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Association of Microsatellite Variations of *Plasmodium falciparum* Na+/H+ Exchanger (*Pfnhe-1*) Gene with Reduced *In Vitro* Susceptibility to Quinine: Lack of Confirmation in Clinical Isolates from Africa

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Abstract. We sought to test the association of polymorphisms in *Plasmodium falciparum nhe-1* (*Pfnhe-1*, gene PF13_0019) with *in vitro* susceptibility to quinine, which was previously reported in a limited number of reference strains or culture-adapted isolates. Determination of *in vitro* susceptibility to quinine, genotyping of *Pfnhe-1* ms4760 microsatellite and polymorphism in codon 76 of *Pfcrt* were performed for 83 isolates obtained from symptomatic malaria-infected travelers returning from various African countries to France or from subjects living in Madagascar. Nineteen different ms4760 microsatellite profiles of *Pfnhe-1* were found including 14 not previously described. Multivariate analysis showed no significant association between the *in vitro* susceptibility to quinine with particular ms4760 profiles. Contrary to previous reports, we only observed that the number of NHNDNHNNDDD repeats was positively associated with the increased IC50 of QN ($P = 0.01$). We concluded that the studied polymorphisms in *Pfnhe-1* did not appear as valid molecular markers of *in vitro* susceptibility to quinine in *P. falciparum* isolates from Africa. Because we did not include any isolate of Asian origin in our series, these results did not exclude the possibility of regional associations, for example in South-East Asia.

INTRODUCTION

Quinine (QN), a natural compound found in *Cinchona* bark, used for four centuries in malaria endemic regions, is still a major antimalarial drug, especially to treat severe malaria cases or malaria in pregnant women. Although the first cases of quinine resistance were reported nearly 100 years ago in Brazil[1,2] and sporadic observations of clinical failures have been successively reported since the 1960s from Asia (Thai-Cambodian border),[3] Western Oceania, or South America,[4] the emergence and spreading of resistance to QN remained particularly low in comparison to other antimalarials as chloroquine or antifolates.

Contrary to those more recent and synthetic drugs, the mechanisms of parasite resistance to QN are not well known. Some polymorphisms in multiple transporters and in the *Plasmodium falciparum chloroquine resistance transporter* gene (*Pfcrt*) have been associated with QN resistance.[4] However, the degree of implication or linkage of those genes in QN resistance remains uncertain. Another candidate gene involved in *in vitro* QN resistance, the *P. falciparum* Na+/H+ exchanger (*Pfnhe-1*) gene (PF13_0019), was identified by Ferdig and others in 2004.[5] The physiological contribution of PfNHE-1 in QN resistance is still evaluated and debated, and could be strain-dependent.[6] Concomitantly, the evaluation of the association of polymorphisms in PF13_0019 with QN susceptibility is under progress. The princeps study by Ferdig and others described three point polymorphisms at three separate codons (790, 894, 950) and microsatellite variations in three different repeat sequences (msR1, ms3580, ms4760). Only variations in ms4760 showed significant association with *in vitro* QN response in 71 *P. falciparum* lines. Among the eight profiles of ms4760 described, one profile, the ms4760-1, was relatively frequent in less quinine-susceptible lines but was also present in fully susceptible parasites. More interestingly, the number of a DNNND repeat motif, shared by the different ms4760 profiles, was reported as associated with QN response. Since then, another study investigated the association of polymorphisms in PF13_0019 with *in vitro* QN susceptibility.[7] In that series of 23 culture-adapted isolates or reference strains, the relationship between the number of DNNND repeats and the inhibitory concentration 50% values (IC_{50}) to QN was confirmed and the increased number of another repeat motif, NHNDNHNNDDD, was associated with decreased IC_{50s} to QN. A limitation of those previous studies was that the *in vitro* QN susceptibility and polymorphisms determinations were performed on culture-adapted cloned isolates or reference strains, which could lead to biased results caused by accumulated mutations selected by *in vitro* conditions.

In this study, we have tried to test, in isolates originating from various African countries, the association of the previously reported polymorphisms of PF13_0019 with *in vitro* QN response. Fresh isolates obtained from symptomatic, malaria-infected travelers returning from Africa to France or from subjects living in Madagascar were used.

MATERIALS AND METHODS

Reference culture-adapted strains. Three culture-adapted and cloned strains of *P. falciparum* (chloroquine-resistant and chloroquine-susceptible strains) were used for quality control (3D7Africa and W2 Indochina in Paris; 3D7Africa and FCM29 Cameroon in Madagascar). These clones were obtained from MR4-ATCC (Manassas, VA). Reference strains were cryopreserved and thawed before each measurement. They were cultivated in erythrocytes resuspended in RPMI 1640 medium supplemented with 10% human serum (Abcys Biowest, Paris, France) at 37°C in a 5% CO_2, 5% O_2, 90% N_2 atmosphere until a minimum parasite density of 1% was

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achieved. The *in vitro* susceptibilities to chloroquine (CQ) and QN were determined after suspension in the same medium and addition of uninfected erythrocytes to obtain 0.3% parasitemia and 1.5% hematocrit. The culture obtained within three asexual cycles after thawing contained a minimum of 90% ring forms and did not require synchronization.

Patients and clinical samples. Clinical isolates of *P. falciparum* from symptomatic malaria-infected travelers returning to France from various African countries in 1997–2007 were obtained at the National Reference Center for Malaria (NRCM), Paris, France. Clinical samples from Madagascar were collected, as part of the surveillance of antimalarial drug resistance, in 2006/2007 from symptomatic patients before treatment in six health centers. The health centers were located in areas exhibiting three of the four epidemiological patterns of malaria transmission: Ihosy in South Madagascar (sub-desert stratum, epidemic prone), Maevatanana and Miandrivazo in West Madagascar (tropical stratum, seasonal and endemic area), and Tsiroanomandidy, Saharevo, and Moramanga in the foothills of the Central Highlands of Madagascar (highlands stratum, low-endemic area). Venous blood samples (10 mL) were collected in tubes coated with EDTA (Vacutainer tubes, Becton Dickinson, Rutherford, NJ), from malaria-positive patients (> 2 years of age). Parents or guardians gave their consent for participation in the study (No. 007/SANPF/2007, registration number from the Ethics Committee of the Ministry of Health of Madagascar). Malaria positivity was evaluated by using a rapid diagnostic test based on the detection of *Plasmodium*-specific lactate dehydrogenase (pLDH) (OptiMAL-IT, DiaMed AG, Cressier sur Morat, Switzerland). Positive patients were treated with artesunate-amodiaquine combination, according to the National Malaria Control Program (NMCP) in Madagascar.

Determination of QN and CQ *in vitro* IC_{50s}. At the NMRC, the determinations of IC_{50s} were routinely done on fresh isolates as part of the surveillance of antimalarial susceptibility of *P. falciparum* isolates obtained in travelers returning to France. *In vitro* assays performed on isolates obtained at the NRMC, Paris, France and at the Malaria Research Unit (MRU), Institut Pasteur de Madagascar, Antananarivo, Madagascar were processed using the isotopic microtest method.[8,9] Antimalarial drugs in the appropriate solvent were distributed on 96-well tissue culture plates and dried: chloroquine disulfate (Sigma Aldrich, Saint-Quentin Fallavier, France), 12 to 3,200 nM (NCRM, Paris) or 12.5 to 1,600 nM (MRU, Antananarivo) and quinine (Sigma Aldrich), 25 to 3,200 nM (NCRM, Paris and MRU, Antananarivo). For each drug tested, three control wells were drug free, and each concentration was studied in duplicate or triplicate. Clinical isolates with at least a 0.1% parasite density were included in the study and were maintained at +4°C (for up to 48 h after collection) before a culture was started. The blood samples were washed three times with a solution of RPMI 1640 (Gibco, Invitrogen Life Technologies, Cergy-Pontoise, France) plus 25 mM HEPES (Sigma) and 25 mM $NaHCO_3$ (Sigma). The blood samples were then resuspended in the same culture medium supplemented with 10% human serum (Abcys Biowest, Paris, France). If necessary, a dilution was performed by adding uninfected O-positive-group erythrocytes (from Etablissement Français du Sang, Rungis, France, or from Blood Transfusion Center, Antananarivo, Madagascar) to obtain a 0.3% parasite density and a 1.5% hematocrit. For *in vitro* isotopic microtest, 200 µL/well of the suspension of parasitized erythrocytes were distributed in 96-well plates predosed with antimalarial agents. Plates were incubated for 42 h at 37°C in an atmosphere of 5% O_2, 5% CO_2, and 90% N_2, and a relative humidity of 95%. Plates were then frozen and kept at –20°C.

Isotopic measurement. For the isotopic assay, 1 mL RPMI 1640 with [8-^{3}H] hypoxanthine (40 mCi/L; Amersham Biosciences, Orsay, France) was added to 20 mL of the homogeneous parasite suspension (~0.4 µCi [8-^{3}H]hypoxanthine/well) at the beginning of the *in vitro* assay. After the plates were thawed, the content of each well was harvested onto fiber filter disks (FilterMAT, Wallac, Turku, Finland). The fiber filter papers were washed and dried and mixed with 2 mL of scintillation fluid (OptiScint, Perkin-Elmer; NBCS 204, Amersham Biosciences, Orsay, France), and the level of parasite incorporation of radioactivity (in counts per minute) was measured with a liquid scintillation counter (Wallac 1410; Perkin-Elmer). The IC_{50s} were determined by nonlinear regression analysis with an inhibitory sigmoid maximum-effect model.[8,9] The cut-off values, defined as > 2 SD above the mean and/or after correlation with clinical failures, for *in vitro* resistance or reduced susceptibility to CQ and QN, were 100 and 800 nM, respectively.

Genotyping of Pfnhe ms4760 microsatellite polymorphisms and of Pfcrt codon76. Parasites DNA from 100 µL infected blood were extracted using the phenol chloroform method as described elsewhere.[10] The polymerase chain reaction (PCR) genotyping of highly polymorphic genetic markers was performed to select monoclonal isolates. The polymorphic regions of *msp-1* (block2) and *msp-2* (block3) were amplified by nested PCR. Second-round appropriate PCR primers were used to amplify the K1, MAD20, and RO33 allelic families of *msp-1* and the 3D7 and FC27 allelic families of *msp-2*. The primers and conditions used for amplifications were those previously reported by Ranford-Cartwright and others.[11] For each sample, 25 µL reactions mixtures were prepared containing 1X PCR buffer, 150 µM of each dNTPs, 100 nM of each primer, and 1.25 U of Amplitaq Gold (Applied Biosystems, Roche). Five microliters (5 µL) of sample DNA were used for outer amplification and 2 µL of the outer PCR product was transferred as template for the nested amplification. Eight microliters (8 µL) of nested PCR products were analyzed by electrophoresis using 2% agarose gels. Isolates showing only one band in each marker were processed for further genotyping studies including genotyping of *Pfnhe-1* ms4760 microsatellite polymorphisms and of *Pfcrt* codon76.

For *Pfnhe-1*, the inner amplification reaction mixture contained 1X PCR buffer, 2.5 mM $MgCl_2$, 1 mM of dNTPs, and 0.25 µM of primers (NHE-1: 5′-TCCTGATAGTAGCG AAGAAGAA-3′ and NHE-2: 5′-CAGTGCATGGACCAA AATTA-3′). Reactions were carried out in 25 µL and 1.25 U DNA polymerases (ExTaq, TaKaRa Bio Inc, Japan). The outer PCR was carried out in 55 µL with identical mixture with nested primers (NHE-3: 5′-AGTCGAAGGCGAATCAGATG-3′ and NHE-4: 5′-CCTGAACAAGCACTGCAAGA-3′). The PCR conditions used to genotype codon 76 of *Pfcrt* were as previously described.[12] After purification by filtration using Macherey-Nagel plate (NucleoFast 96 PCR, Macherey-Nagel, Düren, Germany), sequencing reactions were carried out with the ABI PRISM BigDye Terminator cycle sequencing ready reaction kit run on a 3730xl Genetic Analyser (Applied Biosystems, Courtaboeuf, France). Electrophoregrams were visualized and analyzed with CEQ2000 Genetic Analysis

System software (Beckman Coulter, Sykesville, MD). Amino-acid sequences were compared with wild-type sequence (GenBank accession no. XM_001349726 for *NHE-1* gene). *Pfnhe-1* ms4760 microsatellite genotypes were constructed from full sequence presenting an unambiguous single allele signal at all positions.

Statistical analysis. Epi Info software (version 6.04, CDC, Atlanta, GA) and MedCalc software (version 9.1.0.1, Mariakerke, Belgium) were used for data analysis. Continuous variables were compared using an independent-sample analysis of variance (ANOVA) or Mann-Whitney test. The CQ and QN geometric means IC_{50s} were analyzed with respect to location of the isolates collection, polymorphism in *Pfcrt* gene, *Pfnhe-1* ms4760 profiles (only the four most numerous profiles were included in the analysis), number of DNNND or NHNDNHNNDDD repeats or repeats ratio. A P value < 0.05 was considered statistically significant. For the multivariate analysis, variables with a P value < 0.25 were initially introduced into the model and removed following a backward-stepwise selection procedure to leave only those with a P value < 0.05 in the final model.

Nucleotide sequence accession numbers. The exact sequence of each new ms4760 genotype has been submitted to GenBank (accession no. FJ947067 to FJ947073).

RESULTS

A total of 83 isolates from Madagascar (N = 40) and from 13 other African countries (36 from West Africa, 6 from Central Africa, 1 from East Africa), collected between 1997 and 2007, appearing monoclonal on the basis of *msp-1*/*msp-2* genotyping and with *Pfnhe-1* full sequences presenting only one single allele at all positions, were included in the study (Table 1). The *Pfcrt* 76T mutant-type allele was present in 30 isolates (36.1%) and its prevalence distribution was significantly heterogeneous: from 66.7% to 100% in mainland Africa and absent in Madagascar (P < 0.0001).

The quality controls of batches of plates using reference lines were as follow: (IC_{50s} geometric mean ± SD): for CQ, 3D7 Africa, 28 nM ± 10 nM (N = 116, Paris) and 13 nM ± 3 nM (N = 5, Madagascar), W2 Indochina, 389 nM ± 106 nM (N = 154, Paris) and FCM29 Cameroon, 216 nM ± 19 nM (N = 5, Madagascar); for QN, 3D7 Africa, 130 nM ± 52 nM (N = 43, Paris) and 138 nM ± 30 nM (N = 5, Madagascar), W2 Indochina, 455 nM ± 177 nM (N = 68, Paris) and FCM29 Cameroon, 372 nM ± 86 nM (N = 5, Madagascar).

The IC_{50} median *in vitro* activities of drugs were 137 nM for QN (confidence interval [CI] 95%: 107–183 nM, range: 22–1,600 nM) and 74 nM for CQ (CI 95%: 51–89 nM, range: 3–498 nM), respectively. Contrary to CQ (P = 0.07), the QN IC_{50} geometric means displayed significant differences (P = 0.002) ranging from 121 nM from Madagascar (N = 40) to 171 nM from West Africa (N = 36), 315 nM from Central Africa (N = 6), and 1600 nM from East Africa (N = 1).

Nineteen different ms4760 microsatellite profiles of *Pfnhe-1* were found including 14 not previously described (Figure 1). The profiles ms-1 (31.3%), ms-3 (19.3%), ms-7 (16.9%), and ms-6 (8.4%) were the most common. No significant associations were noticed between the *Pfnhe-1* profiles and the geographical origin of the isolate collection (P = 0.4). The number of DNNND repeats ranged from 0 to 3 (median = 2; 48.2% of the isolates), whereas the number of NHNDNHNNDDD repeats ranged from 1 to 3 (median = 2; 61.5% of the isolates) (Table 1). The median of the DNNND: NHNDNHNNDDD repeats ratio was 1 (range: 0–3).

In univariate analysis, the CQ IC_{50} geometric mean was significantly associated with the *Pfcrt* polymorphism (P < 0.0001, 50 nM for *Pfcrt* K76 versus 138 nM for *Pfcrt* 76T), and the QN IC_{50} geometric mean was significantly associated with the *Pfcrt* polymorphism (P = 0.001, 121 nM for *Pfcrt* K76 versus 242 nM for *Pfcrt* 76T), the number of NHNDNHNNDDD repeats (P = 0.02, 117 nM for one repeat and 192 nM for two repeats) and the location of the samples collection (as previously shown). There was no significant association between *in vitro* QN susceptibility and the number of DNNND repeats (P = 0.4).

In the multivariate analysis performed to control confounding factors, five variables (*Pfcrt* polymorphism, number of NHNDNHNNDDD repeats, DNNND/NHNDNHNNDDD repeats ratio, ms4760 microsatellite profiles, and location of the sample collection) were initially introduced into the model. Backward stepwise selection procedure to leave only variables with a P value < 0.05 in the final model showed that the remaining significant associations were the *in vitro* susceptibility to CQ with the *Pfcrt* polymorphism (P < 0.001) and the *in vitro* susceptibility to QN with the number of NHNDNHNNDDD repeats (P = 0.01).

DISCUSSION

Because no valid molecular marker of QN resistance is currently available, the identification of genes potentially involved in QN resistance is of paramount importance to develop new tools for the surveillance of emergence and spreading of *P. falciparum*-resistant strains. The finding of association of polymorphisms in putative genes with clinical failures and/or *in vitro* susceptibility constitutes a pivotal step in this process. Such associations must be verified on numerous isolates originating from various geographical areas and further molecular studies are required to assess the involvement of the candidate genes in drug resistance. Recent genetic and physiological studies reinforced the observation that QN resistance is a complex trait requiring multiple factors;[6] those studies did not exclude a potential role for PfNHE in QN resistance, but in a strain-dependent manner. The recent study of microsatellite markers flanking *Pfnhe-1* gene reported an absence of selective sweep in 108 Indian *P. falciparum* isolates and also a lack of association of microsatellite markers with DNNND repeats,[13] possibly indicating that there is no strong selection pressure on this target gene. In this context, the validity and reliability of candidate polymorphisms in *Pfnhe-1* gene as molecular markers of QN resistance has to be carefully evaluated.

In this study, we have compiled *in vitro* susceptibility data from France and from Madagascar to perform statistical tests for association. As shown by the IC_{50s} values of reference lines used as quality controls, both drug testing sites had comparable results and reproducibility, which enabled us to merge the two sets of data for analysis. We noticed that isolates having the highest values of QN IC_{50s} (> 600 nM) had also a mutant allele in codon 76 of *Pfcrt (*associated with CQ resistance). The significant relationship found in univariate analysis between *in vitro* QN response and the polymorphism in codon 76 of *Pfcrt* was consistent with that found by Ferdig and others.[5] Henry and others[7] did not report such an association though all strains of their series having QN IC_{50s} > 400 nM, except one,

TABLE 1

Characteristics (country and year of collection, *in vitro* susceptibility, and *Pfcrt* codon 76 and *Pfnhe*-1 polymorphisms) of the 83 *Plasmodium falciparum* isolates from African countries collected in 1997–2007

Isolates	Country	Year of collection	*Pfcrt* codon76†	*In vitro* susceptibility, CI_{50} (nM)*		*Pfnhe-1* ms4760 microsatellite		
				QN	CQ	No. DNNND repeat	No. NHNDNHNNDDD repeat	No. genotype profile‡
565	Mozambique	1997	76T	1600	ND	2	2	ms-1
1785	Togo	1999	76T	1267	ND	3	1	ms-7
1648	Benin	1999	76T	1191	111	1	2	ms-20
1563	Ivory Coast	1999	76T	1179	64	2	2	ms-1
5805	Cameroon	2005	76T	855	498	2	2	ms-1
1553	DRCongo	1999	76T	821	67	2	1	ms-28
TDD01	Madagascar	2006	76K	612	138	1	2	ms-3
4274	Guinea	2003	76T	611	ND	2	2	ms-1
TDD02	Madagascar	2007	76K	435	50	2	2	ms-1
6268	Mali	2006	76K	413	30	1	2	ms-3
IHO01	Madagascar	2007	76K	393	47	2	2	ms-1
MOR01	Madagascar	2006	76K	385	ND	2	2	ms-1
6569	Mali	2006	76T	371	77	2	2	ms-26
MIA01	Madagascar	2006	76K	363	104	2	2	ms-1
TDD03	Madagascar	2006	76K	353	30	1	2	ms-3
5813	DRCongo	2005	76T	330	237	3	2	ms-23
TDD04	Madagascar	2006	76K	327	38	3	1	ms-7
6643	Mali	2006	76T	310	216	1	2	ms-3
6562	Mali	2006	76T	299	167	1	2	ms-3
6685	Mali	2006	76K	285	55	1	2	ms-3
TDD05	Madagascar	2006	76K	275	ND	1	2	ms-3
6514	Mauritania	2006	76K	266	43	2	1	ms-6
3406	DRCongo	2002	76T	260	63	1	2	ms-3
MIA02	Madagascar	2006	76K	247	63	2	2	ms-1
MAE01	Madagascar	2006	76K	240	47	2	1	ms-6
MOR02	Madagascar	2006	76K	222	27	2	2	ms-1
MIA03	Madagascar	2006	76K	222	86	0	2	ms-33
MIA04	Madagascar	2006	76K	221	14	2	2	ms-1
6339	Burkina Faso	2006	76T	196	163	2	2	ms-1
4627	Ivory Coast	2004	76T	187	ND	1	2	ms-24
4275	Cameroon	2003	76T	186	ND	1	2	ms-3
MIA05	Madagascar	2006	76K	185	42	1	2	ms-31
6534	Niger	2006	76T	177	187	2	2	ms-1
5864	Senegal	2005	76T	176	52	2	2	ms-1
MIA06	Madagascar	2006	76K	166	51	1	2	ms-3
6410	Mali	2006	76T	163	139	1	2	ms-3
6588	Mali	2006	76K	159	40	3	1	ms-7
6529	Mali	2006	76T	150	114	2	2	ms-1
5773	Mali	2005	76T	145	154	2	1	ms-25
5832	Ivory Coast	2005	76T	144	294	2	2	ms-1
5774	Senegal	2005	76K	142	40	3	1	ms-7
5833	Benin	2005	76T	137	231	2	1	ms-21
5769	Burkina Faso	2005	76K	135	37	2	3	ms-22
MIA07	Madagascar	2006	76K	125	57	3	1	ms-7
MIA08	Madagascar	2006	76K	125	127	3	1	ms-7
MIA09	Madagascar	2006	76K	123	140	2	2	ms-1
5693	Ivory Coast	2005	76K	123	74	2	1	ms-21
6506	Burkina Faso	2006	76T	115	173	2	1	ms-6
MIA10	Madagascar	2007	76K	112	48	1	2	ms-30
SHV01	Madagascar	2006	76K	110	84	3	1	ms-7
6340	Mali	2006	76T	109	ND	2	2	ms-1
6481	Mali	2006	76T	107	188	2	2	ms-1
MIA11	Madagascar	2006	76K	106	74	1	2	ms-3
IHO02	Madagascar	2007	76K	105	67	1	2	ms-3
6539	Mali	2006	76T	105	120	3	2	ms-27
MIA12	Madagascar	2006	76K	105	79	2	1	ms-32
5322	Benin	2005	76T	104	138	2	2	ms-1
6612	Mali	2006	76K	104	35	1	3	ms-12
6126	Niger	2006	76T	103	212	2	2	ms-1
MIA13	Madagascar	2006	76K	102	85	2	2	ms-1
6659	Mali	2006	76K	97	22	1	2	ms-3
MIA14	Madagascar	2006	76K	96	63	2	1	ms-29
6532	Mali	2006	76K	94	39	1	3	ms-12
MIA15	Madagascar	2007	76K	92	25	3	1	ms-7
IHO03	Madagascar	2007	76K	90	33	2	1	ms-6
6430	Chad	2006	76K	88	29	1	2	ms-3
SHV02	Madagascar	2006	76K	87	126	2	1	ms-6

(*Continued*)

TABLE 1
Continued

Isolates	Country	Year of collection	*Pfcrt* codon76†	*In vitro* susceptibility, CI_{50} (nM)*		*Pfnhe-1* ms4760 microsatellite		
				QN	CQ	No. DNNND repeat	No. NHNDNHNNDDD repeat	No. genotype profile‡
MIA16	Madagascar	2007	76K	76	13	2	2	ms-1
MIA07	Madagascar	2006	76K	74	88	1	2	ms-3
5866	Mali	2005	76T	71	34	2	1	ms-6
MIA18	Madagascar	2007	76K	70	3	3	1	ms-7
MOR03	Madagascar	2006	76K	70	37	3	1	ms-7
MIA19	Madagascar	2006	76K	64	94	1	3	ms-12
SHV03	Madagascar	2006	76K	63	142	1	2	ms-31
5655	Ivory Coast	2005	76T	57	223	2	2	ms-1
SHV04	Madagascar	2006	76K	57	17	3	1	ms-7
IHO04	Madagascar	2007	76K	51	88	3	1	ms-7
6299	Burkina Faso	2006	76K	49	21	1	3	ms-12
MIA20	Madagascar	2006	76K	37	99	3	1	ms-7
6374	Mali	2006	76K	27	30	2	2	ms-1
TDD06	Madagascar	2006	76K	23	87	2	2	ms-1
MOR04	Madagascar	2007	76K	23	142	3	1	ms-7
MOR05	Madagascar	2006	76K	22	126	2	1	ms-6

* QN = quinine, CQ = chloroquine.
† *Pfcrt* 76K (wild-type allele) and *Pfcrt* 76T (mutant-type allele).
‡ *Pfnhe-1* ms4760 microsatellite genotype profiles are detailed in Figure 1.
ND = not done.

had also a mutant allele in codon 76 of *Pfcrt*. Although some CQ IC_{50s} were not performed in our series, results of genotyping of codon 76 of *Pfcrt* were consistent with the measured CQ IC_{50s} in French isolates.[12] A particularity of Malagasy isolates at this point is that *Pfcrt* mutant alleles are almost totally absent from Madagascar[14,15] and thus *in vitro* CQR Malagasy isolates did not present this type of mutations, but they frequently have single nucleotide polymorphisms (SNPs) in the *Pfmdr1* gene.[15,16]

The discovery of the different ms4760 microsatellite profiles of *Pfnhe-1* appeared as a burgeoning process as we reported 14 not previously described, which added to the many already known polymorphisms in this locus.[5,7,17] The only association revealed by the multivariate analysis of our study, except the already known *Pfcrt* 76/*in vitro* CQ response association, was that of the number of NHNDNHNNDDD repeats with increased QN IC_{50s} geometric mean. These results were conflicting with those of Henry and others,[7] who reported an association of a greater number of NHNDNHNNDDD repeats with decreased QN IC_{50s}. We did not confirm the previously reported associations of particular ms4760 profiles with *in vitro* QN response. Indeed, the ms4760-1 that was found by Ferdig and others[5] as associated with higher QN IC_{50s} was not confirmed by Henry and others who claimed that strains having another particular profile (ms4760-7) had reduced susceptibility to QN. In our series, isolates having either ms4760-1 or ms4760-7 had not particularly reduced susceptibility to QN. We did not confirm either the previously reported associations of the number of DNNND repeats with *in vitro* QN response.

Some hypotheses may explain our data. First, the associations previously reported could be geographically restricted for a part. Thus, among the 15 less susceptible strains of the

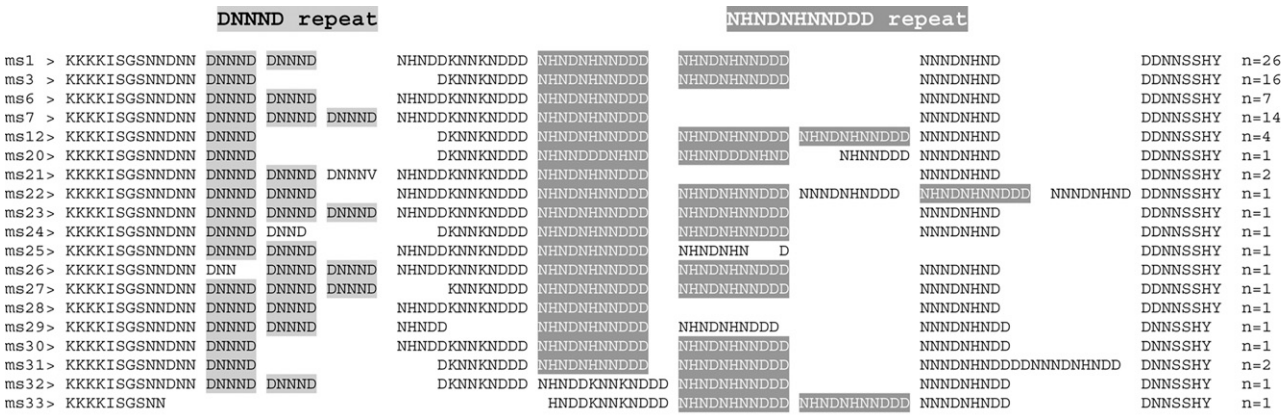

FIGURE 1. Multiple amino acid sequence alignment of the 19 *pfnhe-1* ms4760 microsatellite observed in 83 *Plasmodium falciparum* isolates collected in African countries between 1997 and 2007.The *pfnhe-1* ms4760 microsatellite sequences from ms-1 to ms-12 were previously described.[2,12] The DNNND and NHNDNHNNDDD repeats, previously associated with the *in vitro* quinine susceptibility are shown in the gray box. The number of isolates for each profile is given on the right.

series of Ferdig and others,[5] 11 strains originated from South-East Asia (mainly Thailand, Vietnam, and Cambodia) as only four strains among the 56 other more susceptible ones originated from South-East Asia. In the same way, five out the seven strains having the highest QN IC_{50} values in the work by Henry and others originated from South-East Asia as none of the 16 other more susceptible strains came from this region. Contrary to those previous studies, we did not include any isolate of Asian origin in our series. It may be underlined that at present *P. falciparum* QN resistance is much less frequently found in Africa than in Asia.[4,18] Second, the likely multigenic nature of QN resistance and the probable strain-specific relative role of each molecular actor may hamper the accurate detection of reliable association of genetic polymorphisms with *in vitro* QN response. The contrasted results found by different teams about the associations of *Pfnhe-1* polymorphisms with QN resistance were not without recalling us about the discussed status of the association of *Pfmdr-1* N86Y mutation with CQ resistance, which fluctuated largely according to studies.[19]

In conclusion, the studied polymorphisms in PF13_0019 did not appear as valid molecular markers of *in vitro* QN response in our series of isolates originating from various African countries. At present, the study of these polymorphisms did not appear useful to monitor QN resistance in Africa and help policy markers.

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REFERENCES

1. Neiva A, 1910. Ueber die Bildung einur chininresistenten Rasse des Malariaparasiten. *Mem Inst Oswaldo Cruz 2:* 131–140.
2. Nocht B, Werner H, 1910. Beobachtungen uber eine relative Chininresistenz bei malaria aus Brasilien. *Dtsch Med Wochenschr 36:* 1557–1560.
3. Peters W, 1987. Resistance of human malaria I, III, and IV. In: *Chemotherapy and Drug Resistance in Malaria*. Second edition. London: Academic Press, 543–568, 593–658, 659–786.
4. Wongsrichanalai C, Pickard AL, Wernsdorfer WH, Meshnick SR, 2002. Epidemiology of drug-resistant malaria. *Lancet Infect Dis 2:* 209–218.
5. Ferdig MT, Cooper RA, Mu J, Deng B, Joy DA, Su XZ, Wellems TE, 2004. Dissecting the loci of low-level quinine resistance in malaria parasites. *Mol Microbiol 52:* 985–997.
6. Nkrumah LJ, Riegelphaupt PM, Moura P, Johnson DJ, Patel J, Hayton K, Ferdig MT, 2009. Probing the multifactorial basis of *Plasmodium falciparum* quinine resistance: evidence for a strain-specific contribution of the sodium-proton exchanger PfNHE. *Mol Biochem Parasitol 165:* 122–131.
7. Henry M, Briolant S, Zettor A, Pelleau S, Baragatti M, Baret E, Mosnier J, Amalvict R, Fusai T, Rogier C, Pradines B, 2009. *Plasmodium falciparum* Na+/H+ exchanger 1 transporter is involved in reduced susceptibility to quinine. *Antimicrob Agents Chemother 53:* 1926–1930.
8. Kaddouri H, Nakache S, Houzé S, Mentré F, Le Bras J, 2006. Assessment of the drug susceptibility of *Plasmodium falciparum* clinical isolates from Africa by using a *Plasmodium* lactate dehydrogenase immunodetection assay and an inhibitory maximum effect model for precise measurement of the 50-percent inhibitory concentration. *Antimicrob Agents Chemother 50:* 3343–3349.
9. Rason MA, Randriantsoa T, Andrianantenaina H, Ratsimbasoa A, Menard D, 2008. Performance and reliability of the SYBR Green I-based assay for the routine monitoring of susceptibility of *Plasmodium falciparum* clinical isolates. *Trans R Soc Trop Med Hyg 102:* 346–351.
10. Rakotonirina H, Barnadas C, Raherijafy R, Andrianantenaina H, Ratsimbasoa A, Randrianasolo L, Jahevitra M, Andriantsoanirina V, Menard D, 2008. Accuracy and reliability of malaria diagnostic techniques for guiding febrile outpatient treatment in malaria-endemic countries. *Am J Trop Med Hyg 78:* 217–221.
11. Ranford-Cartwright LC, Taylor J, Umasunthar T, Taylor LH, Babiker HA, Lell B, Schmidt-Ott JR, Lehman LG, Walliker D, Kremsner PG, 1997. Molecular analysis of recrudescent parasites in a *Plasmodium falciparum* drug efficacy trial in Gabon. *Trans R Soc Trop Med Hyg 91:* 719–724.
12. Durand R, Jafari S, Vauzelle J, Delabre JF, Jesic Z, Le Bras J, 2001. Analysis of *pfcrt* mutations and chloroquine susceptibility in isolates of *Plasmodium falciparum*. *Mol Biochem Parasitol 114:* 95–102.
13. Choudhary V, Sharma YD, 2009. Extensive heterozygosity in flanking microsatellites of *Plasmodium falciparum* Na+/H+ exchanger (*pfnhe-1*) gene among Indian isolates. *Acta Trop 109:* 241–244.
14. Randrianarivelojosia M, Fidock DA, Belmonte O, Valderramos SG, Mercereau-Puijalon O, Ariey F, 2006. First evidence of *pfcrt* mutant *Plasmodium falciparum* in Madagascar. *Trans R Soc Trop Med Hyg 100:* 826–830.
15. Andriantsoanirina V, Andrianaranjaka V, Ratsimbasoa A, Bouchier C, Jahevitra M, Rabearimanana S, Radrianjafy R, Randriantsoa T, Rason MA, Tichit M, Rabarijaona LP, Mercereau-Puijalon O, Durand R, Ménard D, 2009. *Plasmodium falciparum* drug resistance in Madagascar: facing the spread of unusual *pfdhfr* and *pfmdr-1* haplotypes and the decrease of the dihydroartemisinin susceptibility. *Antimicrob Agents Chemother 53:* 4588–4597.
16. Rason MA, Andrianantenaina HB, Ariey F, Raveloson A, Domarle O, Randrianarivelojosia M, 2007. Prevalent *Pfmdr-1* N86Y mutant *Plasmodium falciparum* in Madagascar despite absence of *Pfcrt* mutant strains. *Am J Trop Med Hyg 76:* 1079–1083.
17. Vinayak S, Alam MT, Upadhyay M, Das MK, Dev V, Singh N, Dash AP, Sharma YD, 2007. Extensive genetic diversity in the *Plasmodium falciparum* Na+/H+ exchanger 1 transporter protein implicated in quinine resistance. *Antimicrob Agents Chemother 51:* 4508–4511.
18. Jelinek T, Grobusch MP, Loscher T, 2001. Patterns of *Plasmodium falciparum* drug resistance in nonimmune travellers to Africa. *Eur J Clin Microbiol Infect Dis 20:* 284–286.
19. Picot S, Olliaro P, de Monbrison F, Bienvenu AL, Price RN, Ringwald P, 2009. A systematic review and meta-analysis of evidence for correlation between molecular markers of parasite resistance and treatment outcome in falciparum malaria. *Malar J 8:* 89.