Polymorphisms in Pfmdr1, Pfcrtr, and PfNhe1 Genes Are Associated with Reduced In Vitro Activities of Quinine in Plasmodium falciparum Isolates from Western Kenya

Jelagat Cheruiyot,a,b Luicer A. Ingasia,a Angela A. Omondi,a,b Dennis W. Juma,a Benjamin H. Opot,a Joseph M. Ndegwa,a,b Joan Mativo,a Agnes C. Cheruiyot,a,b Redemptah Yeda,a Charles Okudo,a Peninah Muiruri,a Ngalah S. Bidii,a Lorna J. Chebon,a Paul O. Angienda,b Fredrick L. Eyase,a Jacob D. Johnson,a Wallace D. Bullimo,a Ben Andagalu,a Hoseah M. Akala,a Edwin Kamua

Global Emerging Infections Surveillance Program, United States Army Medical Research Unit—Kenya, Kenya Medical Research Institute—Walter Reed Project, Kisumu and Nairobi, Kenya; School of Biological/Physical Sciences, Department of Zoology, Maseno University, Maseno, Kenya

In combination with antibiotics, quinine is recommended as the second-line treatment for uncomplicated malaria, an alternative first-line treatment for severe malaria, and for treatment of malaria in the first trimester of pregnancy. Quinine has been shown to have frequent clinical failures, and yet the mechanisms of action and resistance have not been fully elucidated. However, resistance is linked to polymorphisms in multiple genes, including multidrug resistance 1 gene (Pfmdr1), the chloroquine resistance transporter (Pfcrt), and the sodium/hydrogen exchanger gene (Pfnhe1). Here, we investigated the association between in vitro quinine susceptibility and genetic polymorphisms in Pfmdr1 codons 86 and 184, Pfcrt codon 76, and Pfnhe1 ms4760 in 88 field isolates from western Kenya. In vitro activity was assessed based on the drug concentration that inhibited 50% of parasite growth (the IC50), and parasite genetic polymorphisms were determined from DNA sequencing. Data revealed there were significant associations between polymorphism in Pfmdr1-86Y, Pfmdr1-184F, or Pfcrt-76T and quinine susceptibility (P < 0.0001 for all three associations). Eighty-two percent of parasites resistant to quinine carried mutant alleles at these codons (Pfmdr1-86Y, Pfmdr1-184F, and Pfcrt-76T), whereas 74% of parasites susceptible to quinine carried the wild-type allele (Pfmdr1-N86, Pfmdr1-Y184, and Pfcrt-K76), respectively. In addition, quinine IC50 values for parasites with Pfnhe1 ms4760 3 DNNND repeats were significantly higher than for those with 1 or 2 repeats (P = 0.033 and P = 0.0043, respectively). Clinical efficacy studies are now required to confirm the validity of these markers and the importance of parasite genetic background.

Quinine (QN), a quinoline derivative, is used in many African countries as second-line treatment for uncomplicated malaria, as an alternate first-line treatment for severe malaria, and for treatment of malaria in the first trimester of pregnancy (1). World Health Organization (WHO) guidelines recommend a combination of QN plus doxycycline, tetracycline, or clindamycin for treatment of malaria (2). However, in most African countries, QN is used as a monotherapy (1, 2), probably due to the high cost of antibiotics (3). QN has been shown to have frequent clinical failures in Southeast Asia (4, 5), South America (5), and Africa (6–8). In a clinical trial conducted in Kenya concerning the treatment of severe Plasmodium falciparum malaria, QN was shown to have longer clearance times, longer fever clearance times, and higher recrudescence rates than malarone (9). The high rates of QN clinical failures can be explained by variations in pharmacokinetics, drug quality, and treatment compliance, but less so to resistance. Parasites with high-grade resistance to QN have not been documented (3). However, in vitro analysis has shown reduced sensitivity to QN in Asia (10) and South America (11), and much less so in Africa (12, 13). The high rates of reinfection and recrudescence can also be explained by the short half-life of QN (14).

Although the mechanisms of action and resistance to QN have not been fully elucidated, inhibition of heme detoxification in the parasite digestive vacuole has been implicated in QN antimalarial activity (15), and resistance is linked to polymorphisms in multiple genes, including the multidrug resistance 1 gene (Pfmdr1), chloroquine resistance transporter gene (Pfcrt), and the sodium/hydrogen exchanger gene (Pfnhe1) (16). In particular, mutations in Pfmdr1 at codons 86, 184, 1042, and 1246 and in Pfcrt at codon 76 have been associated with reduced QN sensitivity (17–19). Interestingly, however, in some studies polymorphism in Pfcrt-76 has been shown not to have any effect on QN activity (20, 21), and the Pfmdr1-86 mutation only resulted in a decreased QN activity that did not reach statistical significance (20). Other novel mutations in the Pfcrtr gene that have recently been shown to alter QN sensitivity are Q352K/R and C350R (20, 22, 23).

Pfnhe1 is a 5,760-bp gene that encodes 1,920 amino acids, with a microsatellite polymorphism consisting of variable DNNND repeat units, designated ms4760. Sequence polymorphisms in the Pfnhe1 gene have been analyzed in laboratory strains and field isolates with varied susceptibilities to QN (16, 24–27). Several variants of ms4760 have been described in which ms4760–1, with 2 copies of the DNNND repeat unit, was significantly associated with reduced in vitro QN sensitivity in laboratory clones (16) and in field isolates (24). On the contrary, other studies have not found an association between polymorphisms in the Pfnhe1 gene and

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Address correspondence to Edwin Kamau, Edwin.Kamau@us.army.mil.
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QN reduced susceptibility (26, 27). These conflicting results may indicate that the influence of PfNhe1 on QN susceptibility is strain dependent and can vary depending on the geographic origin of the parasites (28). Polymorphisms in the PfNhe1 ms4760 DDNND NHHND and DDDNNNDHNND repeats have also been associated with varied in vitro QN susceptibilities (24–27, 29, 30).

Similar to most countries in Africa, QN is used as second-line treatment for uncomplicated malaria and treatment of severe malaria in Kenya (9, 12). A recent study analyzed the association of in vitro activities of QN with Pfmdr1 codon 86, Pfctrt codon 76, and PfNhe1 polymorphism in isolates collected from Kilifi District, which is located on the Kenyan coast (24). These authors observed Pfmdr1-86 mutants, and 2 copies of DNNND repeat units of PfNhe1 were associated with a decrease in QN susceptibility. In this study, we investigated the association between in vitro QN susceptibility and genetic polymorphisms in Pfmdr1 codons 86 and 184, Pfctrt codon 76, and PfNhe1 ms4760 in field isolates from western Kenya, where malaria is holoendemic.

**MATERIALS AND METHODS**

*Plasmodium falciparum* parasites. *P. falciparum* field isolates used in this study were collected from patients with uncomplicated malaria, ages 6 months and older, attending outpatient clinics in Kisumu, Kisii, and Kericho District hospitals in western Kenya between January 2010 and December 2011. Details of this protocol have been described elsewhere (31). The research protocol was approved by the Ethical Review Committee of the Kenya Medical Research Institute (KEMRI number 1330) and the Walter Reed Army Institute of Research institutional review board of the Kenya Medical Research Institute (KEMRI number 1330) and W2 (resistant) were used as controls. These clones were obtained by the Walter Reed Army Institute of Research (WRAIR), Silver Spring, MD. Parasites were prepared in 5 ml of 70% ethanol to attain 5 mg/ml, which was lowered to concentrations ranging from 2,554 nM to 5 nM and predosed on 96-well tissue culture plates (Applied Biosystems, Foster City, CA), as directed by the manufacturer on a 3500XL genetic analyzer. Sequences were analyzed with the ABI Prism BigDye Terminator v3.1 cycle sequencing ready reaction kits (Applied Biosystems, Foster City, CA), as directed by the manufacturer on a 3500XL genetic analyzer. Sequences were analyzed with the BioEdit sequence alignment editor (version 7.0.9.0). Sequences were analyzed for the number of DNNND, DDDNNNDHNND, and DDDNNNDHNND repeats in the PfNhe1 ms4760 microsatellite.

**RESULTS**

**QN chemosensitivity.** The QN IC50 values for the two reference strains, 3D7 and W2, were established and used as internal controls in subsequent experiments. The median IC50 values (nM; interquartile ranges and *n* values in parentheses) were 27 (17–32; *n* = 4) for 3D7 and 362 (111–400; *n* = 4) for W2. A total of 88 culture-adapted field isolates were successfully analyzed, and QN IC50s were determined. The median IC50 (interquartile range) for the 88 isolates was 69.01 nM (19.05–336.0).

**Pfmdr1 and Pfctrt mutations.** Genotype analyses revealed 67% and 52% of the isolates carried the wild-type genotype at codons N86 and Y184 of the Pfmdr1 gene, respectively, whereas 35% of the isolates carried the wild-type genotype at codon K76 of the Pfctrt gene. Notably, all the isolates had a single copy of the Pfmdr1 gene.

**Associations of QN in vitro sensitivity with Pfmdr1 and Pfctrt polymorphisms.** Figure 1 shows profiles for in vitro QN sensitivity per genetic polymorphism in the Pfmdr1 and Pfctrt genes. The isolates revealed a significant association between the QN IC50 and mutations in Pfmdr1 codons 86 and 184 and Pfctrt codon 76 (*P* < 0.0001 for each association). The median IC50 for parasites for Pfmdr1 codons 86 and 184 were ~15-fold higher in the mutant genotypes compared to the wild-type, whereas with Pfctrt codon

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76, the median IC_{50} was ~9-fold higher for the mutant genotype than for the wild type.

**Pfhr1 ms4760 polymorphism.** The genetic polymorphisms of ms4760 in Pfhr1 gene was analyzed. Information on the QN IC_{50} values and the genetic profiles is summarized in Table S1 of the supplemental material. These isolates contained 15 different genetic polymorphisms of ms4760 in the Pfhr1 gene. Five of the genetic profiles have not been previously described. The new profiles were deposited with GenBank and were assigned accession numbers KF719182, KF719183, KF719184, KF719185, and KF719186. Previously described profiles that were present in our isolates included the following: ms4760-1, ms4760-2, ms4760-3, ms4760-4, ms4760-6, ms4760-8, ms4760-9, ms4760-16, and ms4760-53. The most common genetic polymorphisms were ms4760-16, KF719184, and KF719186, each of which was present in only one isolate (Fig. 2). Figure 3 shows alignments of the 15 sequences of Pfhr1 ms4760 identified in 88 western Kenya *P. falciparum* isolates.

**Association between QN sensitivity and the number of DNNND repeats in Pfhr1.** There was a significant association between the QN IC_{50} and the number of DNNND repeats (Fig. 4). The QN IC_{50} for parasites with 3 DNNND repeats was significantly higher than for those with 1 or 2 repeats (*P = 0.033 and *P = 0.0043, respectively). There was no statistical difference in QN IC_{50} between parasites with 1 or 2 repeats. Interestingly, there was no association between QN IC_{50} and DDN HNDNHNNDN repeats. The median IC_{50} (interquartile range; n) for parasites with 1 DDNHNNDHNNDN repeat was 80.72 nM (18.71–589.5; n = 28), for parasites with 2 repeats it was 57.79 nM (18.19–253.4; n = 59), and for those with 3 repeats it was 948.7 nM (118.4–1,779; n = 2).

**Associations of QN sensitivity and genotype combination for Pfmdr1, Pfcrt, and Pfhr1.** Our data showed a significant association of genetic polymorphisms in the Pfmdr1 gene at codons 86 and 184, the Pfcrt gene at codon 76, and the Pfhr1 gene with DNNND repeats. To further understand this observation, we analyzed the relationship between the QN IC_{50} and the genotype combination of DNNND repeat number for the Pfmdr1 and Pfcrt genes. In Pfmdr1 codons 86Y (Fig. 5A) and 184F (Fig. 5B), data revealed parasites with 3 DNNND repeats had higher median IC_{50}s than those with 1 or 2 repeats. For codon 86Y, there was a statistically significant difference between parasites with 3 DNNND repeats versus 1 or 2 repeats (*P = 0.0302). For codon 184F, there was a statistically significant difference between parasites with 3 DNNND repeats versus 1 or 2 repeats (*P = 0.0148 and *P = 0.0031, respectively). For Pfcrt codon 76T (Fig. 5C), data revealed parasites with 3 DNNND repeats had median IC_{50}s more than 5-fold higher than in parasites with 1 or 2 DNNND repeats, and there was a statistically significant difference between those with 3 DNNND repeats versus 1 or 2 repeats (*P = 0.0019 and *P = 0.0041, respectively).

**Genetic profiles of parasites with a QN IC_{50} outside the interquartile range.** To further investigate the role of genetic polymorphisms in the Pfmdr1, Pfcrt, and Pfhr1 genes in determining parasite phenotypic characteristics in response to QN sensitivity, the genetic profiles of parasites with QN IC_{50}s below the lower interquartile range or above the upper interquartile range were analyzed. There were 23 parasites with a QN IC_{50} of ≤19.05 and 22 parasites with a QN IC_{50} of ≥336. Table S2 in the supplemental material shows the genetic profiles of parasites with QN IC_{50}s below and above the interquartile range. For the parasites with values below the lower interquartile range, the predominant genotype present in 74% of the parasites was Pfmdr1-N86Y Pfmdr1-Y184F Pfcrt-K76 (all codons carrying the wild-type genotype), whereas above the upper interquartile range, the predominant genotype present in 82% of the parasites was Pfmdr1-86Y Pfmdr1-184F Pfcrt-76T (all codons carrying mutant genotypes). Two genotype profiles, Pfmdr1-N86Y Pfmdr1-Y184F Pfcrt-76T and Pfmdr1-N86Y Pfmdr1-184F Pfcrt-76T were present in parasites both in the lower and above the interquartile ranges. In the parasites with values below the lower interquartile range, 52% of the parasites had 2 DNNND repeats, whereas for parasites above the upper interquartile range, 55% had 3 DNNND repeats.

![Figure 1](image1.png) **FIG 1** In vitro QN sensitivity for polymorphisms at each genetic marker in Pfmdr1-86, Pfmdr1-184, and Pfcrt-76 codons. The analysis comparing alleles at each codon was done using the Mann-Whitney test. The horizontal (blue) bars indicate medians. The numbers of isolates analyzed are shown in brackets. There was a significant association between the QN IC_{50} and mutation at each codon.  

![Figure 2](image2.png) **FIG 2** Prevalence rates of Pfhr1 ms4760 profiles among the 88 western Kenya isolates. Profiles ms-1 to ms-53 have been described previously, while the sequences for KF719182 to KF719186 were first described in this study.
DISCUSSION

In this study, we have clearly shown a significant association between polymorphisms in Pfmdr1 codons 86 and 184 and Pfcrtn76 and QN susceptibility in P. falciparum parasites from western Kenya. Furthermore, the diversity of microsatellite repeats in PfNhe1 ms4760 within these isolates was underscored and also shown to be linked to QN susceptibility. Three repeats of the DNNND polymorphism in the PfNhe1 gene significantly reduced parasite susceptibility to QN. Most importantly, we have described a predominant parasite genotype, Pfmdr1-86Y Pfmdr1-184F Pfcrtn76T, in parasites with high QN IC50s.

The median IC50 (interquartile range) for isolates from our study was 69.01 nM (19.05–336.0). The chemosensitivity threshold for QN has not been clearly defined. However, the historical WHO IC50 against the W2 clone of P. falciparum, which is considered resistant, is 315 nM (39). Other different QN threshold cut-offs have been proposed: 300, 500, and 800 nM (40–42). In studies conducted in Senegal (43) and Kenya (24), only 1% and 7% (respectively) of the isolates tested against QN had IC50 > 500 nM. In the study conducted in Kilifi, Kenya, none of the isolates had an IC50 of >800 nM (24). In field isolates from the Republic of the Congo, 25.7% exceeded the 500 nM cutoff, whereas only 5.4% exceeded the 800 nM cutoff (29). In our study, 18.2% (16/88) of the isolates had IC50 > 800 nM. Although 70.4% (62/88) of the isolates had IC50 estimates below 300 nM, and were therefore considered sensitive to QN, a relatively high number of samples, compared to those collected elsewhere, can be considered resistant to QN, with a threshold cutoff exceeding 800 nM. The in vitro data from our study were supported by the genotypic data. This was expected, since in western Kenya, the prevalence of the Pfmdr1-86Y Pfmdr1-184F Pfcrtn76T genotype, which is also responsible for chloroquine resistance, has remained persistently high (31).

In a study that analyzed parasite isolates from Kilifi, Kenya, parasites carrying the Pfmdr1-86 mutation showed a trend toward decreased QN activity, but there was no significant association (24). Similarly, in a study that used field isolates from Uganda, polymorphisms in Pfmdr1-86 and Pfmdr1-184 were implicated in decreased sensitivity to QN but did not reach significance (P = 0.22 and P = 0.34, respectively) (44). However, the Pfmdr1-1246
mutation was shown to be statistically significant for modulating QN activity ($P = 0.029$). Transfection studies have also shown that Pfmdr1-1034, Pfmdr1-1042, and Pfmdr1-1246 mutations modulate resistance to QN (17). Here, we have shown statistically significant associations of mutations in Pfmdr1 codons 86 and 184 with QN activity. The difference in these observations could be due to the genetic backgrounds of the parasites. QN resistance appears to be dependent on multiple genes, which indicates polymorphisms in Pfmdr1 contribute to the overall phenotype of the parasite but in the backdrop of the genetic background of the parasite (17). It will be important to go back and analyze Pfmdr1 codon 1246 in isolates from our study, as this mutation has been shown to be important in modulating QN activity both in transfection and among in vitro data of field isolates (16, 44).

Transfection studies have shown that K76T contributes to QN resistance, but the extent of its contribution differs between strains (16, 45). Similarly, our data clearly showed a significant association of K76T mutation with QN reduced sensitivity. This is contrary to what other studies have shown (24). However, similarly to the Pfmdr1 gene, the genetic background seems to be important and must therefore ultimately determine parasite phenotype.

Studies of the association of polymorphisms in the Pfne1 ms4670 microsatellite with in vitro susceptibility to QN have had conflicting results (24, 27, 29, 46). In studies that showed a positive correlation, the number of repeats in block 2 (DDNND), block 5 (DDNHNDNHNNDD), and block 6 (DDNNNDNHND) have been associated with modulation of QN resistance (27, 46). In this study, we showed there was a significant association for parasites with 3 repeats of DNNND and QN susceptibility. Polymorphisms in DDNHNDNHNNDD or DDNNNDNHNDD repeats did not have an effect on QN susceptibility. Some studies have shown that an increase in DNNND repeats is associated with reduced susceptibility to QN (16, 46). Interestingly, the study that analyzed isolates from Kilifi, Kenya, showed 2 DNNND repeats, and not 3 DNNND repeats, were associated with reduced QN susceptibility (24). Further, that study showed that 3 DNNND repeats restored QN activity, contrary to our findings. Other studies did not find any association for polymorphisms in DNNND repeats with QN activity when evaluating isolates from African patient populations (27, 29). Interestingly, however, in one of the studies, there was a positive association for NHNDNHNNDDDD repeats with increased QN IC$_{50}$ ($P = 0.01$) among isolates from an African population (27). This is in line with findings from

FIG 5 Relationship between the QN IC$_{50}$ and the genotype combination of DNNND repeats for Pfmdr1-86 (A), Pfmdr1-184 (B), and Pfcr-76 (C). The analysis comparing the number of DNNND repeats with each allele was done using the Mann-Whitney test. The horizontal bars (blue) indicate the medians. The numbers of isolates analyzed are shown in brackets. With one exception, 3 DNNND repeats had statistically significantly higher QN IC$_{50}$ values than did isolates with 1 or 2 repeats for all mutant alleles analyzed. The association of Pfmdr1-86Y with 1 versus 3 DNNND repeats was not statistically significant ($P = 0.21$).
these genetic markers are only relevant in the context of the ge-
phisms with parasite responses to QN. Further, we showed that
strongly suggested polymorphisms in Pf
isolates.
QN can also be explained by differences in parasite genetic back-
in the Pf
posing isolates with IC50 estimates above the WHO cutoff for resis-
amples with results above the upper interquartile range, compris-
in each one of the genotypes. When we analyzed the genotypes of
samples with results above the upper interquartile range, compris-
ing isolates with IC50 estimates above the WHO cutoff for resis-
tance, the majority of these isolates (82%) carried mutant alleles at
the three genetic markers. When analyzed in combination with
DNND repeats, 55% of these isolates carried 3 repeats, as op-
posed to 32% with 2 repeats, and the remainder with 1 repeat. On
the contrary, the majority of the isolates (74%) with values below
the lower interquartile range had wild-type genotypes for all three
codons analyzed, with most (52%) carrying 2 DNND repeats.

Conclusions. In this study, we showed the associations of
Pmdr1-86, Pmdr1-184, Pfcrt-76, and Pinhel ms4670 polymor-
phisms with parasite responses to QN. Further, we showed that
these genetic markers are only relevant in the context of the ge-
etic background of the isolates. Andriantsioarinina et al. (27) strongly suggested polymorphisms in Pinhel might not be valid
molecular markers for in vitro susceptibility to QN in P. falcipar-
um isolates from Africa. Those authors tested only 83 isolates
from a few countries in Africa. Africa is a continent with extremely
diverse geographical landscapes, different malaria ecologies, var-
ied rates of malaria endemicity, and parasites with different ge-
etic structures. The cumulative evidence is clearly showing that
the genetic background of the parasite is critical in determining
QN activity. This will have important implications, because para-
sites from each geographic region must therefore be analyzed to
determine which markers confer reduced QN susceptibility. To
further validate our observations and conclusions, it will be im-
portant that isolates from different malaria ecological zones and
regions of malaria endemicity in Kenya are analyzed. Most impor-
tantly, more clinical efficacy studies must be conducted in Kenya
and other African countries, given the importance of QN in the
management of malaria.

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We declare that there are no competing interests.

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