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Polymorphisms in Pf*mdr1*, Pf*crt*, and Pf*nhe1* Genes Are Associated with Reduced *In Vitro* Activities of Quinine in *Plasmodium falciparum* Isolates from Western Kenya

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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 (Pf*mdr1*), the chloroquine resistance transporter (Pf*crt*), and the sodium/hydrogen exchanger gene (Pf*nhe1*). Here, we investigated the association between *in vitro* quinine susceptibility and genetic polymorphisms in Pf*mdr1*codons 86 and 184, Pf*crt* codon 76, and Pf*nhe1* 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 IC_{50}), and parasite genetic polymorphisms were determined from DNA sequencing. Data revealed there were significant associations between polymorphism in Pf*mdr1*-86Y, Pf*mdr1*-184F, or Pf*crt*-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 (Pf*mdr1*-86Y, Pf*mdr1*-184F, and Pf*crt*-76T), whereas 74% of parasites susceptible to quinine carried the wild-type allele (Pf*mdr1*-N86, Pf*mdr1*-Y184, and Pf*crt*-K76, respectively). In addition, quinine IC_{50} values for parasites with Pf*nhe1* 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 of 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 (Pf*mdr1*), chloroquine resistance transporter gene (Pf*crt*), and the sodium/hydrogen exchanger gene (Pf*nhe1*) (16). In particular, mutations in Pf*mdr1* at codons 86, 184, 1042, and 1246 and in Pf*crt* at codon 76 have been associated with reduced QN sensitivity (17–19). Interestingly, however, in some studies polymorphism in Pf*crt*-76 has been shown not to have any effect on QN activity (20, 21), and the Pf*mdr1*-86 mutation only resulted in a decreased QN activity that did not reach statistical significance (20). Other novel mutations in the Pf*crt* gene that have recently been shown to alter QN sensitivity are Q352K/R and C350R (20, 22, 23).

Pf*nhe1* 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 Pf*nhe1* 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 Pf*nhe1* gene and

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QN reduced susceptibility (26, 27). These conflicting results may indicate that the influence of Pf*nhe1* on QN susceptibility is strain dependent and can vary depending on the geographic origin of the parasites (28). Polymorphisms in the Pf*nhe1* ms4760 DDNHND NHNND and DDNNNDNHNDD 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 Pf*mdr1* codon 86, Pf*crt* codon 76, and Pf*nhe1* polymorphism in isolates collected from Kilifi District, which is located on the Kenyan coast (24). These authors observed Pf*mdr1*-86 mutants, and 2 copies of DNNND repeat units of Pf*nhe1*were associated with a decrease in QN susceptibility. In this study, we investigated the association between *in vitro* QN susceptibility and genetic polymorphisms in Pf*mdr1*codons 86 and 184, Pf*crt* codon 76, and Pf*nhe1* 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 (WRAIR number 1384). *P. falciparum* reference strains3D7 (sensitive) and W2 (resistant) were used as controls. These clones were obtained from cryopreserved stocks and culture adapted for drug sensitivity assays.

***In vitro* drug sensitivity tests.** QN was obtained from the Walter Reed Army Institute of Research (WRAIR), Silver Spring, MD. Parasites were maintained in continuous culture by using a method described elsewhere (32). A previously described SYBR green I-based drug sensitivity assay was used for *in vitro* drug sensitivity testing (33–35). *P. falciparum* parasites in continuous culture attaining 3 to 8% parasitemia (field isolates or reference strains) were adjusted to 2% hematocrit and 1% parasitemia. QN was prepared in 5 ml of 70% ethanol to attain 5 mg/ml, which was lowered to 2,554 nM in tissue culture medium. QN was then diluted across 10 concentrations ranging from 2,554 nM to 5 nM and predosed on 96-well microtiter plates as previously described (36). The assay was initiated by addition of 100 μl reconstituted parasite components to each well of the drug plates and incubation at 37°C as previously described (35). The assay was terminated after 72 h, 100 μl of lysis buffer containing SYBR green I (1×, final concentration) was added directly to the plates, and the mixtures were kept at room temperature in the dark for 24 h. Parasite replication inhibition was quantified by measuring the per well relative fluorescence units (RFU) of SYBR green 1 dye, using the Tecan Genios Plus system with excitation and emission wavelengths of 485 nm and 535 nm. The 50% inhibition concentration (IC_{50}) values for QN were calculated as described previously (31).

DNA extraction. Total genomic DNA of each *P. falciparum* isolate was extracted from filter paper blots (FTA; Whatman Inc., Bound Brook, NJ) by using a QIAamp DNA blood minikit (Qiagen, Valencia, CA), according to the manufacturer's instructions.

Genotypic characterization of Pf*mdr1* and Pf*crt* genes. The $2^{-\Delta\Delta CT}$ method (based on the threshold cycle [C_T]) of relative quantification was used to estimate copy number variation in Pf*mdr1*, as previously published (37). Polymorphisms in Pf*mdr1* codons 86 and 184 and Pf*crt* codon 76 were determined by nested PCR and sequencing as previously described (31, 38).

Pf*nhe1* microsatellite genotyping. Microsatellite repeats in Pf*nhe1* were characterized by nested PCR using 1.5 μl of each primer. Primary run primers were NHE-F (5′-AGTCGAAGGCGAATCAGATG-3′) and NHE-R (5′-GATACTTACGAACATGTTCATG-3′). Secondary run primers NHE-F (5′-ATCCCTGTTGATATATCGAATG-3′) and NHE-R (5′-TTGTCATTAGTACCCTTAGTTG-3′), as previously described (24). The 25-μl reaction volume setup consisted of 10× PCR buffer to a final concentration of 1×, 25 mM $MgCl_2$ to a final concentration of 1.5 mM, 20 mM deoxynucleoside triphosphates mix to a final concentration of 2 mM, 10 μM each of the primers to a final concentration of 100 nM, 5 U/μl AmpliTaq DNA polymerase to a final concentration of 1 U/reaction tube. Amplification of the ms4760 region was done under the following amplification conditions for the first run: initial hybridization at 94°C for 5 min, a subsequent 35 cycles of denaturation at 94°C for 30 s, annealing at 57.0°C for 30 s, and extension at 72°C for 30 s, and a final extension at 72°C for 7 min. Secondary run conditions were the following: initial hybridization at 94°C for 5 min, a subsequent 35 cycles of denaturation at 94°C for 30 s, annealing at 59.0°C for 30 s, and extension at 72°C for 30 s, and a final extension at 72°C for 7 min. The PCR products were resolved by electrophoresis on a 2% agarose gel, stained with 0.5 μg/ml ethidium bromide, and visualized under UV light. Amplicons were purified and sequenced using 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, DDNHNDNHNND, and DDNNND NHNDD repeats in the Pf*nhe1* ms4760 microsatellite.

Statistical analysis. Continuous data in the form of IC_{50} estimates were expressed as medians with interquartile ranges. Further, IC_{50} data for isolates with various genetic polymorphism in Pf*mdr1*, Pf*crt*, and Pf*nhe1* were compared by using nonparametric tests (one-way analysis of variance, Kruskal-Wallis test with Dunn's multiple comparison posttest, and the Mann-Whitney test). Associations between IC_{50} estimates and genetic markers were considered significant when P was <0.05. Analyses were conducted using the Prism program (version 5.0.2; GraphPad Software, Inc., San Diego, CA). For analyses, all isolates with mixed genotypes were considered mutants.

Nucleotide sequence accession numbers. New Pf*nhe1* ms4760 microsatellite profiles were deposited in GenBank under the following accession numbers: KF719182, KF719183, KF719184, KF719185, and KF719186.

RESULTS

QN chemosensitivity. The QN IC_{50} values for the two reference strains, 3D7 and W2, were established and used as internal controls in subsequent experiments. The median IC_{50} values (in 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 IC_{50}s were determined. The median IC_{50} (interquartile range) for the 88 isolates was 69.01 nM (19.05–336.0).

Pf*mdr1* and Pf*crt* mutations. Genotype analyses revealed 67% and 52% of the isolates carried the wild-type genotype at codons N86 and Y184 of the Pf*mdr1* gene, respectively, whereas 35% of the isolates carried the wild-type genotype at codon K76 of the Pf*crt* gene. Notably, all the isolates had a single copy of the Pf*mdr1* gene.

Associations of QN *in vitro* sensitivity with Pf*mdr1* and Pf*crt* polymorphisms. Figure 1 shows profiles for *in vitro* QN sensitivity per genetic polymorphism in the Pf*mdr1* and Pf*crt* genes. The isolates revealed a significant association between the QN IC_{50} and mutations in Pf*mdr1* codons 86 and 184 and Pf*crt* codon 76 ($P < 0.0001$ for each association). The median IC_{50} for parasites for Pf*mdr1* codons 86 and 184 were ~15-fold higher in the mutant genotypes compared to the wild-type, whereas with Pf*crt* codon

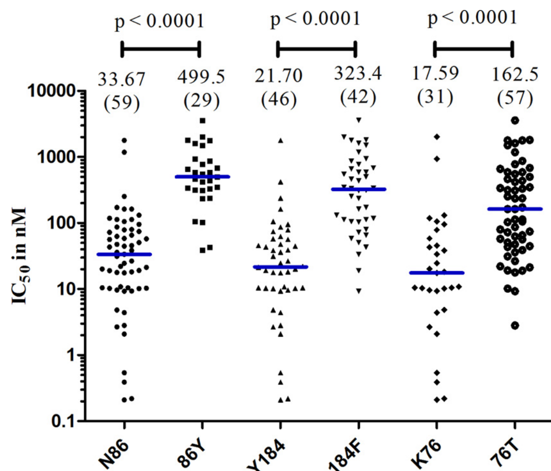

FIG 1 *In vitro* QN sensitivity for polymorphisms at each genetic marker in Pf*mdr1*-86, Pf*mdr1*-184, and Pf*crt*-76 codons. The analysis comparing alleles at each codon was done using the Mann-Whitney test. The horizontal (blue) bars indicate medians. The numbers of isolates analyzed are shown in brackets. There was a significant association between the QN IC_{50} and mutation at each codon.

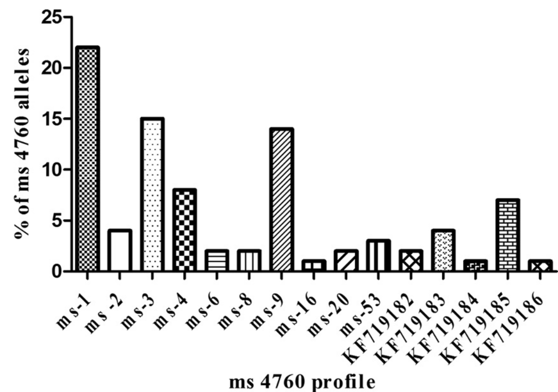

FIG 2 Prevalence rates of Pf*nhe1* ms4760 profiles among the 88 western Kenya isolates. Profiles ms-1 to ms-53 have been described previously, while the sequences for KF719182 to KF719186 were first described in this study.

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

Pf*nhe1* ms4760 polymorphism. The genetic polymorphisms of ms4760 in Pf*nhe1* 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 Pf*nhe1* 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 the following: ms4760-1, ms4760-3, and ms4760-9, in 22, 16, and 12 of the isolates analyzed, respectively. Combined, they represented 60% of the total isolates analyzed. The least 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 Pf*nhe1* ms4760 identified in 88 western Kenya *P. falciparum* isolates.

Association between QN sensitivity and the number of DNNND repeats in Pf*nhe1*. 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}s between parasites with 1 or 2 repeats. Interestingly, there was no association between QN IC_{50} and DDN HNDNHNND repeats. The median IC_{50} (interquartile range; *n*) for parasites with 1 DDNHNDNHNND 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 Pf*mdr1*, Pf*crt*, and Pf*nhe1*. Our data showed a significant association of genetic polymorphisms in the Pf*mdr1* gene at codons 86 and 184, the Pf*crt* gene at codon 76, and the Pf*nhe1* 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 Pf*mdr1* and Pf*crt* genes. In Pf*mdr1* 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 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 Pf*crt* 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 Pf*mdr1*, Pf*crt*, and Pf*nhe1* 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 ranges. For the parasites with values below the lower interquartile range, the predominant genotype present in 74% of the parasites was Pf*mdr1*-N86 Pf*mdr1*-Y184 Pf*crt*-K76 (all codons carrying the wild-type genotype), whereas above the upper interquartile range, the predominant genotype present in 82% of the parasites was Pf*mdr1*-86Y Pf*mdr1*-184F Pf*crt*-76T (all codons carrying mutant genotypes). Two genotype profiles, Pf*mdr1*-N86 Pf*mdr1*-Y184 Pf*crt*-76T and Pf*mdr1*-N86 Pf*mdr1*-184F Pf*crt*-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.

Profile No		I	II	III	IV	V	VI	
MS_4760-1 :	KKKKISGSNN-	DNN-	DNNNDDNNND------	NHND-	DKNNKND-------------------	DDNHNDNHNNDDDNHNDNHNND------------	DDNNNDNHNDD-------	DNNSSHY
MS_4760-2 :	KKKKIRGSNN-	DNN-	DNNND------------	------	DKNNKND-DDNHNDNH-KNDKNNKKD-	DDNHNDNHNND------------------------	DDNNNDNHNDD-------	DNNSSHY
MS_4760-3 :	KKKKISGSNN-	DNN-	DNNND------------	------	DKNNKND-------------------	DDNHNDNHNNDDDNHNDNHNND------------	DDNNNDNHNDD-------	DNNSSHY
MS_4760-4 :	KKKKISGSNN-	DNN-	DNNNDDNNNDDNNND-	NHND-	DKNNKND-------------------	DDNHNDNHNND------------------------	DDNNNDNHNDD-------	DNNSSHY
MS_4760-6 :	KKKKISGSNN-	DNN-	DNNNDDNNND------	NHND-	DKNNKND-------------------	DDNHNDNHNND------------------------	DDNNNDNHNDD-------	DNNSSHY
MS_4760-8 :	KKKKISGSNN-	DNN-	DNNNDDNNNDDNNND-	NHND-	DKNNKND-------------------	DDNHNDNHNNDDDNHNDNHNNDDDNHNDNHNDD-	------------------	DNNSSHY
MS_4760-9 :	KKKKISGSNN-	DNN-	DNNNDDNNNDDNNND-	NHND-	DKNNKND-------------------	DDNHNDNHNNDDDNHNDNHNND------------	DDNNNDNHNDD-------	DNNSSHY
MS_4760-16 :	KKKKISGSNN-	DNN-	DNNNDDNNND------	NHND-	DKNNKND-------------------	DDNHNDNHNNDDDNHNDNHNND------------	DDNNNDNHNDD-DNHNDD-	DNNSSHY
MS_4760-20 :	KKKKKSGSNN-	DNN-	DNNND------------	------	DKNNKND-------------------	DDNHNDNHNNDDDNHNDNHNND------------	DDNNNDNHNDD-------	DNNSSHY
MS_4760-53 :	KKKKKSGSNN-	DNN-	DNNND------------	------	DKNNKND-DDNNNDNHKND--------	DDNHNDNHNNDDDNHNDNHNND------------	DDNNNDNHNDD-------	DNNSSHY
KF719182 :	KKKKKSGSNN-	DNN-	DNNNDDNNND------	NHND-	DKNNNND-------------------	DDNHNDNHNND------------------------	DDNNNDNHNDD-------	DNNSSHY
KF719183 :	KKKKKSGSNN-	DNN-	DNNNDDNNNDDNNND-	NHNH-	NDNHNND-------------------	DDNHNDNHNND------------------------	DDNNNDNHNDD-------	DNNSSHY
KF719184 :	KKKKKSGSNN-	DNN-	DNNNDDNNNDDNNND-	NHNH-	NKNHKND-------------------	DDNHNDNHNND------------------------	DDNNNDNHNDD-------	DNNSSHY
KF719185 :	KKKKKSGSNN-	DNN-	DNNNDDNNND------	NHND-	DKNNKND-------------------	DDNHNDNHNNDDDNHNDNNND-------------	DDNNNDNHNDD-------	DNNSSHY
KF719186 :	KKKKISGSNN-	DNN-	DNNND------------	------	DKNNKND-DDNNNDNHNND--------	DDNHNDNHNND------------------------	DDNNNDNHNDD-------	DNNSSHY

FIG 3 Alignment of 15 sequences of Pf*nhe1* ms4760 identified in 88 western Kenya *P. falciparum* isolates. Blocks I to VI have been described previously (29). The DNNND repeats are in block II, the DDNNNDNHNDD repeats are in block V, and the DDNNNDNHNDD repeats are in block VI. Profiles ms4760-1 to ms4760-53 have been described previously (25, 27, 32); sequences KF719182 to KF719186 are the described for the first time in this study. Other differences among sequences are highlighted.

DISCUSSION

In this study, we have clearly shown a significant association between polymorphisms in Pf*mdr1* codons 86 and 184 and Pf*crt* codon 76 and QN susceptibility in *P. falciparum* parasites from western Kenya. Furthermore, the diversity of microsatellite repeats in Pf*nhe1* ms4760 within these isolates was underscored and also shown to be linked to QN susceptibility. Three repeats of the DNNND polymorphism in the Pf*nhe1*gene significantly reduced parasite susceptibility to QN. Most importantly, we have described a predominant parasite genotype, Pf*mdr1*-86Y Pf*mdr1*-184F Pf*crt*-76T, in parasites with high QN IC_{50}s.

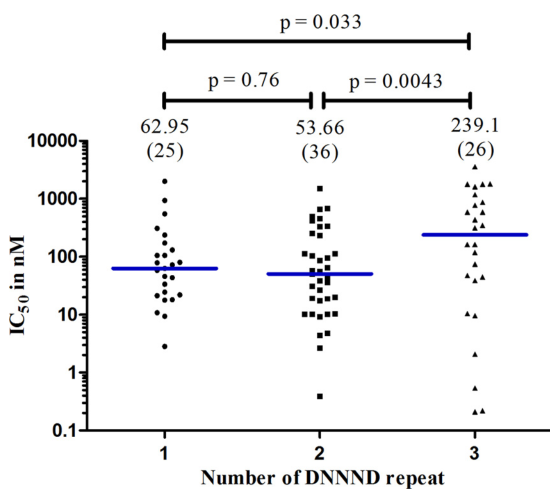

FIG 4 Association between the QN IC_{50} and the number of DNNND repeats. The analysis comparing polymorphisms in DNNND repeats was done using the Mann-Whitney test. The horizontal (blue) bars indicate the medians. The numbers of isolates analyzed are shown in brackets. *P* values comparing the repeats are indicated; 3 DNNND repeats had statistically higher QN IC_{50} values, compared to isolates with 1 or 2 repeats.

The median IC_{50} (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 IC_{50} against the W2 clone of *P. falciparum*, which is considered resistant, is 315 nM (39). Other different QN threshold cutoffs 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 IC_{50}s of $>$500 nM. In the study conducted in Kilifi, Kenya, none of the isolates had an IC_{50} 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 exceeded the 500 nM cuttoff and 11.4% (10/88) exceeded the 800 nM cutoff. Although 70.4% (62/88) of the isolates had IC_{50} 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 Pf*mdr1*-86Y Pf*mdr1*-184F Pf*crt*-76T genotype, which is also responsible for chloroquine resistance, has remained persistently high (31). Also, *in vitro* data for field isolates from Uganda have shown a tight correlation between sensitivities to chloroquine and QN (44).

In a study that analyzed parasite isolates from Kilifi, Kenya, parasites carrying the Pf*mdr1*-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 Pf*mdr1*- 86 and Pf*mdr1*-184 were implicated in decreased sensitivity to QN but did not reach significance (P = 0.22 and P = 0.34, respectively) (44). However, the Pf*mdr1*-1246

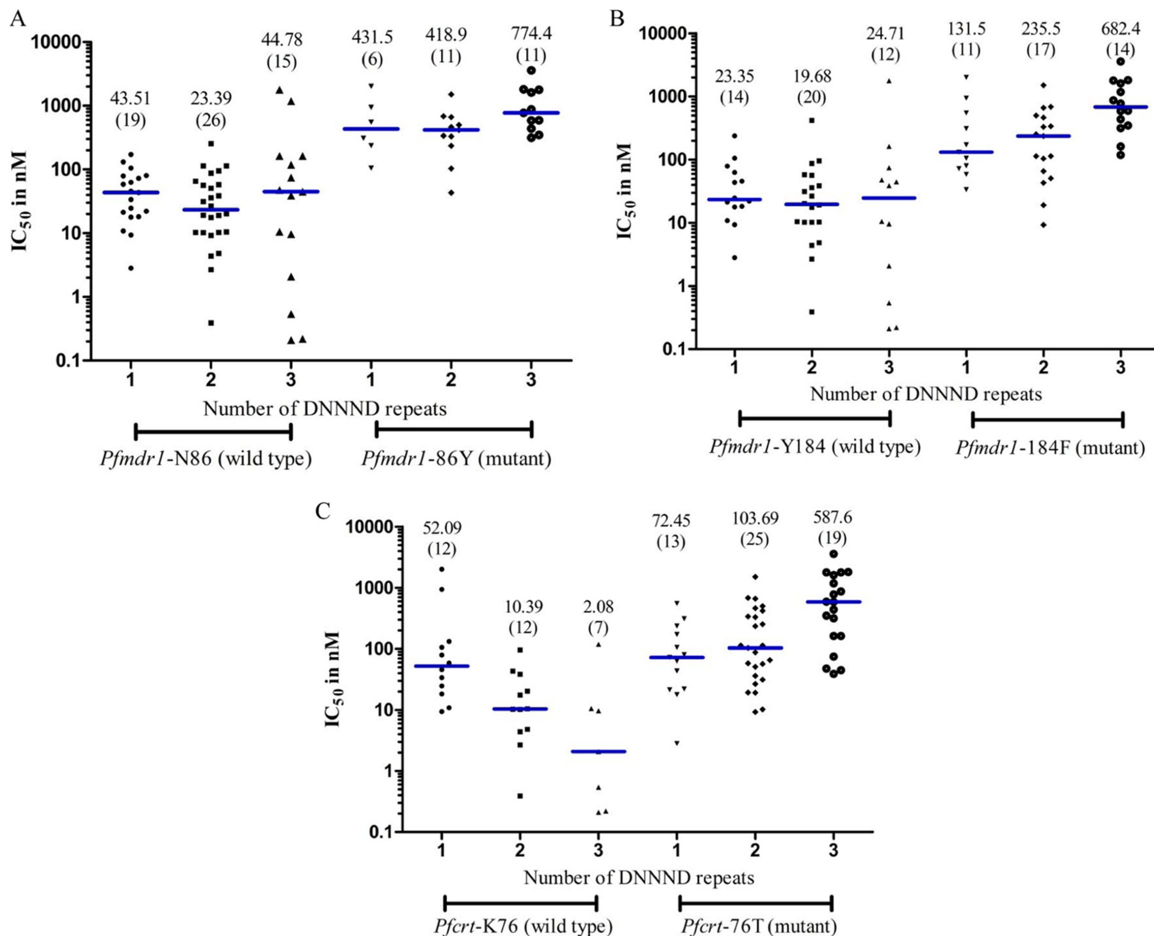

FIG 5 Relationship between the QN IC_{50} and the genotype combination of DNNND repeats for Pf*mdr1*-86 (A), Pf*mdr1*-184 (B), and Pf*crt*-76 (C). The analysis comparing the number of DNNND repeats with each allele was done using the Mann-Whitney test. The horizontal bars (blue) indicate the medians. 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 Pf*mdr1*-86Y with 1 versus 3 DNNND repeats was not statistically significant ($P = 0.21$).

mutation was shown to be statistically significant for modulating QN activity ($P = 0.029$). Transfection studies have also shown that Pf*mdr1*-1034, Pf*mdr1*-1042, and Pf*mdr1*-1246 mutations modulate resistance to QN (17). Here, we have shown statistically significant associations of mutations in Pf*mdr1* 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 Pf*mdr1* 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 Pf*mdr1* 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 Pf*mdr1*gene, the genetic background seems to be important and must therefore ultimately determine parasite phenotype.

Studies of the association of polymorphisms in the Pf*nhe1* 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 (DNNND), block 5 (DDNHNDNHNNDD), and block 6 (DDNNNDNH NDD) 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 NHNDNHNNDDD repeats with increased an QN IC_{50} ($P = 0.01$) among isolates from an African population (27). This is in line with findings from

studies conducted using isolates from Vietnam and the China-Myanmar border, where an increased number of DDNHNDNH NNDD repeats was associated with high *in vitro* QN susceptibility (30, 43). The seemingly conflicting association of polymorphisms in the Pf*nhe1* ms4670 microsatellite with *in vitro* susceptibility to QN can also be explained by differences in parasite genetic backgrounds, which can be associated with geographic origins of the isolates.

Lastly, we established the contributions of Pf*mdr1*-86, Pf*mdr1*-184, and Pf*crt*-76 in the context of DNNND polymorphisms to QN susceptibility. For all three genotypes (Pf*mdr1*-86Y, Pf*mdr1*-184F, and Pf*crt*-76T), the cumulative effect was highest in the background of 3 DNNND repeats, reaching statistical significance in each one of the genotypes. When we analyzed the genotypes of samples with results above the upper interquartile range, comprising isolates with IC_{50} estimates above the WHO cutoff for resistance, the majority of these isolates (82%) carried mutant alleles at the three genetic markers. When analyzed in combination with DNNND repeats, 55% of these isolates carried 3 repeats, as opposed 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 DNNND repeats.

Conclusions. In this study, we showed the associations of Pf*mdr1*-86, Pf*mdr1*-184, Pf*crt*-76, and Pf*nhe1* ms4670 polymorphisms with parasite responses to QN. Further, we showed that these genetic markers are only relevant in the context of the genetic background of the isolates. Andriantsoanirina et al. (27) strongly suggested polymorphisms in Pf*nhe1* might not be valid molecular markers for *in vitro* susceptibility to QN in *P. falciparum* 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, varied rates of malaria endemicity, and parasites with different genetic 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 parasites 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 important that isolates from different malaria ecological zones and regions of malaria endemicity in Kenya are analyzed. Most importantly, 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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