polymorphisms at *pfnhe1*, *pfmdr1*, and *pfcrt*. Two *pfmdr1* fragments (bp 19 to 606 and bp 2902 to 3867 of the open reading frame) were amplified by PCR with two primer pairs as described previously to include the codons 86, 184, 1034, 1042, and 1246 (3). To determine the polymorphisms in codons 72 to 76 and codon 220 of the *pfcrt* gene, a nested PCR was performed with primers described previously (16). A fragment containing the ms4760 minisatellite in the *pfnhe1* gene was amplified using primers pfnhe-3802F and pfnhe-4322R (9). All PCR products were verified on 1% agarose gels. Amplified DNA was purified using the High Pure PCR cleanup microkit (Roche) and sequenced using BigDye Terminator v3.1. Sequences were aligned with the Clustal X 2.0.12 with manual editing.

Statistical analysis. All statistical analyses were performed using SPSS for Windows, version 8. The *in vitro* drug response data were entered into the SPSS data editor, and the geometric mean of the half-maximal inhibitory concentration (IC_{50}) and standard deviation (SD) were calculated for all isolates from a regression-probit analysis. The associations between IC_{50}s and polymorphisms in *pfmdr1*, *pfcrt*, and *pfnhe1* were assessed using a nonparametric Kruskal-Wallis test with Bonferroni correction, and the significance cutoff level was set at $P = 0.0045$. The differences between IC_{50}s of parasite isolates with different *pfnhe1* ms4760 haplotypes were compared using a *t* test.

RESULTS

***In vitro* QN responses.** We have collected a total of 260 clinical *P. falciparum* isolates at the China-Myanmar border. Genotyping the parasites at three highly polymorphic loci (*msp1*, *msp2*, and *glurp*) identified 118 samples with monoclonal infections, of which 60 were successfully culture adapted and assayed for *in vitro* sensitivity to QN. In addition, QN sensitivity of eight laboratory strains was also measured in order to define the cutoff values of QN responses. The IC_{50}s of QN-sensitive (QNS) strains 3D7 and HB3 were 108 and 319 nM, respectively, whereas the laboratory QNR strains 7G8, W2, and Dd2 had much higher QN IC_{50}s, 619, 720, and 602 nM, respectively. This result was consistent with the earlier separation of these strains into the QNS and QNR categories.

TABLE 1. QN responses of *P. falciparum* strains and polymorphisms in the *pfnhe1*, *pfmdr1*, and *pfcrt* genes

Parasite isolate	IC_{50} (nM) (mean ± SD)	*pfnhe1* ms4760[a]			*pfmdr1* codon[b]					*pfcrt* codon(s)[b]	
		Haplotype	Rep1	Rep2	86	184	1034	1042	1246	72–76	220
F07-7	456 ± 33	1	2	2	N	Y	S	N	D	CVIET	S
F07-10	306 ± 16	1	2	2	N	Y	S	N	D	CVIET	S
F07-23	447 ± 71	1	2	2	N	Y	S	N	D	CVIET	S
F07-50	204 ± 13	1	2	2	N	**F**	S	N	D	CVIET	S
F07-59	197 ± 15	1	2	2	N	**F**	S	N	D	CVIET	S
F08B53	468 ± 68	1	2	2	N	Y	S	N	D	CVIET	S
F09N1	191 ± 46	1	2	2	N	Y	S	**D**	D	CVIET	S
F09N18	284 ± 45	1	2	2	N	Y	S	N	D	CVIET	S
F07-13	365 ± 61	3	1	2	N	Y	S	N	D	CVIET	S
F09A54	243 ± 32	3	1	2	N	**F**	S	N	D	CVIET	S
F09A55	149 ± 26	3	1	2	N	**F**	S	N	D	CVIET	S
F09A9	562 ± 128	5	4	1	N	Y	S	N	D	CVIET	S
F09N72	500 ± 47	5	4	1	N	Y	S	N	D	CVIET	S
F07-3	875 ± 44	6	2	1	N	Y	S	N	D	CVIET	S
F07-9	321 ± 101	6	2	1	N	**F**	S	N	D	CVIET	S
F07-11	629 ± 81	6	2	1	N	Y	S	N	D	CVIET	S
F07-46	894 ± 56	6	2	1	N	Y	S	N	D	CVIET	S
F07-56	427 ± 52	6	2	1	N	Y	S	N	D	CVIET	S
F08B32	302 ± 45	6	2	1	N	**F**	S	N	D	CVIET	S
F09N35	905 ± 28	6	2	1	N	**F**	S	N	D	CVIET	S
F09N58	448 ± 109	6	2	1	N	**F**	S	N	D	CVIET	S
F09N66	711 ± 78	6	2	1	N	**F**	S	N	D	CVIET	S
F09N68	288 ± 82	6	2	1	N	**F**	S	N	D	CVIET	S
F07-6	543 ± 39	7	3	1	N	Y	S	N	D	CVIET	S
F07-8	874 ± 12	7	3	1	N	**F**	S	**D**	D	CVIET	S
F07-25	502 ± 45	7	3	1	N	**F**	S	N	D	CVIET	S
F07-27	650 ± 44	7	3	1	N	Y	S	N	D	CVIET	S
F07-28	473 ± 25	7	3	1	N	Y	S	N	D	CVIET	S
F07-29	282 ± 5	7	3	1	N	Y	S	N	D	CVIET	S
F07-31	433 ± 32	7	3	1	N	**F**	S	N	D	CVIET	S
F07-35	821 ± 224	7	3	1	N	Y	S	N	D	CVIET	S
F07-40	298 ± 53	7	3	1	**Y**	Y	S	N	D	CVIET	A
F07-42	388 ± 95	7	3	1	N	Y	S	N	D	CVIET	S
F07-58	680 ± 91	7	3	1	N	Y	S	N	D	CVIET	S
F08B2	1,016 ± 196	7	3	1	N	**F**	S	N	D	CVIET	S
F08B9	218 ± 31	7	3	1	N	**F**	S	N	D	CVIET	S
F08B27	870 ± 111	7	3	1	N	Y	S	N	D	CVIET	S
F08B41	886 ± 61	7	3	1	N	Y	S	N	D	CVIET	S
F08B61	1,128 ± 106	7	3	1	N	Y	S	N	D	CVIET	S
F08B63	579 ± 20	7	3	1	N	Y	S	N	D	CVIET	S
F08B72	583 ± 78	7	3	1	N	Y	S	N	D	CVIET	S
F09A10	716 ± 8	7	3	1	N	Y	S	N	D	CVIET	S
F09A41	1,295 ± 99	7	3	1	N	Y	S	N	D	CVIET	S
F09A61	373 ± 27	7	3	1	N	Y	S	N	D	CVIET	S
F09N6	710 ± 45	7	3	1	N	Y	S	**D**	D	CVIET	S
F09N22	535 ± 37	7	3	1	N	**F**	S	N	D	CVIET	S
F09N29	853 ± 45	7	3	1	N	Y	S	**D**	D	CVIET	S
F09N33	490 ± 28	7	3	1	N	Y	S	N	D	CVIET	S
F09N40	2,123 ± 93	7	3	1	N	Y	S	N	D	CVIET	S
F09N44	514 ± 26	7	3	1	N	Y	S	N	D	CVIET	S
F09N64	475 ± 66	7	3	1	N	Y	S	N	D	CVIET	S
F09N78	513 ± 18	7	3	1	N	Y	S	N	D	CVIET	S
Feng	371 ± 44	7	3	1	N	Y	S	N	D	CVIET	
PF18	300 ± 48	7	3	1	N	Y	S	N	D	SVMNT	S
F07-34	574 ± 33	9	3	2	N	Y	S	N	D	CVIET	S
F08B7	1,099 ± 283	9	3	2	N	Y	S	N	D	CVIET	S
F08B60	767 ± 32	14	3	1	N	Y	S	N	D	CVIET	S
F09A21	258 ± 26	18	2	2	N	Y	S	N	D	CVIET	S
F07-47	324 ± 21	20	4	1	N	**F**	S	N	D	CVIET	S
F08B40	260	21	1	1	N	**F**	S	N	D	CVIET	S
SM	188 ± 33	6	2	1	**Y**	Y	S	N	D	CVIET	S
FCC1/HN	179 ± 68	6	2	1	N	Y	S	N	D	CVIET	S
W2	720 ± 221	1	2	2	**Y**	Y	S	N	D	CVIET	S
3D7	108 ± 48	2	1	2	N	Y	S	N	D	CVMNK	A
7G8	619 ± 73	1	2	2	N	**F**	**C**	**D**	**Y**	SVMNT	S
Dd2	602 ± 87	1	2	2	**Y**	Y	S	N	D	CVIET	S
K1	407 ± 75	5	4	1	**Y**	Y	S	N	D	CVIET	S
HB3	319 ± 55	5	4	1	N	**F**	S	**D**	D	CVMNK	A

[a] The Rep 1 column indicates the number of DNNND repeats; the Rep 2 column shows the number of NHNDNHNNDDD repeats.
[b] Letters in bold indicate point mutations.

The K1 strain that originated from Thailand showed an intermediate level of QN sensitivity (IC_{50}, 407 nM). The two laboratory parasite strains (FCC1/HN and SM) collected in China in 1983 and 1998, respectively, had *in vitro* QN IC_{50}s among the lowest in this study, below 200 nM. In comparison, a substantial proportion of the isolates (>50%) from the China-Myanmar border showed reduced QN sensitivity, with IC_{50}s above 500 nM. Remarkably, one China-Myanmar border isolate exhibited significantly elevated *in vitro* resistance to QN, with an IC_{50} above 2,000 nM (Table 1).

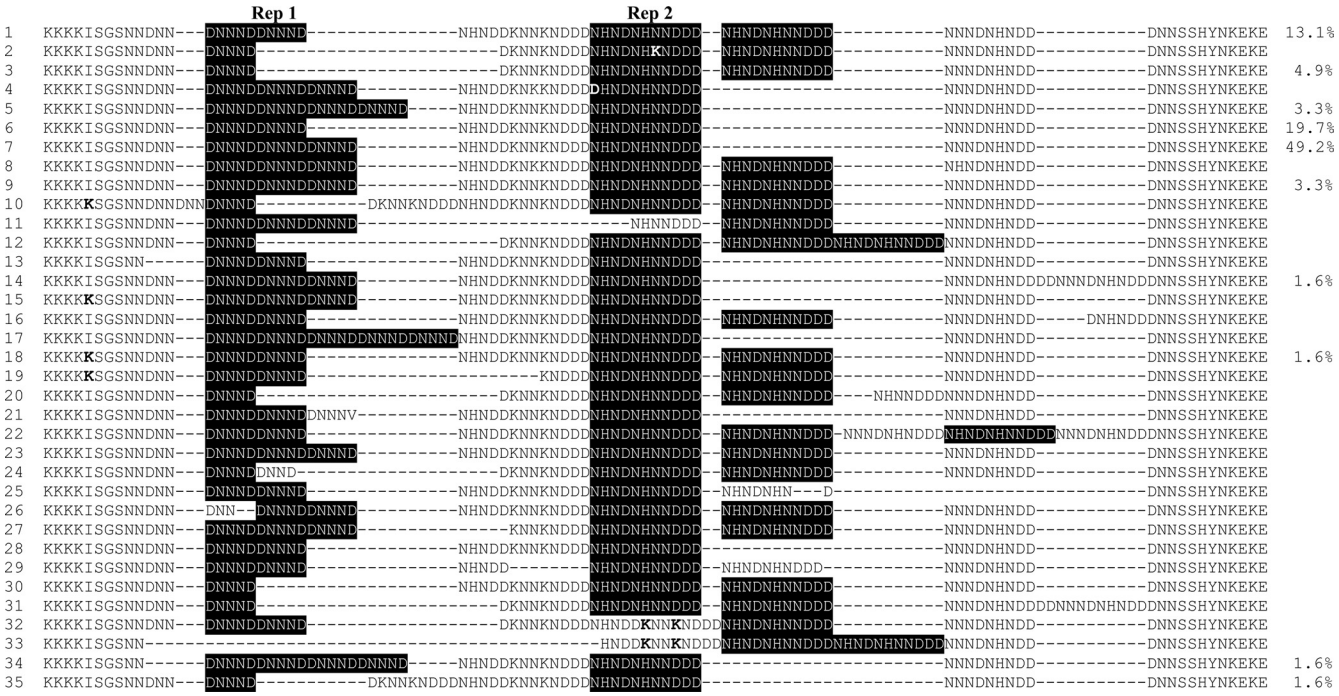

FIG. 1. Alignment of the 35 *pfnhe1* ms4760 haplotypes identified to date. Two types of repeats (Rep 1, DNNND, and Rep 2, NHNDNHNNDDD) are highlighted. Bold letters indicate mutated amino acids.

Molecular polymorphisms at *pfcrt*, *pfmdr1*, and *pfnhe1*. To study the genetic polymorphisms in the *pfcrt*, *pfmdr1*, and *pfnhe1* genes in the parasite population at the China-Myanmar border, we genotyped these three genes in 60 fresh parasite isolates and eight reference strains. For *pfcrt*, genotypes around codons 76 and 220 were determined. The major CQR determinant PfCRT 76T was present in all of these field samples. The majority (98%) of the parasite isolates around codon 76 had a mutant genotype of CVIET, which is most often associated with CQR in Southeast Asia. All but 1 parasite isolate from the 59 isolates successfully genotyped at codon 220 had the A220S mutation.

We genotyped *pfmdr1* at codons 86, 184, 1034, 1042, and 1246. For the 60 isolates collected from the China-Myanmar border area, most *pfmdr1* point mutations except that at codon 184 were rare (Table 1). Only one isolate harbored two mutations, at codons 184 and 1042, whereas the rest of the isolates from this area had no more than one mutation at these five codons. The frequencies of the 86Y, 184F, and 1042D mutations were 1.7, 30, and 6.7%, respectively. All these field isolates were wild type at codons 1034 and 1246 (Table 1).

We sequenced the ms4760 fragment of the *pfnhe1* gene and found 10 ms4760 haplotypes (Fig. 1). These included two new haplotypes (ms4760-34 and -35) and eight haplotypes (ms4760-1, -3, -5, -6, -7, -9, -14, and -18) that were described previously in *P. falciparum* samples collected from different regions of the world (1, 9, 40). The most prevalent ms4760 haplotype was ms4760-7, found in nearly half of the isolates (Fig. 1). The ms4760 haplotypes 6 and 1 were relatively abundant, accounting for 16.7 and 13.3% of the isolates, respectively. In comparison, the two new haplotypes, ms4760-34 and -35, were found in only one isolate each (Fig. 1). In the 60 field samples, the copy number of the DNNND sequence varied from one to four repeats, with two and three copies being more common and accounting for 32 and 57% of parasite samples, respectively. In addition, all parasite isolates studied possessed either one copy (76.7%) or two copies (23.3%) of the longer repeat, NHNDNHNNDDD (Fig. 1).

Associations of genetic polymorphisms with QN response. Genetic studies have detected associations of polymorphisms in *pfcrt*, *pfmdr1*, and *pfnhe1* genes with QN susceptibility (9, 13). To determine whether the observed polymorphisms in these three genes are associated with QN responses in parasite isolates examined in this study, we tested for a correlation between each polymorphic allele and the QN IC_{50}, and the results are summarized in Table 2. For the 60 newly established parasite isolates from the China-Myanmar border area, the QN IC_{50} was not significantly associated with any polymorphism in *pfmdr1* ($P = 0.022$ to 0.501) or *pfcrt* ($P = 0.040$ to 0.433). In contrast, when the more common *pfnhe1* ms4760 haplotypes (profiles 1, 3, 5, 6, and 7) were used for statistical analysis, a significant association was detected between the QN IC_{50} and ms4760 haplotype ($P = 0.003$). These five ms4760 haplotypes accounted for more than 90% of the isolates included in this study. Based on the *in vitro* QN IC_{50}s, these five haplotypes could be divided into two groups. Isolates with ms4760 haplotype 1 or 3 (group 1) were more susceptible to QN, with IC_{50}s of 319 ± 122 nM (mean ± SD) and 223 ± 106 nM, respectively. In comparison, isolates with ms4760 haplotype 5, 6, or 7 (group 2) had reduced QN sensitivity, with IC_{50}s of 531 ± 44 nM, 580 ± 254 nM, and 661 ± 374 nM, respectively. The IC_{50}s were not significantly different between haplotypes within each group ($P > 0.05$; *t* test), whereas they were significantly different between the two groups ($P < 0.05$; *t* test).

TABLE 2. Summary of the association of *in vitro* QN response (IC_{50}) with polymorphisms in the *pfnhe1*, *pfmdr1*, and *pfcrt* genes from *P. falciparum* isolates

Genotype and basis of analysis	*P* value[a]		Significance
	All samples[b]	60 field isolates	
pfnhe1			
ms4760 haplotype	0.010	0.003	S
His/Asp ratio	0.003	0.001	S
No. of DNNND repeats	0.001	0.003	S
No. of DDNHNDN HNND repeats	0.012	0.001	S
pfmdr1			
Codon 86	0.573	0.400	NS
Codon 184	0.067	0.022	NS
Codon 1034	0.647		NS
Codon 1042	0.519	0.501	NS
Codon 1246	0.647		NS
pfcrt			
Codons 72 to 76	0.198	0.433	NS
Codon 220	0.407	0.040	NS

[a] *P* values were determined with the Kruskal-Wallis test. The variables analyzed were the QN IC_{50}s. S, significant; NS, not significant (cutoff of $P = 0.0045$ [Bonferroni correction]).
[b] All samples included 60 new isolates, 8 laboratory standard strains, and 2 strains established earlier in China.

Two types of repeats in ms4760 have been associated with the *in vitro* QN response (1, 9, 13). In parasite isolates collected from the China-Myanmar border, we have also observed significant associations between the copy numbers of two repeat types and the QN response (for the DNNND repeat, $P = 0.003$; for the NHNDNHNNDDD repeat, $P = 0.001$) (Table 2; Fig. 2). Four isolates had one DNNND repeat, and they tended to be more susceptible to QN (254 ± 89 nM). In comparison, isolates with two or three DNNND repeats tended to have significantly reduced susceptibility to QN (IC_{50}, 453 ± 239 nM for two repeats [$n = 19$]; IC_{50}, 674 ± 365 nM for three repeats [$n = 34$]; $P < 0.0045$, Kruskal-Wallis test). However, the three isolates with four DNNND repeats showed intermediate susceptibility to QN (IC_{50}, 462 ± 123 nM) (Fig. 2). As for the longer repeat, a parasite isolates having one copy of the repeat was associated with reduced QN sensitivity (IC_{50}, 624 ± 337 nM [$n = 46$ isolates]), whereas an isolate having two copies of this longer *pfnhe1* repeat was associated with intermediate QN sensitivity (IC_{50}, 374 ± 244 nM [$n = 14$ isolates]; $P < 0.0045$, Kruskal-Wallis test).

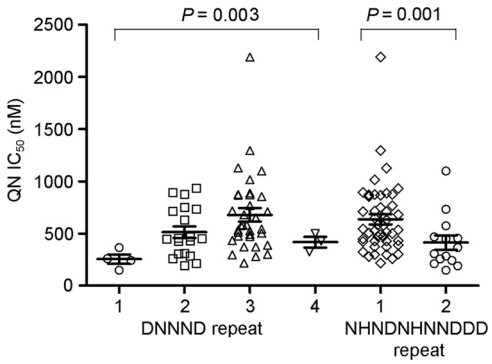

FIG. 2. Scatter plot of mean IC_{50} values (± SD) of the parasite isolates for QN, categorized by the number of copies Rep 1 (1, 2, 3, or 4) or Rep 2 (1 or 2). For Rep 1 and Rep 2, the *P* values were determined by using a nonparametric Kruskal-Wallis test with Bonferroni correction.

TABLE 3. Summary of *pfnhe-1* ms4760 haplotypes, His/Asp ratios, DNNND copy numbers, and *in vitro* QN responses (IC_{50}) of 60 field *P. falciparum* isolates

His/Asp ratio[a]	ms4760 haplotype(s)	No. of isolates	No. of DNNND copies	IC_{50} (nM) (mean ± SD)
5:8	5, 34	3	4	462 ± 123
5:6	7	31	3	661 ± 374
6:6	14	1	3	767
7:6	9	2	3	837 ± 372
5:4	6	10	2	580 ± 254
7:4	1, 18	9	2	312 ± 115
5:2	35	1	1	260
6:2	3	3	1	223 ± 106

[a] ms4760 haplotypes found among the China-Myanmar border parasite population are grouped here by His/Asp ratios, which are arranged from low to high values.

The copy numbers of the two types of repeats influence the ratios of two charged amino acids, histidine and aspartic acid, in the C-terminal domain of *pfnhe1*. The 10 ms4760 haplotypes found in the parasite population from the China-Myanmar border could be grouped according to eight His/Asp ratios (Table 3). Statistical analysis of the more abundant His/Asp groups found that the His/Asp ratio was significantly associated with QN response ($P = 0.001$). Increased His/Asp ratios (5:2 or 6:2) were associated with higher QN sensitivity, whereas strains with lower His/Asp ratios (5:4 or lower) had intermediate or reduced QN susceptibility (Table 3).

DISCUSSION

QN has been used to treat malaria for centuries, and development of resistance in *P. falciparum* is slow. However, a decrease in efficacy and emergence of clinical resistance in *P. falciparum* in some areas of endemicity have been observed (12, 26). In Thailand, QN was used extensively in the 1980s due to the spread of parasites resistant to CQ and antifolates (32). Although QN remains an effective treatment for severe MDR falciparum malaria in this area, there is evidence of a decline in its therapeutic response (26). In China's Yunnan province and bordering malarious regions, *in vitro* drug resistance surveys have also detected reduced QN responses in *P. falciparum* (48, 49). Further, there are significant geographical variations in *in vitro* QN responses reported: parasites from western Yunnan bordering Myanmar (IC_{50}, 608 nM) exhibited much lower QN susceptibility than parasites from southern Yunnan (480 nM) and southeastern Yunnan (352 nM) (47). This appears to agree with the malaria epidemiology in this region, where Myanmar is the country with the highest malaria endemicity in the Greater Mekong subregion and remains 1 of the 31 high-burden malarious countries in the world (43). In this study, we culture adapted 60 parasite clinical isolates from the China-Myanmar border area and grew parasites *in vitro* for 10 to 14 days before performing *in vitro* drug assays. While different *in*

vitro studies have used different cutoff values for QNR without clinical verification (5, 28), the range of the IC_{50}s of the three laboratory QNR strains 7G8, W2, and Dd2 (602 to 720 nM) suggests that 500 nM is an appropriate cutoff IC_{50} for QNR. Our results showed that 50% of the parasite samples had an *in vitro* QN IC_{50} above 500 nM, among which 13 parasites had IC_{50}s of >800 nM. It is noteworthy that an African parasite isolate recently obtained from a malaria patient with QN treatment failure had an *in vitro* QN IC_{50} of 829 nM (23). Therefore, our findings suggest that a large proportion of parasite isolates from the China-Myanmar border *P. falciparum* population may have developed increased resistance to QN.

While many drug resistance phenotypes are complex and involve multiple genes, a small number of genes usually play more important roles in determining the phenotype. *In vitro* QN responses in cloned *P. falciparum* parasites showed a smooth, continuous distribution of IC_{50}s, suggesting that QNR is a multigene trait (18). *In vitro* correlation studies of putative transporter genes in *P. falciparum* have found evidence of associations of mutations in *pfmdr1*, *pfcrt*, and three additional genes with higher QN IC_{50}s (18). One such transporter, *pfmrp*, was later shown to modulate the responses to multiple antimalarial drugs, including QN (27). Analysis of the Dd2 × HB3 cross further confirmed the contribution of *pfcrt* and *pfmdr1* to QN susceptibility and identified *pfnhe1* as another candidate associated with the QN response (9). While these four genes are associated with QN susceptibility, they do not appear to be the major genetic determinants of QNR. Besides, the degrees of their contributions to QNR seem to depend on the genetic background of the parasite (6, 7, 19, 30, 35), which varies greatly among regions of malaria endemicity due to different drug selection pressures. To validate these genes as genetic markers of QNR, Henry et al. performed an evaluation of 23 *P. falciparum* laboratory clones and found a strong association of *pfnhe1* polymorphism with QN response (13). Our study using 60 parasite samples collected from a region of malaria hyperendemicity at the China-Myanmar border further corroborated this finding (9, 13). However, a similar study using 40 samples from Madagascar and 36 samples from Africa did not identify such a correlation but found a significant positive correlation between *in vitro* QN response and number of the ms4760 long repeat (1). Those authors speculated that this discrepancy might be due to differences in parasite origins, since most of the strains with the highest QN IC_{50}s examined in earlier studies originated from Southeast Asia (9, 13).

The frequency of the ms4760 haplotype displays regional variations, which may be a result of geographically different natural histories of drug selection. In India, haplotype 6 is the predominant one, followed by haplotypes 7 and 3 (40). In Madagascar, hapotypes 1 and 7 are the most predominant (1). The predominant *pfnhe1* haplotype at the China-Myanmar border was haplotype 7, accounting for almost 50% of the parasite isolates. Parasites with this most abundant ms4760 haplotype were associated with an increased QN IC_{50} (>600 nM). The ms4760 polymorphism depends largely on the numbers of two types of repeats, DNNND and NHNDNHNNDDD. Our study showed that an increased number of DNNND repeats was correlated with increased QN IC_{50}, whereas parasites with one copy of the NHNDNHNNDDD repeat tended to have higher QN IC_{50}s than those with two copies of this repeat. Besides, 10 of the 13 parasite isolates for which the IC_{50} was over 800 nM had three DNNND repeats, and 12 had one NHNDNHNNDDD repeat. With regard to the influence of the ms4760 repeats on QN response, Bennett et al. speculated that the number of DNNND repeats, together with the proximal C-terminal poly-His region, might affect the His/Asp ratio and the pI of PfNHE1 and lead to altered Na^+/H^+ set point regulation in QNR (4). In support of this hypothesis, we found a significant association of the His/Asp ratio with the QN IC_{50}. More specifically, parasites with higher His/Asp ratios were likely to have increased QN sensitivity.

We consider our samples as not ideal for vigorously testing the contributions of *pfcrt* and *pfmdr1* polymorphisms to QN responses, since the overwhelming majority of our parasite isolates contained the CQR *pfcrt* genotype (CVIET at codons 72 to 76 and codon 220S) and wild-type *pfmdr1*. The CQR genotype was consistent with our earlier study in two neighboring counties in Yunnan Province (50), which all indicated that CQR parasites were still highly prevalent in this large region despite the withdrawal of CQ in treatment for *P. falciparum* decades ago. Since *pfcrt* point mutations have strong associations with QNR (18), it has been argued that CQ and QN may both interact with *pfcrt*. It is thus plausible that the homogeneous CQR *pfcrt* genotype in our studied parasite population may be partially responsible for the observed prevalence of reduced QN sensitivity. However, with regard to the point mutations at the *pfmdr1* C-terminal domain, all parasite isolates from the study area were wild type with 1034F and 1246D, while <7% of the parasite isolates had the 1042D mutation. The triple mutation 1034C/1042D/1246Y, commonly found in South America and which is associated with reduced QN response (35), was not found in our studied parasite isolates. Despite the Y184F mutation being present in ~30% of the field parasite isolates, no association with QN response was detected. Furthermore, in areas where MQ has been heavily deployed, increased *pfmdr1* copy number is associated with decreased susceptibility to MQ and a number of other antimalarials, including QN (24). However, *pfmdr1* amplification was found in only 1 of the 60 parasite isolates (data not shown), suggesting neither *pfmdr1* polymorphisms nor the copy number is likely to play a major role in the reduced QN response observed in this region.

The association of the two ms4760 haplotypes with decreased QN susceptibility requires further investigation, as the sample size of our study was relatively small. Since our samples were collected from a relatively small geographic region, the influence of population structure should be minimal. Yet, we cannot rule out this possibility, because we did not test the studied parasite population. We also genotyped polymorphic sites in *msp1*, *msp2*, and *glurp*, and none of the sites was associated with the parasite response to QN (data not shown). Similarly, substitutions in *pfcrt* and *pfmdr1* were not associated with the response to QN. These data suggest that the association of the repeats in *pfnhe1* and QN response is unlikely to be the result of a structured population.

Molecular techniques are increasingly being used for drug resistance surveillance, because many of the target gene polymorphisms are predictive of drug resistance and clinical drug failures. The combination of *in vitro* drug assay and molecular work will generate a more detailed picture of the epidemiology

of drug resistance. Yet, the molecular targets for a number of drugs are not completely understood, making molecular diagnosis of drug resistance difficult. In this study, we evaluated the validity of three candidate genes for QN susceptibility in the China-Myanmar border area. Our result found further evidence of an association between *pfnhe1* polymorphisms and *in vitro* QN response. However, conflicting results obtained from another study have cast doubts on the validity of *pfnhe1* polymorphisms for predicting *in vitro* QN response (1). Nonetheless, these studies have detected relationships for either the short or long repeat in ms4760 with QN response, suggesting a possibility that the *pfnhe1* polymorphisms might be associated with the QN susceptibility phenotype by a hitchhiking effect. Taken together, the degree of contribution of *pfcrt*, *pfmdr1*, and *pfnhe1* to QN susceptibility seems to vary geographically and should be examined in diverse parasite populations. Given that the China-Myanmar border area has a totally different drug use history from other areas where *P. falciparum* is endemic and that drug resistance surveillance efforts are very weak in this region, focused research on drug resistance mechanisms and extensive surveillance are highly desired to guide regional malaria control.

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