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Limited Ability of *Plasmodium falciparum pfcrt*, *pfmdr1*, and *pfnhe1* Polymorphisms To Predict Quinine *In Vitro* Sensitivity or Clinical Effectiveness in Uganda[▽]

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Quinine is a standard drug for treating severe malaria in Africa, and it is also increasingly used to treat uncomplicated disease. However, failures of quinine therapy are common, and it is unknown if failures in Africa are due to drug resistance. Recent studies have identified associations between *in vitro* quinine sensitivity and polymorphisms in genes encoding putative transporters, including well-described polymorphisms in *pfcrt* and *pfmdr1* and varied numbers of DNNND or DDNHNDNHNND repeats in microsatellite 4760 (ms4760) of the predicted sodium-hydrogen exchanger, *pfnhe1*. To better characterize mediators of quinine response, we assessed associations between genetic polymorphisms, *in vitro* quinine sensitivity, and quinine treatment responses in Kampala, Uganda. Among 172 fresh clinical isolates tested *in vitro*, decreasing sensitivity to quinine was associated with accumulation of *pfmdr1* mutations at codons 86, 184, and 1246. Nearly all parasites had *pfcrt* 76T, preventing analysis of associations with this mutation. *pfnhe1* ms4760 was highly polymorphic. Parasites with 2 copies of either ms4760 repeat showed modest decreases in quinine sensitivity compared to those with 1 or ≥3 repeats, but the differences were not statistically significant. None of the above polymorphisms predicted treatment failure among 66 subjects treated with quinine for uncomplicated malaria. Our data suggest that quinine sensitivity is a complex trait and that known polymorphisms in *pfcrt*, *pfmdr1*, and *pfnhe1*, while associated with quinine sensitivity, are not robust markers for quinine resistance.

Quinine was the first established antimalarial drug, and it has been used to treat malaria for centuries (26). Intravenous quinine is the standard therapy for severe *Plasmodium falciparum* malaria in Africa and many other areas, although intravenous artesunate recently showed superior efficacy in Asia (15). Quinine is also a second-line regimen used for the treatment of uncomplicated malaria in many countries after failure of initial therapy. Recent WHO guidelines suggest second-line use of quinine in combination with an antibiotic (55), but monotherapy is still commonly used (59). In addition, with failures of older therapies and limited availability of new artemisinin-based combination therapies, quinine is increasingly used as a first-line drug for treatment of uncomplicated malaria in Africa (1).

The use of quinine to treat uncomplicated malaria is problematic for a number of reasons. First, the efficacy of quinine for the treatment of uncomplicated malaria is uncertain. A number of studies and case reports have demonstrated apparent failures after quinine therapy for falciparum malaria in Asia (9, 41), South America (46, 60), and Africa (22, 31, 38). Recent studies incorporating detailed assessments of treatment efficacy or effectiveness have shown 28-day failure rates of >10% after 7-day courses of quinine for uncomplicated malaria in Sudan (3), Thailand (40), and Uganda (1). In Uganda, the 28-day genotype-corrected failure rate was 23%; some failures may have been due to poor treatment compliance, but no association between measures of compliance and treatment efficacy was identified (1). On the other hand, some studies have shown excellent efficacy for quinine, including over 95% success after 14 to 28 days for the treatment of falciparum malaria in Equatorial Guinea (45), in Venezuela (2), and in returned travelers in France (32). Second, quinine is poorly tolerated, especially later in the course of 7 days of treatment, suggesting that noncompliance with full treatment courses is common (19, 50). Noncompliance may limit treatment efficacy and help to select for drug-resistant parasites. Third, widespread quinine use engenders risks of serious toxicities, including cardiac effects, hypoglycemia, hemolysis, and thrombocytopenia (50). Fourth, quinine pharmacokinetics are variable, and metabolism can be altered by coadministration of a number of other drugs (42).

Diminished *in vitro* responsiveness of *P. falciparum* to quinine has been documented in parasites from Asia, South America, and Africa (57). Available studies have suggested that parasites from Africa generally remain sensitive to quinine (4, 21, 24, 37, 43, 51), but methodologies have varied, and strict cutoffs for *in vitro* drug resistance have not been established. Despite relatively low 50% inhibitory concentrations (IC_{50}s), it is noteworthy that absolute sensitivities of African *P. falciparum* strains have varied greatly. For example, in a recent study in Uganda, the IC_{50}s for quinine against freshly isolated parasites varied about 50-fold (29).

Mediators of *P. falciparum* resistance to quinine are poorly understood (58). The resistance phenotype is consistent with a complex genetic basis, as changes in sensitivity have appeared

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to evolve gradually. Quantitative trait locus analysis identified three genes predicted to play roles in the responsiveness of *P. falciparum* to quinine (18): *pfcrt*, which encodes a predicted transporter in which the 76T mutation is the principal mediator of resistance to chloroquine (12, 49); *pfmdr1*, which encodes a P-glycoprotein homolog for which different polymorphisms are associated with altered responses to a number of antimalarials (34, 44, 52); and *pfnhe1*, which encodes a putative sodium-hydrogen exchanger (18). Among known variations, an increased copy number of *pfmdr1* is most clearly associated with the response to quinine, with its amplification being associated with 2- to 3-fold decreases in sensitivity (33, 47, 58). Increased *pfmdr1* copy number has been very uncommon in Africa, although it has recently been reported in West Africa (56). Single nucleotide polymorphisms (SNPs) in *pfcrt* and *pfmdr1* are also associated with alterations in quinine sensitivity (10, 27, 44, 49). In Ugandan field isolates, the *pfmdr1* 1246Y mutation was associated with diminished sensitivity to quinine (29). However, unlike analyses of chloroquine sensitivity, associations between quinine sensitivity and polymorphisms in *pfcrt* and *pfmdr1* have been modest, suggesting important roles for additional polymorphisms, including *pfnhe1* and other loci (18).

Considering variation in *pfnhe1*, reducing its expression by about 50% using allelic exchange led to a 30% increase in quinine sensitivity in some but not other parasite strains (28). Four studies have recently evaluated associations between polymorphisms at *pfnhe1* microsatellite 4760 (ms4760) and *in vitro* sensitivity of parasites from individuals infected in countries where malaria is endemic. In 23 culture-adapted strains from multiple countries (20) and 60 freshly isolated strains from the China-Myanmar border (25), increased numbers of the DNNND repeat were associated with decreased quinine sensitivity, while increased numbers of the DDNHNDN HNND repeat within the same microsatellite were associated with increased quinine sensitivity (20). In 29 fresh isolates from the Kenyan coast, 2 DNNND repeats were associated with decreased sensitivity to quinine compared to the sensitivities of isolates with 1 or 3 copies, and there was no association between the number of DDNHNDNHNND repeats and quinine susceptibility (30). In 83 freshly isolated strains from individuals infected in Africa, multiple polymorphisms were seen in ms4760, but, in contrast to the above reports, significant associations were not seen between the numbers of the DNNND repeat and quinine sensitivity, and increased copies of the DDNHNDNHNND repeat were associated with decreased quinine sensitivity (5). Taken together, recent results suggest an uncertain role for *pfnhe* polymorphisms in quinine sensitivity.

To further characterize associations between polymorphisms in *pfcrt*, *pfmdr1*, and *pfnhe1* and sensitivity to quinine, we evaluated key sequences in *P. falciparum* parasites from two studies in Kampala, Uganda. The first study provided access to a large number of clinical isolates for which *in vitro* sensitivity to quinine was determined (29). The second study provided samples from a recent assessment of the clinical effectiveness of quinine for the treatment of uncomplicated malaria (1). Our results are consistent with the conclusion that quinine responsiveness is a complex trait, with polymorphisms in multiple genes contributing to drug sensitivity.

MATERIALS AND METHODS

Clinical trials. *Plasmodium falciparum* DNA was available from two clinical trials in Kampala. The first was a longitudinal comparison of the efficacies of three combination regimens against uncomplicated malaria in children aged 1 to 10 years at enrollment (601 enrolled from November 2004 to June 2005 and an additional 89 enrolled from January to May 2007) (11, 17). In this trial, patients were offered all medical care through the study clinic, so use of antimalarial drugs outside the study protocol was uncommon. Children were treated with quinine only for complicated malaria or after failure of a combination regimen, both uncommon events (17). Samples were collected for parasite culture between August 2006 and May 2008. The second trial was a randomized comparison of quinine and artemether-lumefantrine for the treatment of uncomplicated falciparum malaria in children aged 6 to 59 months (1); 86 children were enrolled into the quinine treatment arm of the study. Responses over 28 days were graded on the basis of standard WHO criteria, with genotyping used to distinguish recurrences due to reinfection from those due to recrudescence (1). In both trials, when falciparum malaria was diagnosed on the basis of fever or a history of fever and parasites on Giemsa-stained blood smears, blood spots were collected on filter paper (Whatman 3MM) for subsequent molecular studies. All study subjects provided written informed consent. Both trials and the analysis of cultured parasites were approved by the Uganda National Council for Science and Technology, the Makerere University Research and Ethics Committee, and the University of California, San Francisco, Committee on Human Research.

Parasite culture and measurement of *in vitro* quinine sensitivity. For the first trial described above, at the time of diagnosis of an episode of malaria and before the initiation of therapy, blood was collected in heparinized tubes and transported within 30 min to our laboratory. Giemsa-stained thin blood smears were examined, and if *P. falciparum* monoinfection was confirmed, culture was initiated. Blood was centrifuged, plasma and buffy coat were removed, and the erythrocyte pellet was washed twice with RPMI 1640 medium at 37°C. Parasites were diluted with 2% group O uninfected erythrocytes to obtain a density of 0.05%. Aliquots (200 μl) were then cultured in 10 ml RPMI 1640 medium supplemented with 25 mM HEPES, 0.2% $NaHCO_3$, 0.1 mM hypoxanthine, 100 μg/ml gentamicin, and 0.5% Albumax II serum substitute to produce a packed cell volume of ~2%. Sensitivities were measured for multiple drugs, including quinine, as previously described utilizing 96-well culture plates predosed with serial dilutions of drugs and a histidine-rich protein 2 (HRP-2)-based enzyme-linked immunosorbent assay (ELISA) (29). For this assay, optical density values were fitted to normal curves based on serial dilutions of HRP-2 standards, and IC_{50}s were calculated on the basis of a nonlinear regression model.

Analysis of parasite genetic polymorphisms. DNA was extracted from filter paper samples by extraction with Chelex, as previously described (35). We screened for polymorphisms at *pfcrt* K76T and *pfmdr1* N86Y, Y184F, S1034C, N1042D, and D1246Y by PCR amplification of flanking sequences, sequence-specific restriction enzyme digestion, and evaluation of the digested fragments by agarose gel electrophoresis, all as previously reported (12, 16). Estimates of *pfmdr1* gene copy number were carried out by quantitative PCR as previously reported (14, 39), using a 7500 real-time PCR system (Applied Biosystems).

The ms4760 locus of the *pfnhe1* gene was amplified with primers NHE-A (5′-AGTCGAAGGCGAATCAGATG-3′) and NHE-B (5′-GATACTTACGAA CATGTTCATG-3′) (53), using a Titanium PCR kit (Clontech). Each 20-μl PCR mixture included 14.8 μl H_2O, 2 μl 10× buffer, 0.4 μl deoxynucleoside triphosphate mix (10 mM each), 0.2 μl of each 20 μM primer, 0.4 μl 50× Titanium *Taq*, and 2 μl of target DNA template. A touchdown PCR approach (23) used the following parameters: 94°C for 120 s; 10 cycles of 94°C for 15 s, 63°C for 30 s, and 68°C for 90 s; 25 cycles of 94°C for 15 s, 58°C for 30 s, and 68°C for 90 s; and 1 cycle of 68°C for 7 min. The PCRs were performed on a Bio-Rad C1000 or S1000 thermocycler. PCR products were purified using ExoSAP-IT (USB). About 150 ng of each purified DNA was sequenced using 15 pmol primers NHE-C (5′-A TCCCTGTTGATATATCGAATG-3′) and NHE-D (5′-TTGTCATTAGTACC CTTAGTTG-3′) (53) at the University of California, San Francisco, Genomics Core Facility using ABI BigDye (version 3.1) Terminator sequencing chemistry and an ABI Prism 3730xl capillary DNA analyzer (Applied Biosystems).

For analysis of *pfnhe1* ms4760, nucleotide traces were physically inspected using the SeqMan tool (DNAStar Lasergene 8 software). The sequences were then translated using the Edit Sequence tool and aligned using the MEGALIGN program (DNAStar). Microsatellite 4760 profiles that were not among the 35 previously reported profiles (5, 18, 25, 53), were confirmed by repeat template amplification and sequencing.

Statistical analysis. Quinine IC_{50}s were calculated using a polynomial regression model and HN-NonLin software (http://malaria.farch.net) and were considered continuous variables. Differences in quinine IC_{50}s were examined using the

TABLE 1. *pfmdr1* polymorphisms seen in Kampala and *in vitro* quinine sensitivities of isolates

Parameter	Result for isolates with the following polymorphisms:					
	N86Y		Y184F		D1246Y	
	WT[a]	Mutant	WT	Mutant	WT	Mutant
No. of isolates	24	143	143	21	37	133
Median IC_{50} (nM)	84	123	112	132	84	125
25th-75th percentile IC_{50} (nM)	24–223	38–259	34–255	33–242	23–180	38–263
Geometric mean IC_{50} (nM)	74	101	95	108	71	103
95% CI[b] (nM)	45–121	84–121	79–114	64–181	49–102	85–125

[a] WT, wild type.
[b] CI, confidence interval.

Mann-Whitney U test for comparisons of two groups or the Kruskal-Wallis test with Dunn's multiple-comparison posttest for comparisons of more than two groups. Infections with mixed mutant/wild-type genotypes at the *pfcrt* and *pfmdr1* loci were analyzed as mutant, in view of the expected phenotype of the infection. Associations between alleles and treatment outcomes were assessed using Fisher's exact two-tailed test. Tests were carried out using GraphPad Prism software. In all cases the statistical significance level was set at a P value of <0.05.

Nucleotide sequence accession numbers. Nucleotide sequences for the Ugandan isolates were deposited in the GenBank database under accession numbers HQ412347 to HQ412386.

RESULTS

Samples available for study. We evaluated polymorphisms in two sets of samples from patients infected with *P. falciparum* in Kampala. First, we evaluated samples from a cohort of children who were followed in a drug efficacy trial comparing three combination regimens for uncomplicated falciparum malaria (17). Blood samples collected upon presentation with uncomplicated falciparum malaria and before the initiation of therapy were used to inoculate cultures, and *in vitro* sensitivities to quinine were determined immediately after sample collection (29). Second, we evaluated 79 samples from the quinine treatment arm of a trial with children randomized to treatment of uncomplicated falciparum malaria with either artemether-lumefantrine or quinine (1). Patients were followed over 28 days after presentation, and treatment outcomes were classified on the basis of WHO criteria (54); *in vitro* sensitivities were not assessed for samples from this trial.

Associations between polymorphisms and *in vitro* quinine sensitivity. The *in vitro* sensitivities to quinine of isolates collected in Kampala varied widely, with a geometric mean IC_{50} of 94.4 nM and an IC_{50} range of from 15 to 761 nM (29). We first assessed known polymorphisms in *pfcrt* and *pfmdr1* and searched for associations with the sensitivity of cultured parasites to quinine. The *pfcrt* 76T mutation was seen in all except 1 of 169 tested samples. Thus, many parasites were highly sensitive to quinine, despite the presence of *pfcrt* 76T, and it was not possible to assess the role of this polymorphism in quinine sensitivity. For *pfmdr1*, the N86Y and 1246Y mutations were also very common, although they were not fixed; the 184F mutation was less common; and mutations at positions 1034 and 1042, which have principally been described in samples from regions other than Africa, were not seen (Table 1). For the 86Y, 184F, and 1246Y polymorphisms, mutant parasites had diminished sensitivity to quinine (Table 1), but none of these differences attained statistical significance. Considering combinations of polymorphisms, there was a trend toward decreased quinine sensitivity with increased numbers of mutations (Table 2). The difference was statistically significant, after adjustment for multiple comparisons, between isolates having any 1 of the *pfmdr1* mutations and isolates having all 3 *pfmdr1* mutations ($P = 0.02$). Increased copy number of *pfmdr1* has been associated with diminished quinine sensitivity in Asia but has been seen uncommonly in African isolates. To consider a potential contribution of *pfmdr1* copy number on results from Uganda, we analyzed 58 samples with a range of quinine sensitivities (26 samples with IC_{50}s of from 15 to 22 nM, 10 with IC_{50}s of from 41 to 298 nM, 22 with IC_{50}s of from 308 to 755 nM). All of these parasites contained only a single copy of the *pfmdr1* gene.

We next evaluated associations between polymorphisms in *pfnhe1* ms4760 and quinine sensitivity. We sequenced 240 isolates, and *in vitro* quinine sensitivity data were obtained for 172 isolates. We found a high degree of polymorphism at this locus. We found 46% (16/35) of the previously described ms4760 haplotypes. In addition, we identified 24 previously undescribed ms4760 haplotypes, now designated ms4760-36 to ms4760-59 (Table 3 and Fig. 1). Nucleotide sequences for the Ugandan isolates were deposited in the GenBank database under the accession numbers indicated

TABLE 2. Impact of combinations of *pfmdr1* polymorphisms on *in vitro* quinine sensitivity[a]

Parameter	Result for isolates with the following polymorphisms:			
	All WT	1 mutation	2 mutations	3 mutations
No. of isolates	12	23	125	5
Median IC_{50} (nM)	64	84	123	278
25th-75th percentile IC_{50} (nM)	20–244	22–170	37–259	179–601
Geometric mean IC_{50} (nM)	65	65	101	312
95% CI[b] (nM)	30–140	40–105	83–123	142–687

[a] In *vitro* IC_{50}s are shown for parasites with all wild-type (WT) sequences at the five *pfmdr1* alleles studied or with one, two, or three mutations at *pfmdr1* codon 86, 184, or 1246.
[b] CI, confidence interval.

TABLE 3. *pfnhe1* microsatellite 4760 profiles detected among Ugandan *P. falciparum* isolates

ms4670 profile[a]	No. of repeats		Quinine IC_{50} (nM)	No. of isolates
	DNNND	DDNHNDNHNND		
35, 36, 42, 58	1	1	22.3–298.0	3
2, 3, 39, 47, 48, 53, 54, 59	1	2	15.4–760.9	39
12, 45, 52	1	3	63.5–96.8	2
6, 24, 37, 55	2	1	16.6–527.1	11
1, 18, 27, 44, 46, 51	2	2	15.4–754.9	58
38, 57	2	3	15.4–193.6	2
7, 15, 21, 43	3	1	15.4–447.5	24
9, 23, 49, 56	3	2	15.4–483.0	19
8, 40	3	3	15.4–275.2	9
5, 50	4	1	15.4–292.3	4
41	6	1	278.4	1

[a] Previously reported profiles 4, 10, 11, 13, 14, 16, 17, 19, 20, 22, 25, 26, 28, 29, 30, 31, 32, 33, and 34 (5, 18, 25, 53) were not observed in this study. Sensitivities for isolates with profiles 23, 24, 35, 52, and 59 were not determined.

above and in Fig. 1. The number of DNNND repeats ranged from 1 to 6 (mean ± standard deviation [SD], 2.12 ± 0.86), and that of DDNHNDNHNND repeats ranged from 1 to 3 (mean ± SD, 1.83 ± 0.54). The quinine sensitivities of isolates with different numbers of DNNND and DDNHNDHNND repeats were compared. Sensitivities varied greatly within each category, but parasites with 2 copies of either repeat were somewhat less sensitive to quinine than those with 1 or 3 or more repeats (Table 4), although the associations between repeat number and quinine IC_{50} were not significant ($P = 0.20$ and 0.15 for DNNND and DDNHNDHNND, respectively). Considered together, the impacts of known polymorphisms in *pfcrt*, *pfmdr1*, and *pfnhe1* did not lead to identification of significant associations. Considering the combinations leading to the least and the most sensitive parasites, those with the wild-type sequence at *pfmdr1* positions 86 and 1246 and 3 DNNND repeats were more sensitive to quinine (IC_{50}, 55 nM) than parasites with mutant *pfmdr1* sequences and 2 DNNND repeats (IC_{50}, 151 nM), but the differences were not statistically significant ($P = 0.13$; Table 5).

Associations between polymorphisms and quinine treatment responses. Of 79 samples available from the quinine treatment arm of a recent drug effectiveness trial in Kampala (1), we excluded 12 samples from patients classified by genotyping as clinical failures due to new infection during follow-up and 1 due to failed amplification, leaving 66 samples for analysis, including 46 (70%) from patients who experienced adequate clinical and parasitological responses and 20 (30%) from treatment failures due to recrudescent parasites (2 early treatment failures, 11 late clinical failures, and 7 late parasitological failures). All evaluable isolates (2 failed to amplify) had the *pfcrt* 76T mutation. For *pfmdr1*, the prevalence rates of the 86Y, 184F, and 1246Y mutations were very similar between

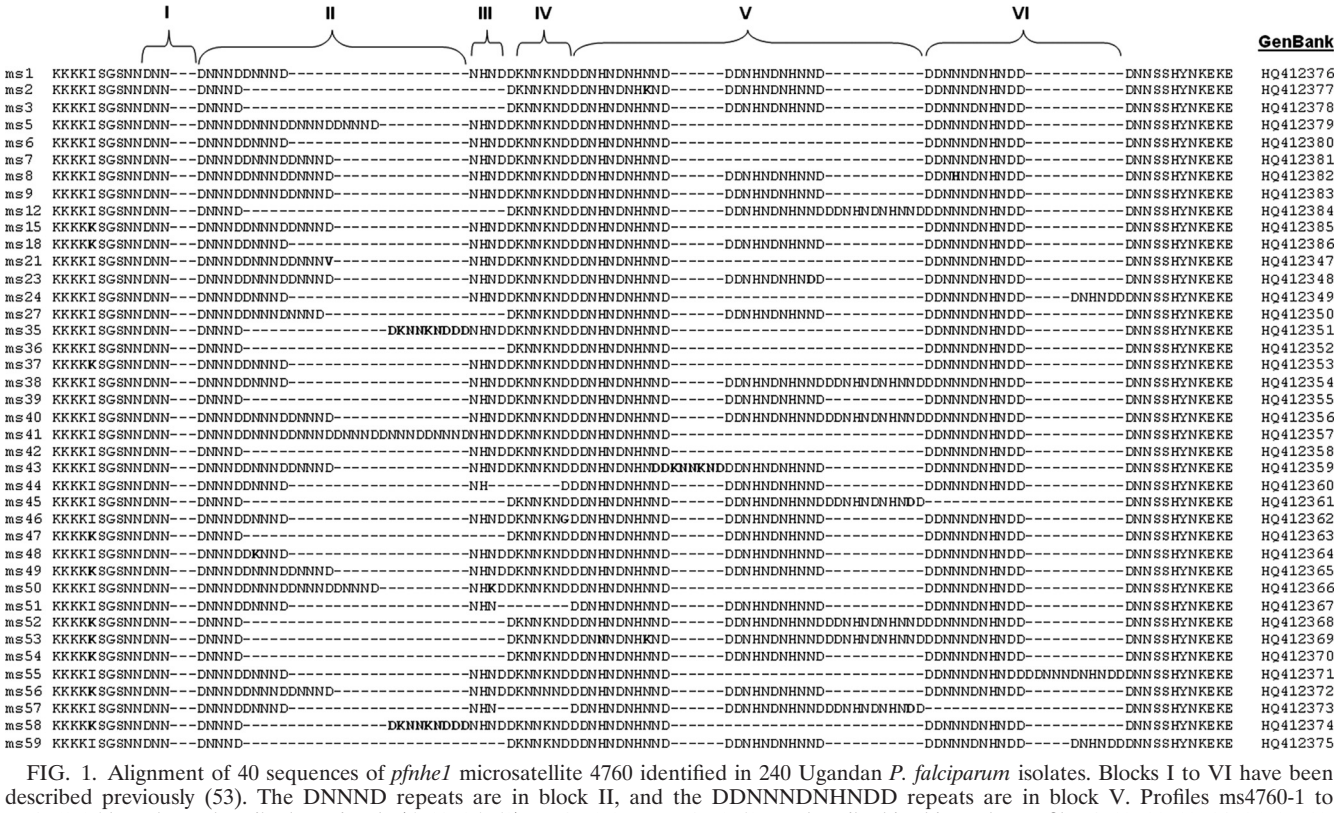

FIG. 1. Alignment of 40 sequences of *pfnhe1* microsatellite 4760 identified in 240 Ugandan *P. falciparum* isolates. Blocks I to VI have been described previously (53). The DNNND repeats are in block II, and the DDNNNDNHNDD repeats are in block V. Profiles ms4760-1 to ms4760-35 have been described previously (5, 18, 25, 53); ms4760-36 to ms4760-59 are described in this study. Profiles 4, 10, 11, 13, 14, 16, 17, 19, 20, 22, 25, 26, 28, 29, 30, 31, 32, 33, and 34 were not observed in the present study. Other differences among sequences are indicated in boldface. Gaps were created for optimal alignment.

TABLE 4. *In vitro* quinine sensitivities of parasites with different numbers of repeats in *pfnhe1* ms4760

Parameter	Result for the indicated no. of repeats of:					
	DNNND			DDNHNDNHNND		
	1	2	≥3	1	2	3
No. of isolates	44	71	57	43	116	13
Median IC_{50} (nM)	105	124	72	93	124	55
25th-75th percentile IC_{50} (nM)	32–233	55–273	25–217	25–271	36–253	16–166
Geometric mean IC_{50} (nM)	91	113	76	87	103	55
95% CI[a] (nM)	64–130	87–147	56–104	61–123	84–127	28–108

[a] CI, confidence interval.

isolates that led to adequate response or treatment failure (Fig. 2). Combinations of any 2 or 3 *pfmdr1* mutations were also not associated with treatment response. As with isolates collected for *in vitro* analyses, mutations at codons 1034 and 1042 (tested in all 66 isolates) or increases in copy number (tested in 55 samples) of *pfmdr1* were not seen in any of the clinical isolates.

We next considered the impact of polymorphisms in *pfnhe1* on treatment responses. The numbers of DNNND and DDN HNDHNND repeats varied somewhat between groups, but there was no significant association between the number of repeats and clinical response (Fig. 3). In particular, there was no significant difference in quinine treatment response between individuals harboring parasites with one versus two or more DNNND or DDNHNDNHNND repeats.

DISCUSSION

Polymorphisms in three *P. falciparum* genes have been linked to varied responses of malaria parasites to quinine. We considered associations between these polymorphisms and *in vitro* quinine sensitivity in samples from children with uncomplicated malaria in Uganda. We found modest associations between known polymorphisms in *pfmdr1* and quinine sensitivity, including a significant decrease in sensitivity with an increased prevalence of combinations of the 86Y, 184F, and 1246Y mutations. Considering newly described polymorphisms in *pfnhe1*, we found a modest and statistically insignificant association between two copies and one or three or more copies of two adjacent repeats and decreased quinine sensitivity. We also assessed associations between *P. falciparum* polymorphisms and clinical outcomes using samples from a clinical trial of quinine for the treatment of uncomplicated malaria in Uganda. None of the tested polymorphisms was predictive of quinine treatment failure. Taken together, our results confirm prior findings showing modest associations between polymorphisms in *pfmdr1* and quinine sensitivity, suggest that associations between *pfnhe1* repeat numbers and quinine sensitivity are not as great as suggested in a recent paper (20), and indicate that the known polymorphisms evaluated in the present study are unlikely to serve as reliable markers of quinine treatment outcomes.

Although quinine has retained good antimalarial efficacy in most areas, its clinical efficacy has decreased in some regions, especially in Asia (51). Further, some reports have identified worrisome limitations in the efficacy of quinine in Africa, including our recent identification of a 23% failure rate for the treatment of uncomplicated falciparum malaria in Uganda (1). The recent Ugandan study did not include directly observed therapy, methodologies have varied among other studies, and the true incidence of quinine treatment failure in Africa is uncertain. Further, the relative contributions of different factors to treatment failure, including parasite resistance to quinine, poor compliance with therapeutic regimens, varied drug absorption and pharmacokinetics, and other factors, are unknown.

Of note, even in areas where quinine efficacy has appeared to remain good, such as sub-Saharan Africa, the sensitivity of individual *P. falciparum* isolates to the drug has varied widely.

TABLE 5. *In vitro* quinine sensitivities of isolates with various combinations of polymorphisms[a]

Parameter	Result for isolates with polymorphisms at codons 86 and 1246 and the indicated no. of DNNND repeats:					
	Mutant			WT		
	1	2	3	1	2	3
No. of isolates	33	49	42	4	10	5
Median IC_{50} (nM)	115	151	124	111	111	55
25th-75th percentile IC_{50} (nM)	33–275	60–292	30–249	42–200	18–238	27–182
Geometric mean IC_{50} (nM)	99	129	90	90	75	61
95% CI[b] (nM)	65–153	96–175	64–128	20–410	29–194	18–206

[a] *In vitro* IC_{50}s are shown for parasites with mutant or wild-type (WT) sequences at *pfmdr1* codons 86 and 1246 and with the indicated number of *pfnhe1* repeats.
[b] CI, confidence interval.

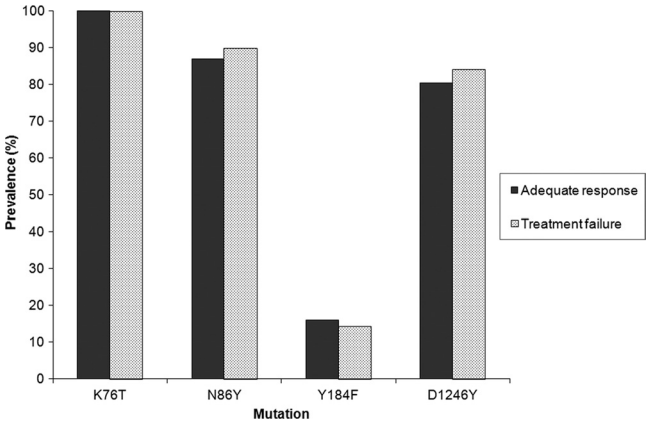

FIG. 2. Prevalence of *pfcrt* and *pfmdr1* mutations among children who experienced adequate response or treatment failure after therapy with quinine.

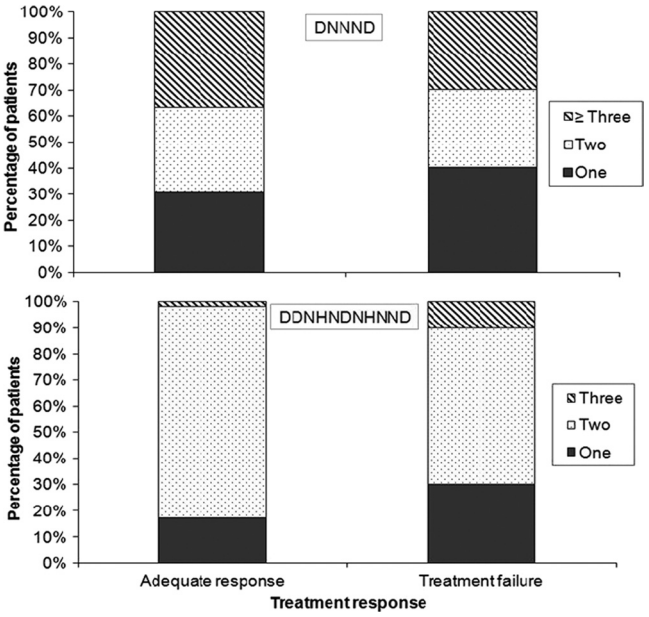

FIG. 3. Percentage of isolates from patients treated with quinine with the indicated numbers of *pfnhe1* ms4760 DNNND and DDNHN DNHNND repeats.

Thus, in older studies the IC_{50}s for isolates collected in Senegal were 31 to 765 nM (7), and those for isolates collected in Cameroon were 23 to 780 nM (8). Many other studies did not report full ranges of sensitivity, but wide variations have commonly been seen. In the samples from Uganda utilized in this study, quinine IC_{50}s ranged from 15 to 761 nM (29). Methodologies for *in vitro* assessments have varied, and associations between *in vitro* values and clinical outcomes are uncertain. Nonetheless, the wide range in quinine sensitivity seen across Africa and recent evidence for quinine treatment failure in Africa suggest that the evolution of parasites with decreased drug sensitivity may be contributing to diminished clinical responses to the drug.

The mediators of decreased quinine sensitivity of *P. falciparum* are uncertain. The well-described K76T polymorphism in the gene encoding the putative transporter *pfcrt*, which is the principal mediator of resistance to chloroquine (13, 49), also appears to play a role in mediating quinine sensitivity, on the basis of the results of laboratory studies (49, 58). The *pfcrt* 76T mutation was nearly universal in Ugandan isolates, so associations between this polymorphism and *in vitro* or clinical outcomes could not be studied. Polymorphisms in the gene encoding the multidrug resistance homolog *pfmdr1* also play roles in resistance to multiple drugs (52, 58). Amplification of *pfmdr1* decreases sensitivity to quinine (39, 58). The single nucleotide polymorphisms in *pfmdr1* that are common in Africa, N86Y, Y184F, and D1246Y, have had less marked impacts on quinine sensitivity in laboratory studies (44, 48). In evaluations of clinical isolates, associations between *pfmdr1* polymorphisms and *in vitro* quinine sensitivity have been uncertain, but evaluations considering the common polymorphisms seen in Africa have been limited (6). We recently evaluated *pfmdr1* polymorphisms in the most and least sensitive of 196 isolates for which quinine sensitivity was measured in Uganda. The most resistant parasites were significantly more likely to harbor the *pfmdr1* 1246Y mutation, but not 86Y or 184F (29). Considering overall results, parasites with any of the three *pfmdr1* polymorphisms seen in our isolates were somewhat more resistant to quinine than those without the polymorphism (Table 1), but the differences were not statistically significant. Considering the mutations together, an increasing number of mutations was associated with progressively greater quinine IC_{50}s (Table 2).

Recently, quantitative trait locus analysis identified variations in the *pfnhe1* gene, in addition to *pfcrt* and *pfmdr1*, as potentially mediating quinine sensitivity (18). Specifically, varied numbers of repeats in the ms4760 microsatellite were associated with quinine sensitivity. Following up on this report, four groups evaluated associations between ms4760 repeat numbers and *in vitro* quinine sensitivity. In 23 culture-adapted strains from multiple regions (20) and in 60 fresh isolates from the China-Myanmar border (25), increasing numbers of the DNNND repeat and decreasing numbers of the DDNHNDN HNND repeat were associated with decreased quinine sensitivity. In contrast, in 83 freshly isolated strains from Africa, a significant association was not seen between quinine sensitivity and the number of DNNND repeats, and an increased number of DDNHNDNHNND repeats was associated with decreased quinine sensitivity (5). Lastly, in 29 fresh isolates from Kenya, two DNNND repeats were associated with decreased sensitivity to quinine compared to the sensitivity of parasites with one or three copies, and there was no association between the number of DDNHNDNHNND repeats and quinine sensitivity (30). Our study adds the analysis of a larger number of isolates from Africa. We found only a modest trend toward decreased quinine sensitivity in isolates with two copies of either repeat. We also offer the first consideration of *pfnhe1* polymorphisms and clinical outcomes. The number of either repeat was not associated with response to quinine therapy. Our results add to uncertainty regarding the importance of *pfnhe1* polymorphisms in mediating quinine sensitivity and treatment response. More broadly, our finding of only modest associations between *pfmdr1* and *pfnhe1* polymorphisms and quinine sensitivity and treatment response are consistent with the con-

clusion that quinine sensitivity is a complex trait, with polymorphisms in multiple genes probably contributing to the phenotype (18, 28).

Simple molecular markers for resistance of malaria parasites to available drugs are valuable, as they allow the ready characterization of the sensitivity of parasites without the complex infrastructure required for clinical trials or *in vitro* analysis of parasites (36). Such data can help to guide treatment policy. For quinine, our results and prior studies suggest contributions of polymorphisms in multiple genes to drug sensitivity, but no single mutation or set of polymorphisms has consistently been a robust marker for *in vitro* quinine sensitivity. Consistent with this result, we saw, at best, only modest associations between previously identified polymorphisms and *in vitro* quinine sensitivity or quinine treatment outcomes. Thus, at present, straightforward markers for the responsiveness of *P. falciparum* to quinine are not available. Our analysis was limited to known markers of interest, and it is likely that additional polymorphisms contribute to quinine sensitivity. Further studies, including whole-genome sequencing strategies, may be needed to better characterize the molecular basis of quinine sensitivity and allow a better appreciation of the extent to which alterations in quinine sensitivity are limiting the antimalarial efficacy of this important drug.

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