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# Association between Polymorphisms in the *Pfmdr6* Gene and *Ex Vivo* Susceptibility to Quinine in *Plasmodium falciparum* Parasites from Dakar, Senegal

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**ABSTRACT** Polymorphisms and the overexpression of transporter genes, especially of the ATP-binding cassette superfamily, have been involved in antimalarial drug resistance. The objective of this study was to use 77 Senegalese *Plasmodium falciparum* isolates to evaluate the association between the number of Asn residues in the polymorphic microsatellite region of the *Plasmodium falciparum* multidrug resistance 6 gene (*Pfmdr6*) and the *ex vivo* susceptibility to antimalarials. A significant association was observed between the presence of 7 or 9 Asn repeats and reduced susceptibility to quinine.

**KEYWORDS** malaria, *Plasmodium falciparum*, antimalarial drug, *in vitro*, resistance, molecular marker

The resistance of malaria to most antimalarial drugs has developed in Southeast Asia and has spread to Africa. The World Health Organization (WHO) has recommended artemisinin-based combination therapy (ACT) as the first-line treatment for malaria since 2005. As recently described in Southeast Asia, the emergence of *Plasmodium falciparum* resistance to artemisinin and its derivatives manifests as delayed parasite clearance following treatment with either artesunate (AS) monotherapy or ACT (1, 2). In areas where artemisinin resistance is emerging, the partner drugs within the combination are under increasing pressure for the selection of resistance. In this context, the identification of molecular markers of resistance to these partner drugs is urgently needed to monitor the emergence and spread of resistance to antimalarial drugs.

Polymorphisms and the overexpression of transporter genes, especially of the ATP-binding cassette (ABC) superfamily, have been involved in antimalarial drug resistance (3). The N86Y mutation of the *P. falciparum* multidrug resistance 1 gene (*Pfmdr1*) is associated with *in vitro* susceptibility to dihydroartemisinin (DHA), lumefantrine (LMF), monodesethylamodiaquine (DQ), and mefloquine (MQ) (4). Field studies in East Africa have also shown selection of the N86 allele in recurrent infections after treatment with artemether plus lumefantrine (5, 6) or artesunate plus mefloquine (7). A single

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mutation (F423Y) in the heavy metal transporter *Pfmdr2* was linked to *in vitro* resistance to pyrimethamine (8). Additionally, deletion of *Pfmdr2* in *P. falciparum* parasites resulted in a minor decrease in susceptibility to mefloquine, quinine (QN), and atovaquone (9). Repetitive amino acid motifs in *Pfmdr5* were associated with reduced *in vitro* susceptibility to lumefantrine (10). In another report, the presence of 9 Asn residues in the polymorphic microsatellite region of *Pfmdr6* (amino acid positions 103 to 109 in 3D7) appeared to modulate the *in vitro* dihydroartemisinin susceptibility of parasites from the China-Myanmar border area (11). Further, the presence of 7 Asn repeats was significantly associated with reduced susceptibility to lumefantrine. Another study on 16 reference strains from different sources worldwide and parasites collected in Kenya showed that parasites with 6 Asn repeats were significantly less susceptible to lumefantrine, whereas those with 8 Asn repeats were less susceptible to piperazine (PPQ) (10). There was no association of 9 Asn repeats with reduced susceptibility to dihydroartemisinin.

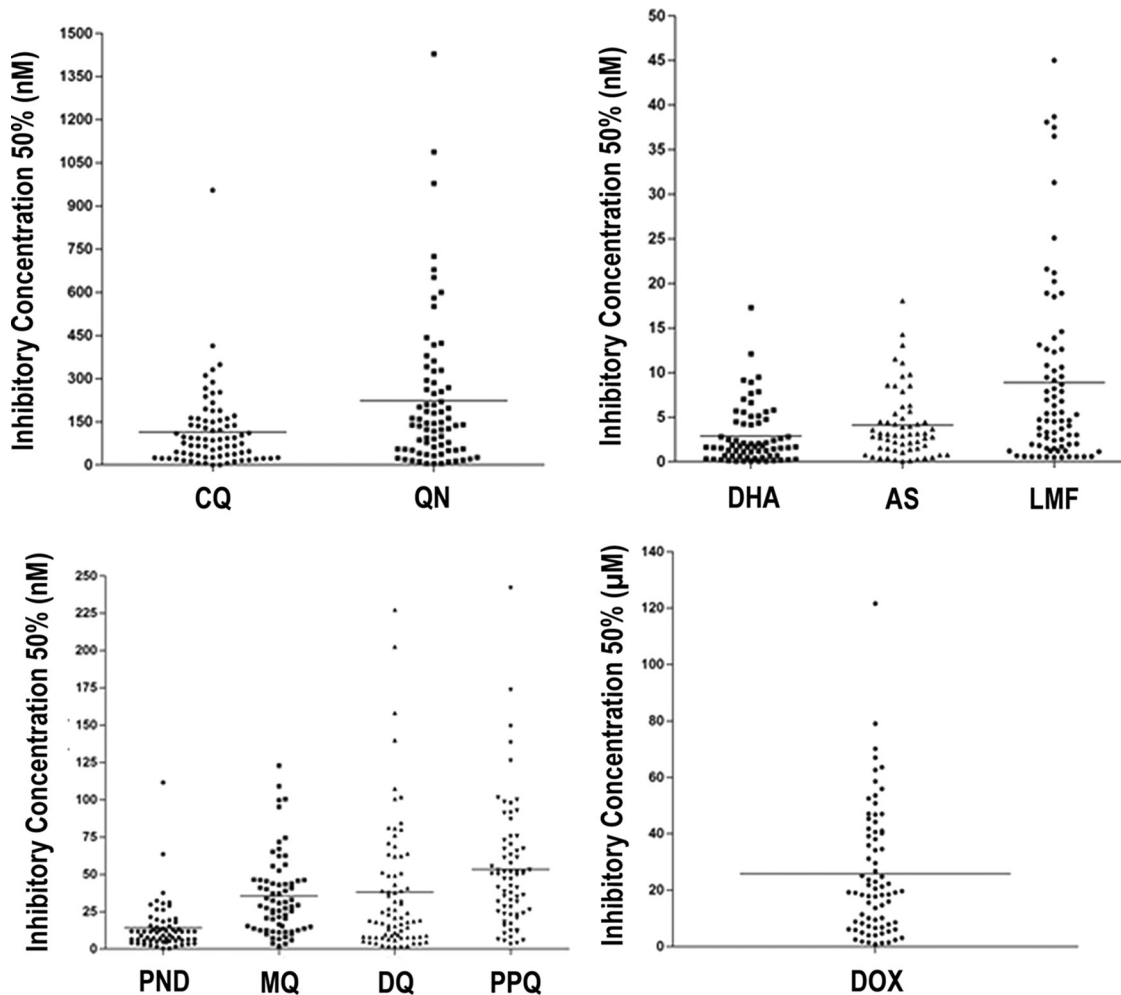
The objective of this study was to evaluate the association between the Asn number in *Pfmdr6* (accession number [PF3D7\\_1352100](https://www.ncbi.nlm.nih.gov/nuccore/PF3D7_1352100)) from Senegalese isolates of *P. falciparum* and *ex vivo* susceptibilities to chloroquine (CQ), quinine (QN), monodesethylamodiaquine (DQ), mefloquine (MQ), lumefantrine (LMF), piperazine (PPQ), pyronaridine (PND), dihydroartemisinin (DHA), artesunate (AS), and doxycycline (DOX).

Seventy-seven *P. falciparum* isolates from *falciparum* malaria patients attending the Hôpital Principal de Dakar from November 2013 to January 2014, August 2014 to December 2014, and September to November 2015 were successfully evaluated (13 from 2013, 32 from 2014, and 32 from 2015). Seventy-four percent of the patients were recruited from the emergency department. The other patients were recruited from the pediatric department (8%), the intensive care unit (5%), and other units (13%). There was no information available on antimalarial treatment prior to admission. The patients were treated with quinine or artesunate or artemether-lumefantrine, with or without doxycycline, at the Hôpital Principal de Dakar. Informed verbal consent was obtained from the patients or their parents/guardians before blood collection. The study was approved by the ethical committee of the Hôpital Principal de Dakar.

Venous blood samples were collected in Vacutainer ACD tubes prior to patient treatment. A malaria diagnosis was confirmed using a thin blood smear and a rapid diagnostic test. Thin blood smears were stained using a RAL kit (Réactifs RAL, Paris, France) based on eosin and methylene blue and were examined to determine *P. falciparum* density and to confirm species monoinfection. The level of parasitemia ranged from 0.06% to 14.1%.

The susceptibility of the isolates was assessed without culture adaptation. For the *in vitro* MicroTests, 100  $\mu$ l of parasitized red blood cells (final parasitemia, 0.5%; final hematocrit, 1.5%) was aliquoted into 96-well plates predosed with antimalarial drugs (CQ, QN, MQ, DQ, LMF, DHA, AS, PPQ, PND, and DOX). The plates were incubated in a sealed bag for 72 h at 37°C with atmospheric generators for capnophilic bacteria using Genbag CO<sub>2</sub> at 5% CO<sub>2</sub> and 15% O<sub>2</sub> (bioMérieux, Marcy l'Etoile, France). The *ex vivo* HRP2 enzyme-linked immunosorbent assay (ELISA) using the commercial Malaria Ag competitive enzyme-linked immunosorbent assay (CELISA) kit (reference number KM2159; Cellabs Pty Ltd., Brookvale, Australia) was previously described (12). The batches of plates were tested and validated on the chloroquine-resistant W2 strain (Indochina) (MR4, VA, USA) in three to six independent experiments using the same conditions. The mean 50% inhibitory concentrations (IC<sub>50</sub>s) in the *in vitro* chemosusceptibility assay for the chloroquine-resistant W2 strain for the different batches used during the 3 years were 292 nM for CQ, 275 nM for QN, 72 nM for DQ, 13.7 nM for LMF, 15.4 nM for MQ, 32.5 nM for PPQ, 26.4 nM for PND, 1.27 nM for DHA, 1.43 nM for AS, and 10.7  $\mu$ M for DOX.

*Pfmdr6* (accession number [PF3D7\\_1352100](https://www.ncbi.nlm.nih.gov/nuccore/PF3D7_1352100)) was amplified by PCR using the following primer pair: 5'-GAG-AAG-TAA-TAG-AAT-AAG-CG-3' and 5'-CCC-ATA-CAT-AAA-ATC-TTC-CT-3'. The reaction mixture consisted of 200 ng of genomic DNA, 0.32  $\mu$ M of each primer, 1 $\times$  final concentration of the reaction buffer [750 mM Tris-HCl, 200 mM



**FIG 1** Distribution of *ex vivo* responses of 77 *P. falciparum* clinical field isolates from Dakar to dihydroartemisinin (DHA), artesunate (AS), lumefantrine (LMF), mefloquine (MQ), pyronaridine (PND), monodesethylamodiaquine (DQ), piperiaquine (PPQ), chloroquine (CQ), quinine (QN), and doxycycline (DOX).

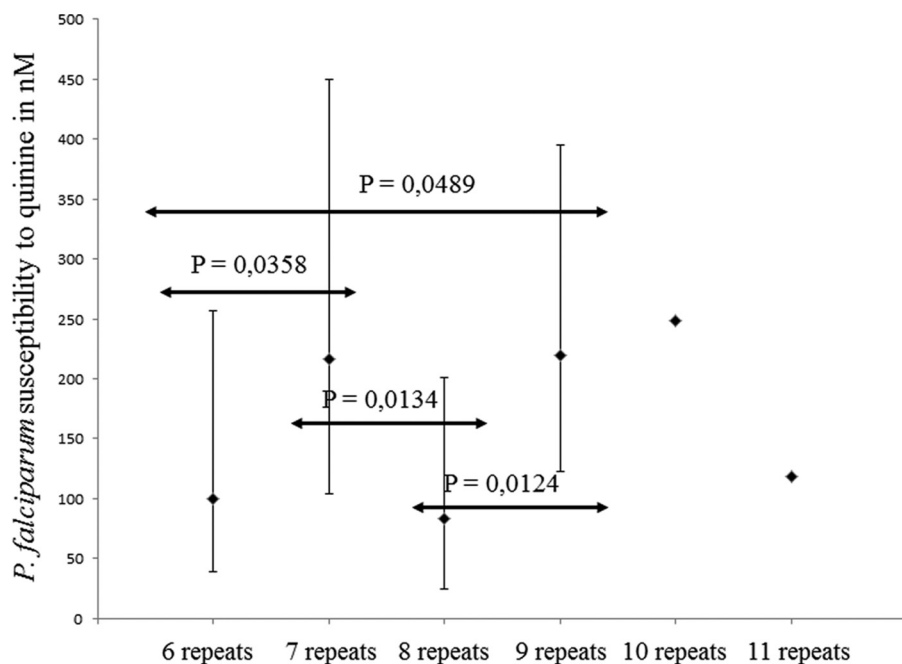
( $\text{NH}_4$ )<sub>2</sub>SO<sub>4</sub>, 0.1% (vol/vol) Tween 20, and stabilizer, pH 8.8], 2.5 mM MgCl<sub>2</sub>, 200  $\mu\text{M}$  deoxynucleoside triphosphate (dNTP) mixture, and 0.2 U of Red Diamond *Taq* polymerase (Eurogentec) in a final volume of 25  $\mu\text{l}$ . The thermal cycler (T3 Biometra) was programmed as follows: 10 min at 94°C followed by 40 cycles of 30 s at 95°C, 45 s at 52°C, and 1 min at 72°C and a final extension for 10 min at 72°C. The purified amplicons were sequenced using the appropriate PCR primers on an ABI Prism 3100 analyzer (Applied Biosystems) according to the manufacturer's instructions. The sequences were analyzed using Vector NTI Advance software (version 11; Invitrogen, Cergy-Pontoise, France) to identify the number of asparagine repeats in *Pfmdr6* and their association with antimalarial drug resistance.

The IC<sub>50</sub>s in the *ex vivo* chemosusceptibility assay ranged from 6.3 to 954.9 nM for CQ, 6.2 to 1,429.8 nM for QN, 1.9 to 227.3 nM for DQ, 0.6 to 45.0 nM for LMF, 3.4 to 123.0 nM for MQ, 3.9 to 241.9 nM for PPQ, 0.4 to 111.6 nM for PND, 0.1 to 17.3 nM for DHA, 0.1 to 18.1 nM for AS, and 0.9 to 121.6  $\mu\text{M}$  for DOX. The distributions of the IC<sub>50</sub>s for the 10 antimalarial drugs are shown in Fig. 1. Six different poly(Asn) repeat profiles, with 6 to 11 repeats, were detected in *Pfmdr6* among the 77 *P. falciparum* isolates from Dakar. The highest proportion of isolates in our study had 6 Asn repeats (41.6% of the samples), contrary to results for Asian isolates, in which 8 repeats predominated (76.5% of the samples) (11). The proportions of isolates with 8, 7, 9, 10, and 11 repeats were 31.1%, 14.3%, 10.4%, 1.3%, and 1.3%, respectively. There was no significant difference

**TABLE 1** *Ex vivo* susceptibility of 77 *Plasmodium falciparum* isolates to quinine, chloroquine, monodesethylamodiaquine, lumefantrine, mefloquine, piperazine, pyronaridine, dihydroartemisinin, artesunate, and doxycycline according to the number of Asn repeats in *Pfmdr6*<sup>a</sup>

Drug	Values according to no. of repeats						P value
	6 repeats	7 repeats	8 repeats	9 repeats	10 repeats	11 repeats	
<b>Quinine</b>							
IC <sub>50</sub> geometric mean (nM) (no.)	99.5 (32)	216.4 (11)	82.9 (24)	219.7 (8)	248.0 (1)	218.0 (1)	0.0291
Median (nM)	123.8	294.0	115.6	171.7			
25 intercentile to 75 intercentile range	50.3–240.5	150.5–402.0	33.6–230.6	145.2–318.1			
% of resistant parasites	9.4	9.1	4.2	12.5			
<b>Chloroquine</b>							
IC <sub>50</sub> geometric mean (nM) (no.)	67.7 (32)	38.4 (11)	80.9 (24)	30.5 (8)	311.0 (1)	266.0 (1)	0.1230
Median (nM)	93.2	65.6	94.0	31.2			
25 intercentile to 75 intercentile range	24.5–190.5	34.1–113.5	46.0–139.3	30.6–35.0			
% of resistant parasites	56.3	36.4	58.3	12.5			
<b>Monodesethylamodiaquine</b>							
IC <sub>50</sub> geometric mean (nM) (no.)	19.7 (32)	24.9 (11)	22.1 (24)	11.8 (8)	71.0 (1)	43.2 (1)	0.5857
Median (nM)	23.8	19.1	23.0	11.8			
25 intercentile to 75 intercentile range	8.0–49.6	8.8–65.7	12.4–52.5	8.2–18.3			
% of resistant parasites	21.9	27.3	25.0	12.5			
<b>Lumefantrine</b>							
IC <sub>50</sub> geometric mean (nM) (no.)	3.9 (32)	6.1 (11)	5.3 (24)	2.4 (8)	13.9 (1)	4.6 (1)	0.5017
Median (nM)	4.3	4.7	6.2	2.4			
25 intercentile to 75 intercentile range	1.4–12.4	2.3–23.0	2.4–9.5	0.6–7.8			
% of resistant parasites	0	0	0	0			
<b>Mefloquine</b>							
IC <sub>50</sub> geometric mean (nM) (no.)	24.9 (31)	22.4 (10)	31.9 (22)	19.4 (8)	46.6 (1)	43.9 (1)	0.6229
Median (nM)	29.1	25.1	33.0	28.8			
25 intercentile to 75 intercentile range	13.8–41.1	15.6–40.8	10.4–36.4	10.4–36.4			
% of resistant parasites	41.9	40.0	50.0	50.0			
<b>Piperazine</b>							
IC <sub>50</sub> geometric mean (nM) (no.)	42.7 (31)	24.2 (10)	38.7 (22)	44.1 (8)	51.5 (1)	51.7 (1)	0.4444
Median (nM)	50.4	33.6	37.5	47.1			
25 intercentile to 75 intercentile range	34.9–91.1	8.8–57.7	20.8–72.9	30.9–64.1			
% of resistant parasites	6.5	0	9.1	0			
<b>Pyronaridine</b>							
IC <sub>50</sub> geometric mean (nM) (no.)	8.4 (30)	10.8 (10)	8.5 (22)	10.1 (8)	21.4 (1)	13.4 (1)	0.6135
Median (nM)	9.4	12.6	7.8	9.3			
25 intercentile to 75 intercentile range	6.4–14.0	8.8–26.8	5.4–14.0	6.4–16.2			
% of resistant parasites	0	10.0	4.5	0			
<b>Dihydroartemisinin</b>							
IC <sub>50</sub> geometric mean (nM) (no.)	1.0 (30)	2.6 (10)	1.6 (22)	2.0 (8)	5.6 (1)	1.7 (1)	0.2913
Median (nM)	1.5	2.6	1.8	3.3			
25 intercentile to 75 intercentile range	0.1–2.3	1.6–5.1	0.7–3.2	1.3–4.4			
% of resistant parasites	3.3	0	4.5	0			
<b>Artesunate</b>							
IC <sub>50</sub> geometric mean (nM) (no.)	1.8 (28)	2.1 (10)	2.8 (22)	3.8 (8)	4.5 (1)	4.2 (1)	0.4010
Median (nM)	2.2	2.8	3.1	4.4			
25 intercentile to 75 intercentile range	0.8–3.9	2.2–3.7	1.4–5.9	2.9–6.8			
% of resistant parasites	3.6	0	9.1	0			
<b>Doxycycline</b>							
IC <sub>50</sub> geometric mean ( $\mu$ M) (no.)	16.5 (32)	17.0 (11)	13.1 (23)	18.3 (8)	121.6 (1)	21.9 (1)	0.8828
Median ( $\mu$ M)	20.5	17.9	14.7	22.9			
25 intercentile to 75 intercentile range	8.8–36.1	9.8–29.9	6.0–40.6	15.3–38.4			
% of resistant parasites	21.9	27.3	30.4	37.5			

<sup>a</sup>The threshold values for the reduced *in vitro* susceptibility or resistance were the following: 611 nM, 77 nM, 61 nM, 115 nM, 30 nM, 135 nM, 60 nM, 12 nM, 12 nM, and 37  $\mu$ M for quinine, chloroquine, monodesethylamodiaquine, lumefantrine, mefloquine, piperazine, pyronaridine, dihydroartemisinin, artesunate, and doxycycline, respectively (12, 13).



**FIG 2** Distribution of quinine  $IC_{50}$  of 77 *P. falciparum* isolates according to the number of Asn repeats in *Pfmdr6*.

in *in vivo* susceptibility among the four different poly(Asn) repeat profiles for CQ, PPQ, PND, DQ, MQ, LMF, DHA, AS, and DOX (Table 1). We observed a significant association between 7 or 9 Asn repeats and reduced susceptibility to QN ( $P = 0.0291$ , Kruskal-Wallis) (Table 1 and Fig. 2). Six isolates were over the threshold of 611 nM for *in vitro* resistance to QN (12), but they were distributed in each group. There was no significant difference between the four groups (6, 7, 8, or 9 Asn repeats) in terms of percentage of isolates resistant to QN ( $P = 0.849$ ). However, the isolate with the higher  $IC_{50}$  for quinine (1,429.8 nM) had 9 Asn repeats. This was the first observation of an association between *Pfmdr6* polymorphisms and reduced susceptibility to QN. The observations reported by Okombo et al. linking 6 and 8 Asn repeats with reduced susceptibility to LMF and PPQ, respectively (10), or by Wang et al. linking 7 and 9 Asn repeats with reduced susceptibility to LMF and DHA, respectively (11), were not replicated in our *P. falciparum* parasite population from Dakar. Additionally, the observations reported by Wang et al. and Okombo et al. were not replicated: reduced susceptibility to LMF was associated with 7 or 8 Asn repeats. A hypothesis explaining these differences is that Asn variations were mostly parasite population markers. The susceptibility to QN was associated with poly(Asn) repeat profiles of *Pfmdr6*. All of the patients treated with QN during the 3 years of collection were successfully treated. All of the isolates from these successfully treated patients contained 6 or 8 Asn repeats in the microsatellite region of *Pfmdr6*, which were associated with a low  $IC_{50}$  to QN. QN susceptibility was previously associated with microsatellite repeats in *P. falciparum*  $Na^+/H^+$  exchanger 1 (*Pfnhe-1*) (14, 15). However, this association was location specific and differed between African and Asian *P. falciparum* isolates (15–17). The low number of *P. falciparum* isolates and the modest association limited the interpretation of the present results. It is difficult to extrapolate these findings to other parts of the world without new data sets. Another limitation was the use of standard *in vitro* tests for exploring resistance to artemisinin. The standard *in vitro* test is not adapted to follow resistance to artemisinin derivatives. The clinical resistance to artemisinin was manifested by an increase in the ring-stage survival rate after contact with artemisinin (ring survival test) (18). It is imperative to further assess more isolates from different geographical areas, to associate those results with functional biochemical studies, and to assess samples from clinical failure with QN to ascertain the role of this repeat polymorphism in *Pfmdr6*.

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We declare that we have no competing interests.

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