Short Report: *Plasmodium falciparum* Na⁺/H⁺ Exchanger (*pfnhe-1*) Genetic Polymorphism in Indian Ocean Malaria-Endemic Areas

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Abstract. To date, 11 studies conducted in different countries to test the association between *Plasmodium falciparum* Na⁺/H⁺ exchanger gene (*pfnhe-1*; PF13_0019) polymorphisms and *in vitro* susceptibility to quinine have generated conflicting data. In this context and to extend our knowledge of the genetic polymorphism of *Pfnhe* gene, we have sequenced the *ms4760* locus from 595 isolates collected in the Comoros ($N = 250$; an area with a high prevalence of chloroquine and sulfadoxine-pyrimethamine resistance) and Madagascar ($N = 345$; a low drug-resistance area). Among them, 29 different alleles were observed, including 8 (27%) alleles not previously described. Isolates from the Comoros showed more repeats in block II (DNNND), which some studies have found to be positively associated with *in vitro* resistance to quinine, compared with isolates from Madagascar. Additional studies are required to better define the mechanisms underlying quinine resistance, which involve multiple gene interactions.

Quinine (QN), a natural quinoline derivative compound found in *Cinchona* bark, has been used for centuries in malaria-endemic regions.¹ To date, resistance to QN remains particularly patchy and rare,^{2–12} and only few cases of clinical failure have been reported in Asia and South America. The mechanism underlying QN resistance is not well-understood, and it is probably complex and multigenic. Since the seminal work by Ferdig and others¹³ in 2004, 11 studies have been conducted in different countries to evaluate implication of *ms4760* polymorphisms in QN resistance,^{14–24} and conflicting data have been reported, likely because of the different geographical origin of parasites (implying different genetic backgrounds), the type of parasites used (fresh isolates, culture-adapted strains, and reference lines), and the method used to assess *in vitro* QN susceptibility.^{21,25}

In this context and to extend our previous work regarding *ms4760* polymorphisms in *Plasmodium falciparum* parasites circulating in malaria-endemic areas in the Indian Ocean,¹⁴ we have analyzed *ms4760* sequences from 595 isolates (Madagascar, $N = 345$; Comoros, $N = 250$).

P. falciparum isolates from Madagascar were collected in 2006 and 2007 as part of the surveillance of antimalarial drug resistance from symptomatic malaria-infected patients before treatment in 14 health centers (northwest: Antohihy, Analalava, Mahajanga, and Maevatanana; central west: Tsiroanomandidy, Miandrivazo, and Morondava; southwest: Ihosy, Ejeda, and Toliara; northeast: Andapa; central east: Toamasina and Moramanga; southeast: Faranfagana). Comorian isolates were collected from finger prick onto filter paper in 2006 in six different sites (Grande Comore: Moroni and Fombouni; Mohéli: Fomboni and Wanani; Anjouan: Pomoni and Domoni) (Figure 1). Informed written consent was provided by all patients or their parents/guardians before inclusion in the study, and blood collections were conducted in accordance to the Ethics Committee of the Ministries of Health of Madagascar

and the Comoros (N°007/SANPF/2007; registration number ISRCTN36517335).

Parasite DNA was extracted from blood spots with Instagene matrix (Bio-Rad, Marnes la Coquette, France) according to the manufacturer's instructions or directly from 100 μ L infected blood by the phenol-chloroform method.²⁶ The parasite species was confirmed by real-time polymerase chain reaction (PCR) as described in the work by Mangold and others.²⁷ Amplification and sequencing of the *ms4760* locus in the *P. falciparum* Na⁺/H⁺ exchanger gene (*pfnhe-1*) was performed in accordance with the protocol described earlier.¹⁴ *pfnhe-1 ms4760* alleles were constructed from a full sequence presenting an unambiguous single-allele signal at all positions and used *P. falciparum* 3D7 (sodium/hydrogen exchanger, Na⁺, H⁺ antiporter, PF13_0019, XM_001349726) as the reference.

Genetic diversity was assessed by Nei's unbiased expected heterozygosity (H_e) from haploid data and calculated as $H_e = [n/(n-1)][1 - p_i]$ (n = the number of isolates sampled; p_i = the frequency of the i th allele).²⁸ Population genetic differentiation was measured using Wright's F statistics (F_{st}).²⁹ A P value < 0.05 was considered statistically significant.

Nucleotide sequences of new *ms4760* haplotypes were deposited in the GenBank database under accession numbers from JX472441 to JX472448.

Among the 595 *P. falciparum* isolates (Madagascar, $N = 345$; Comoros, $N = 250$), 29 different alleles were observed, including 8 alleles not previously described (*ms4760-90* to *ms4760-97*) (Table 1). *ms4760-1* was the most prevalent (180/595; 30.3%) followed by *ms4760-3* (96/595; 16.1%), *ms4760-7* (77/595; 12.9%), and *ms4760-6* or *ms4760-9* (43/595; 7.2%); 15 *ms4760* alleles (*ms4760-1*, *ms4760-2*, *ms4760-3*, *ms4760-6*, *ms4760-7*, *ms4760-8*, *ms4760-9*, *ms4760-12*, *ms4760-27*, *ms4760-29*, *ms4760-30*, *ms4760-35*, *ms4760-91*, *ms4760-95*, and *ms4760-96*) were distributed in both countries, whereas others were exclusively found in Madagascar ($N = 10$; *ms4760-19*, *ms4760-22*, *ms4760-31*, *ms4760-32*, *ms4760-33*, *ms4760-42*, *ms4760-92*, *ms4760-93*, *ms4760-94*, and *ms4760-97*) or the Comoros ($N = 4$; *ms4760-5*, *ms4760-14*, *ms4760-34*, and *ms4760-90*). Details are given in Table 1, and multiple amino acid sequence alignments are shown in Figure 2.

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FIGURE 1. Samples collection sites in the Comoros Islands and Madagascar from 2006 to 2007.

The distribution and prevalence of the alleles were significantly different between countries ($P < 0.0001$) and within country only for isolates from the Comoros (between Anjouan/Mohéli and Grande Comore, $P = 0.003$ and $P = 0.02$, respectively).

According to the number of repeats in block II (DNNND) and block V (NHNDNHNNDDDD), which have been associated with modulation of *in vitro* susceptibility to QN,¹³ *ms4760* alleles were grouped in 12 different profiles (from *ms4760-A* to *ms4760-L*) as presented in Table 2. The number of repeats in block II (DNNND) varied from zero (*ms4760-A*) to four (*ms4760-K* and *ms4760-L*), whereas the number of repeats in block V (NHNDNHNNDDDD) varied from zero (*ms4760-E*) to three (*ms4760-D* and *ms4760-H*); 73% of isolates were grouped in three profiles: *ms4760-C* ([DNNND]₁; [NHNDNHNNDDDD]₂; 20.5%), *ms4760-G* ([DNNND]₂; [NHNDNHNNDDDD]₂; 32.3%), and *ms4760-I* ([DNNND]₃; [NHNDNHNNDDDD]₁; 20.3%). Seven profiles (*ms4760-B*, -C, -D, -F, -G, -I, and -J) were found in both countries, four profiles (*ms4760-A*, -E, -H, and -L) were found only in Madagascar, and one profile (*ms4760-H*) was found only in the Comoros. The mean number of DNNND repeats was significantly higher in the Comoros (2.21) compared with Madagascar (1.93; $P < 0.001$). Inversely, the number of NHNDNHNNDDDD repeats was significantly lower in the Comoros (1.60) compared with Madagascar (1.73; $P = 0.005$). Consequently, the mean ratio of DNNND/NHNDNHNNDDDD repeats was significantly higher in the Comoros (1.77 versus 1.47; $P < 0.001$). The prevalence of the *pfprhe-1* *ms4760* profiles according to the geographical location of the isolates (country and region) significantly differed between the two countries ($P <$

0.0001). In both countries, three profiles were predominant (*ms4760-G*, 32.3%; *ms4760-I*, 20.3%; *ms4760-C*, 20.5%). Two profiles (*ms4760-I*, $P < 0.0001$; *ms4760-K*, $P = 0.006$) were significantly more frequent in the Comoros, and one profile (*ms4760-C*, $P = 0.02$) was significantly more frequent in Madagascar.

Genetic diversity, assessed by Nei's unbiased expected heterozygosity (H_e), was similar between countries (Madagascar = 0.84, ranging from 0.75 for the southeast area to 0.85 for the central west area; Comoros = 0.85, ranging from 0.80 for Grande Comore to 0.87 for Mohéli). However, the degree of genetic differentiation of the *ms4760* profiles within parasite populations, estimated by F_{st} values, indicated a large divergence between Grande Comore populations and Malagasy populations from the northwest, central east, west, and southwest areas (Table 3).

The data represented here are an extension of our previous study performed in 2010.¹⁴ By using a large number of *P. falciparum* isolates from Indian Ocean malaria-endemic areas (Comoros and Madagascar), we confirm the extended polymorphisms of *ms4760* allele in *pfprhe-1* gene in this region. Among the 595 studied sequences, we have observed 29 different alleles, including 8 new alleles (27%). By compiling our data with previous published sequences available in GenBank,^{14–24} we estimate that 101 different *ms4760* alleles have been described to date. However, in most publications, the numbering of the *ms4760* alleles did not always taking into account the previously described alleles, making data comparison difficult. This finding raises the need to establish a standard nomenclature for *ms4760* alleles.

FIGURE 2. Amino acid alignment of 29 *ms4760* haplotypes found in the Comoros Islands and Madagascar from 2006 to 2007.

As expected and found in previous studies, four alleles (*ms4760-1*, *ms4760-3*, *ms4760-6*, and *ms4760-7*) were predominant in both countries.^{14,15,17-22} However, significant differences in the distribution and prevalence of allele were observed both between countries and within sites in the Comoros (Table 1). The genetic diversity of the *ms4760* allele observed in our study was similar between both countries (0.84 and 0.85), and it was comparable with the genetic diversity previously described in African (Congo = 0.76, Uganda = 0.79, and Kenya = 0.66)^{15,17,20} and Indian isolates (0.68)²³ and significantly higher than the diversity found in Asian isolates (China/Myanmar = 0.68, $P = 0.04$; Vietnam = 0.49, $P < 0.0001$).^{19,22} This situation is likely reflecting the level of malaria transmission, but it also could be related to the prevalence of resistant parasites to quinoline antimalarial drugs. This latter hypothesis is strengthened by our data, which show that isolates from the Comoros (an area with a high prevalence of antimalarial drugs resistance, although specific data about QN resistance are lacking) had significantly more repeats in block II (DNNND) than those isolates from Madagascar (a low drug-resistance area); these findings are consistent with some previous findings observed in culture-adapted parasites from Asia,^{13,18,19,21,22} India,²³ and East Africa.²⁰

In conclusion, current observations from molecular surveys that aimed to define an association between potential contributors to QN resistance, such as *ms4760* allele polymorphism, have generated conflicting data and do not allow for proposing a simple molecular typing methodology of global applica-

tion based on this molecular marker. The level of genetic diversity observed in the present study was comparable with the level found in African countries and not comparable with the level found in Asian countries, where the *pfhne-1* polymorphisms seemed more often usable as molecular markers of QN resistance.^{19,22} The higher mean number of DNNND repeats found in isolates from the Comoros compared with Madagascar underlined the importance of the geographical origin of parasites, even at this regional level. Additional studies are required to better define the mechanisms underlying QN resistance, which involve multiple gene interactions.

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TABLE 2

*Pf*hne-1 *ms4760* profile groups according to the number of repeats in block II (DNNND) and block V (DDNHNDNHND) and the geographical location of the isolates

<i>ms4760</i> alleles number	<i>ms4760</i> profiles	No. of allele	Block II (DNNND)	Block V (DDNHNDNHND)	Frequency (%)	
					Comoros Islands	Madagascar
33	<i>ms4760-A</i>	1	0	2	0	0.3
2, 35, 42, 92	<i>ms4760-B</i>	4	1	1	1.2	4.9
3, 30, 31, 91, 93	<i>ms4760-C</i>	5	1	2	13.6	25.5
12	<i>ms4760-D</i>	1	1	3	3.6	2.3
94	<i>ms4760-E</i>	1	2	0	0	0.6
6, 29, 32	<i>ms4760-F</i>	3	2	1	11.2	9.0
1, 19, 27	<i>ms4760-G</i>	3	2	2	34.0	31.0
22	<i>ms4760-H</i>	1	2	3	0	0.6
7, 14, 90, 95, 96	<i>ms4760-I</i>	5	3	1	28	14.8
8, 9	<i>ms4760-J</i>	2	3	2	5.6	10.4
5, 34	<i>ms4760-K</i>	2	4	1	2.8	0
97	<i>ms4760-L</i>	1	4	2	0	0.6

TABLE 3
Pairwise population genetic distances (F_{st} according to Weir and Cockerham)²⁹

	Comoros Islands		Madagascar					
	Mohéli	Anjouan	Northeast	Northwest	Central east	Central west	Southeast	Southwest
Comoros Islands								
Grande Comore	0.037	0.003	0.033	0.0014*	0.0014*	0.0014*	0.020	0.0014*
Mohéli		0.346	0.490	0.018	0.114	0.0389	0.232	0.109
Anjouan			0.58	0.119	0.019	0.003	0.125	0.018
Madagascar								
Northeast				0.115	0.843	0.785	0.479	0.540
Northwest					0.051	0.037	0.380	0.061
Central east						0.183	0.193	0.115
Central west							0.680	0.443
Southwest								0.305
Southeast								

* $P > 0.05$.

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