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Baseline *Ex Vivo* and Molecular Responses of *Plasmodium falciparum* Isolates to Piperaquine before Implementation of Dihydroartemisinin-Piperaquine in Senegal

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ABSTRACT Dihydroartemisinin-piperaquine, which was registered in 2017 in Senegal, is not currently used as the first-line treatment against uncomplicated malaria. A total of 6.6% to 17.1% of *P. falciparum* isolates collected in Dakar in 2013 to 2015 showed *ex vivo*-reduced susceptibility to piperaquine. Neither the exonuclease E415G mutation nor the copy number variation of the *plasmepsin II* gene (*Pfpm2*), associated with piperaquine resistance in Cambodia, was detected in Senegalese parasites.

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

Since 2005, the World Health Organization (WHO) has recommended artemisinin-based combination therapy (ACT) as the first-line treatment against malaria. The emergence of resistance to artemisinins, manifested by delayed parasite clearance after monotherapy with artesunate or ACT, was recently described in Southeast Asia (1, 2). Dihydroartemisinin-piperaquine is one of the most recent ACTs to be commercialized. However, multidrug resistance to dihydroartemisinin-piperaquine is currently emerging in Cambodia, where recrudescence infections have rapidly increased (3–5). Additionally, *in vitro* resistance to piperaquine was detected and increased rapidly between 2013 and 2015 in Cambodia (6).

Very few cases of resistance to dihydroartemisinin-piperaquine have been described in Africa (7). Two cases of late treatment failure after 30 and 32 days was reported in Italian travelers returning from Ethiopia and who were treated with dihydroartemisinin-piperaquine (8, 9).

Dihydroartemisinin-piperaquine obtained marketing authorization in June 2017 in Senegal. Artemether-lumefantrine and artesunate-amodiaquine combinations are currently the ACTs that are most used in Senegal. Dihydroartemisinin-piperaquine demonstrated a high efficacy in the treatment of uncomplicated malaria or in seasonal intermittent preventive treatment in children in Senegal (10–13).

Recently, an increase in the copy number of the *plasmepsin II* gene (*Pfpm2*)

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(PF3D7_1408000), which encodes a protease involved in hemoglobin degradation, has been associated with dihydroartemisinin-piperaquine treatment failures and raised piperaquine 50% inhibitory concentrations (IC₅₀s) in Cambodia (14, 15). The presence of parasites carrying 2 copies of *Pfpm2* was confirmed in 7 infections out of 65 clinical failures to dihydroartemisinin-piperaquine in Mali (16).

The exonuclease gene (PF3D7_1362500) was one of the 40 candidate drug targets for *Plasmodium falciparum* identified by *in silico* prediction (17). The mutation E415G in the exonuclease gene (*exo-E415G*) was correlated with the high IC₅₀ of piperaquine in 297 isolates from Cambodia (Pursat, Preah, and Ratanakiri) obtained from 2011 to 2013 (15). This *exo-E415G* mutation was associated with parasite recrudescence following dihydroartemisinin-piperaquine treatment.

The aim of this study was to establish a susceptibility baseline of *P. falciparum* parasites to piperaquine before the implementation of the dihydroartemisinin-piperaquine combination in Senegal by evaluating *P. falciparum* parasite *ex vivo* susceptibility to piperaquine and the prevalence of the molecular markers potentially involved in piperaquine resistance.

A total of 76 *P. falciparum* clinical isolates collected by venous blood samples before treatment from falciparum malaria patients treated at the Hôpital Principal de Dakar from November 2013 to January 2014, August to December 2014, and September to November 2015 were assessed for *ex vivo* susceptibility to piperaquine and evaluated for the copy number of *Pfpm2* and the *exo-E415G* mutation. Seventy-five percent of the patients were recruited from the emergency department. The patients were treated with quinine until 2014 and then with artesunate, artemether-lumefantrine, or artesunate-amodiaquine.

Thin blood smears were stained using a RAL kit (Réactifs RAL, Paris, France) based on eosin and methylene blue and were examined to determine the infection by *P. falciparum* and parasitemia. Parasitemia ranged from 0.06% to 14.4%. The diagnosis of monospecies infection by *P. falciparum* was confirmed by real-time PCR (LightCycler 2.0; Roche Group, Basel, Switzerland), as previously described (18).

The susceptibility of the isolates was assessed without culture adaptation. The parasitized red blood cells (final maximum parasitemia, 0.5%; final hematocrit, 1.5%) were incubated in 96-well plates predosed with antimalarial drugs (piperaquine [PPQ], chloroquine [CQ], quinine [QN], monodesethylamodiaquine [DQ], mefloquine [MQ], lumefantrine [LMF], and dihydroartemisinin [DHA]) for 72 h at 37°C with atmospheric generators for capnophilic bacteria using Genbag CO2 at 5% CO₂ and 15% of O₂ (bioMérieux, Marcy l'Etoile, France), as previously described (19, 20). PPQ was first dissolved in methanol and then diluted in water to final concentrations that ranged from 1.9 to 998 nM. An *ex vivo* histidine-rich protein 2 (HRP2)-based enzyme-linked immunosorbent assay (ELISA) was then performed using a commercial Malaria Ag competitive enzyme-linked immunosorbent assay (CELISA) kit (reference no., KM2159; Cellabs PTY Ltd., Brookvale, Australia), as previously described (19, 20). Each batch of 96-well plates predosed with antimalarial drugs was validated on the CQ-resistant W2 strain (isolated in Indochina; obtained from MR4, VA, USA) in four independent experiments using the same conditions described below. The mean 50% effective concentration (EC₅₀) value (effective concentration of a drug that prevents 50% of the growth observed in an untreated control) for the W2 strain for the different batches used during the 3 years was 32.5 nM for PPQ (and 292 nM for CQ, 275 nM for QN, 72 nM for DQ, 13.7 nM for LMF, 15.4 nM for MQ, and 1.27 nM for DHA). A comparison of W2 susceptibility data for the 7 antimalarial drugs indicated that there was no significant difference in the responses to antimalarial drugs over the 3 years (0.39 < *P* < 0.95). Additionally, the polymorphic genetic markers *msp1* and *msp2* and microsatellite markers specific to *P. falciparum* were genotyped at least once a month to verify W2 clonality (21, 22).

The copy number of *Pfpm2* (PF3D7_140800) was assessed by quantitative PCR on the LightCycler 2.0 (Roche, Germany), as previously described (23). Isolates with a copy number greater than 1.6 were classified as isolates with 2 copies (14). An isolate

collected from a patient with imported malaria after returning from Cambodia and harboring 2 copies of *Pfpm2* was used as positive control (sample kindly provided by S. Houzé).

The two exons of the exonuclease gene (PF3D7_1362500) were amplified by PCR using the following primer pairs: Exo1_F, 5'-GGAACATAAGAAGGGTACTGAGC-3' and Exo1_R, 5'-ATCTGATGATAGGAGGTGCAA-3' for exon 1; and Exo2_F, 5'-GAATGGAGTCATTTAGCAGCAA-3' and Exo2_R, 5'-CATTCCCATGTACTATCTTTGAACTT-3' for exon 2. For each exon, a standard PCR was carried out with a 25- μ l total reaction mixture containing 200 ng of genomic DNA, 0.32 μ M each primer, 1 \times final concentration of reaction buffer (20 mM Tris-HCl, 1 mM dithiothreitol [DTT], 0.1 mM EDTA, 0.1 M KCl, 0.5% [vol/vol] Nonidet P40, 0.5% [vol/vol] Tween 20; pH 8.0), 2.5 mM MgCl₂, 200 μ M dNTP mixture, and 1 U of Red Diamond *Taq* polymerase (Eurogentec, Belgium). The thermal cycler (T3 Biometra) was programmed as follows: 5 min at 95°C; 40 cycles of 30 sec at 95°C, 30 sec at 50°C, and 1 min 15 sec at 72°C; and a final extension for 10 min at 72°C. The amplified fragments were sequenced using the two PCR forward primers on an ABI Prism 3100 analyzer (Applied Biosystems) according to the manufacturer's instructions. The sequences were analyzed using Vector NTI advance software (v11; Invitrogen, Cergy Pontoise, France) to identify the mutations recently shown to be involved in the *ex vivo* susceptibility to PPQ.

The Mann-Whitney U test was used to compare susceptible and resistant groups. Differences were considered statistically significant if the *P* value was <0.05. Analyses and graphics were performed using XLSTAT software v19.4 (Addinsoft).

Biobanking of human clinical samples used for malaria diagnostics and secondary uses for scientific purposes is possible as long as the corresponding patients are informed and have not indicated any objections. This requirement was fulfilled here by giving verbal information to the patients, and no immediate or delayed patient opposition was reported to the hospital clinicians. Verbal consent was obtained from all the patients or their parents/guardians before blood collection in Dakar. The ethical committee of the Hôpital Principal de Dakar approved the study.

None of the 76 *P. falciparum* Senegalese isolates exhibited a bimodal dose-response curve when exposed to PPQ, unlike the Cambodian parasites, and displayed usual sigmoidal dose-response curves (24–26). The distribution of the EC₅₀ values for PPQ and the other six antimalarial drugs is shown in Fig. 1. The *ex vivo* PPQ EC₅₀ of the 76 isolates ranged from 2.5 to 242 nM. This wide range of PPQ responses is in accordance with previous studies on African parasites assessed using a 42-h isotopic test or a 72-h HRP2 ELISA detection (23, 27, 28). Only the isolates collected in 2016 in Uganda and assessed using a 72-h fluorescence assay with SYBR green I detection presented a narrow range for PPQ EC₅₀, from 1.8 to 26.6 nM (29), unlike those collected in 2010 to 2013, but they were evaluated using HRP2 ELISA detection (3.1 to 188.9 nM) (28). EC₅₀ values depend on methodology, i.e., incubation conditions (gas and time) or detection method (30–33). A limitation could be the use of the standard *ex vivo* assay to assess PPQ susceptibility and the not PPQ survival assay (PSA) (24). However, PPQ susceptibility is currently more frequently estimated by a standard assay than PSA (15, 25, 28, 29, 34, 35). Amato et al. showed that *P. falciparum* parasites that were resistant *in vitro* to PPQ using PSA and associated with amplification of *Pfpm2* also had high EC₅₀ values ranging from 89.3 to 159.6 nM using the *in vitro* standard assay (15).

A previous Bayesian analysis on African isolates estimated that parasites with EC₅₀ values greater than 135 nM were considered parasites with reduced susceptibility to PPQ (27). In the present study, 6.6% of the Senegalese isolates were considered parasites with reduced susceptibility to PPQ and showed EC₅₀ values ranging from 139 to 242 nM. In another study on Cambodian isolates, *P. falciparum* strains resistant *ex vivo* to PPQ using PSA and associated with amplification of *Pfpm2* were also evaluated using the *in vitro* standard assay (15). These Cambodian *P. falciparum* strains, which were resistant to PPQ, showed EC₅₀ values ranging from 89.3 to 159.6 nM using the *in vitro* standard assay, suggesting that parasites with EC₅₀ values above 90 nM were resistant *ex vivo* to PPQ and associated with *in vitro* resistance using PSA and amplifi-

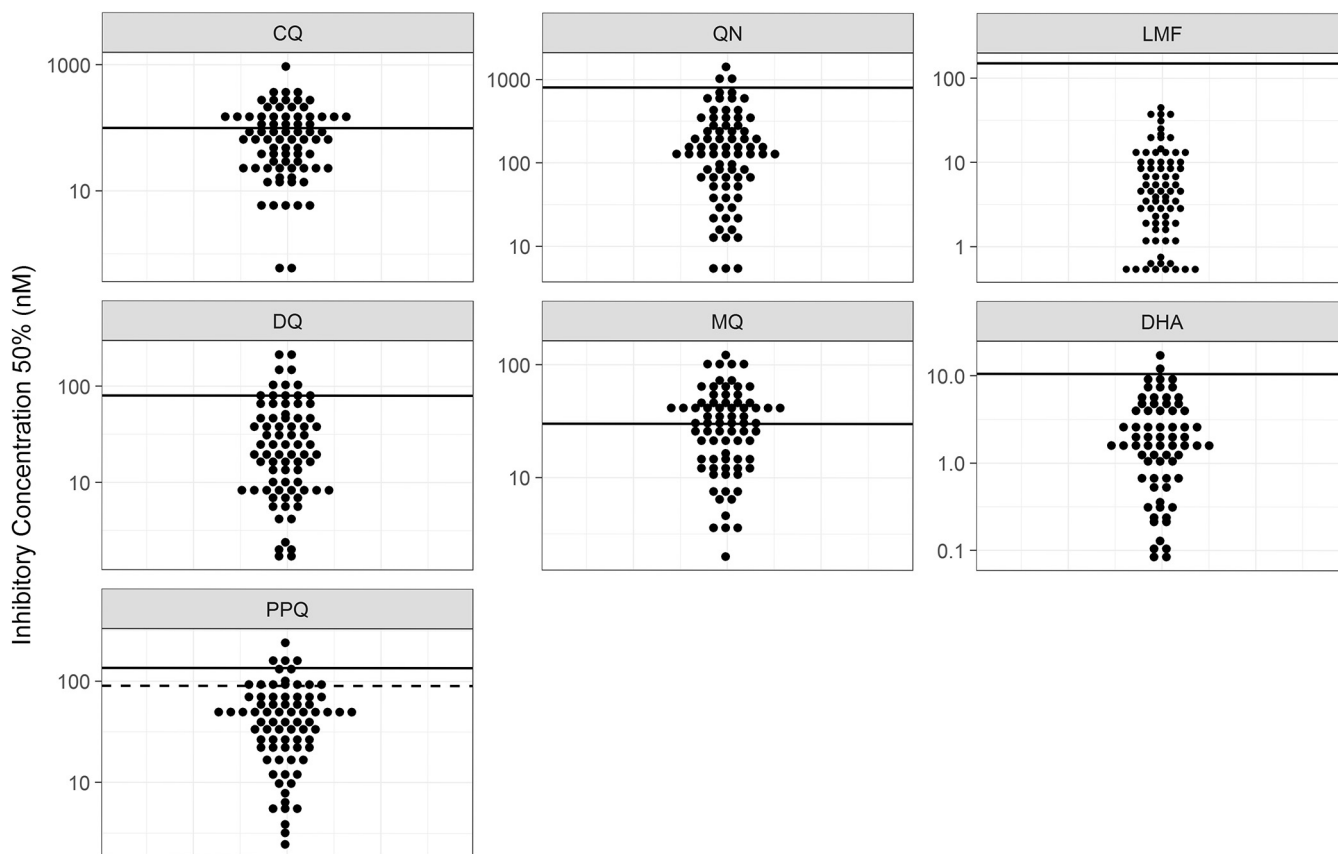


FIG 1 Distribution of *ex vivo* responses of 76 *P. falciparum* clinical field isolates from Dakar, Senegal, to piperazine (PPQ), monodesethylamodiaquine (DQ), mefloquine (MQ), chloroquine (CQ), quinine (QN), lumefantrine (LMF), and dihydroartemisinin (DHA). The black line represents the cutoff for *ex vivo*-reduced susceptibility.

cation of *Pfpm2*. We deduced a new cutoff at 90 nM for PPQ reduced susceptibility. In the present study, a total of 17.1% of Senegalese isolates showed an EC_{50} above 90 nM. We analyzed the data from Dakar by considering the two potential thresholds for PPQ reduced susceptibility. These data would suggest that 6.6% to 17.1% of the *P. falciparum* isolates from Dakar would have reduced susceptibility to PPQ without any current use of DHA-PPQ in Senegal. Significant associations were found between PPQ and DHA (Pearson's coefficient of correlation of 0.56, $P = 0.00002$) and MQ ($r = 0.46$, $P = 0.00003$). Associations between PPQ and LMF ($r = 0.03$, $P = 0.770$), DQ ($r = 0.07$, $P = 0.568$), QN ($r = 0.05$, $P = 0.678$), or CQ ($r = 0.09$, $P = 0.397$) were weak and not significant. Thirty-one percent and 22% of the variation in the *ex vivo* response to PPQ could be explained by variation in response to DHA and MQ, respectively. A significant association between PPQ and MQ was already estimated with African parasites ($r = 0.45$) (23). However, the values of the coefficient of determination remain low to fear cross-resistance between PPQ and DHA or MQ and suggest that parasites that have reduced susceptibility to artemisinin derivatives would also be less susceptible to PPQ. Moreover, all the patients infected with parasites with EC_{50} values ranging between 90 and 100 nM were successfully treated with artesunate or artemether (36). All the patients infected with parasites with EC_{50} values above 100 nM were treated with quinine. The reduced susceptibility to PPQ observed in this study is not linked to cross-resistance to other quinolone-related drugs, such as amodiaquine or lumefantrine, which are both included in the ACTs that have been used in Senegal for a long time. The values of the coefficient of determination between PPQ and LMF ($r^2 = 0.0009$) or PPQ and DQ ($r^2 = 0.0049$) were too low to explain the cross-resistance.

The copy number variation of *Pfpm2* was successfully analyzed for 64 isolates (11

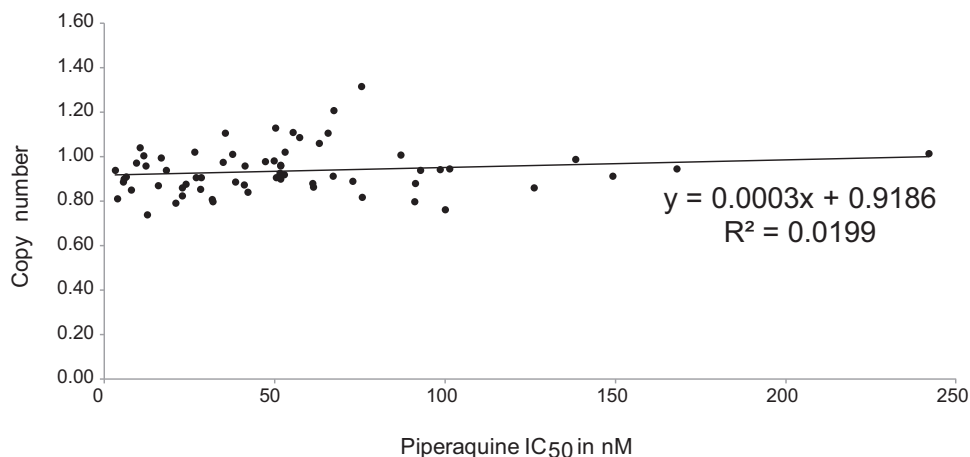


FIG 2 Distribution of the estimated copy numbers of *Pfpm2* according to piperaquine (PPQ) EC₅₀.

from 2013, 29 from 2014, and 24 from 2015). The calculated copy numbers ranged from 0.74 to 1.31, with a mean of 0.94 ± 0.09 in the PPQ susceptible group (EC₅₀ under 135 nM) and 0.96 ± 0.05 in the group with PPQ reduced susceptibility (EC₅₀ above 135 nM). Copy numbers were not significantly different between the susceptible and the resistant group ($P = 0.286$) (Fig. 2). Additionally, there was no difference in copy number variation between *P. falciparum* isolates with an EC₅₀ under or above 90 nM (0.94 ± 0.11 versus 0.91 ± 0.08).

Sequencing of exon 1 and 2 of the exonuclease gene was successfully performed for 76 and 77 isolates, respectively. None of the 77 Senegalese isolates successfully sequenced on exon 2 harbored the *exo-E415G* mutation, although three other mutations were detected in this gene, including S114C (PPQ IC₅₀, 6.4 nM), N419H (PPQ IC₅₀, 41.1 nM), and T435A (PPQ IC₅₀, 65.6 nM). All isolates with a PPQ EC₅₀ above the threshold of 135 nM were wild type. The E415G mutation and, more generally, polymorphisms in the exonuclease gene in Senegalese isolates are not associated with *ex vivo* reduced susceptibility to PPQ, contrary to what has been previously shown in Cambodian parasites (15).

None of the isolates showed amplification of *Pfpm2*, suggesting the absence of PPQ resistance in Dakar. These data are in concordance with those observed in Sierra Leone in 2016 (0%) and in Mozambique in 2015 (1.1%) (37, 38). Rasmussen et al. reported the prevalence of multicopy *Pfpm2* of more than 10% in Uganda (29).

Our results show a prevalence of 6.6% (EC₅₀ above 135 nM) to 17.1% (EC₅₀ above 90 nM) of *P. falciparum* isolates with reduced susceptibility to PPQ in Dakar associated with an absence of *plasmepsin II* amplification.

Only 10% of *P. falciparum* isolates collected from clinical failures to DHA-PPQ in Mali carried 2 copies of *Pfpm2* (16). Additionally, no correlation was observed between the copy number of *Pfpm2* and *ex vivo* susceptibility to PPQ in *P. falciparum* Ugandan isolates (29). The three patients harboring parasites with two copies of *Pfpm2* were successfully treated with DHA-PPQ in Tanzania (39). The use of DHA-PPQ as an intermittent preventive treatment during pregnancy (IPTp) did not select for genotypes associated with resistance in Cambodia, such as the *Pfpm2* or the exonuclease gene (40). No amplification of *Pfpm2* and no *exo-E415G* mutation were found in Cameroonian recrudescing *P. falciparum* parasites 2 years after treatment by DHA-PPQ (41). Additionally, parasites collected from a malaria case imported from Ethiopia after DHA-PPQ failure had a single copy of *Pfpm2* (9). All these data suggest that *Pfpm2* is not the only gene involved in PPQ resistance in Africa. Other genes must be studied, such as the RING E3 ubiquitin protein ligase gene (42) or the *P. falciparum* multidrug resistance 6 gene (*Pfmdr6*) (43). The K76T mutation in the *P. falciparum* chloroquine resistance transporter gene (*Pfcrtr*) which is involved in CQ resistance is not involved in *in vivo* or

ex vivo PPQ resistance (44, 45), but new mutations in this gene, such as H97Y, F145I, M343L, or G353V, could also be involved (25, 35).

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We declare we have no competing interests.

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