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To cite this version:
Bécaye Fall, Cheikhou Camara, Mansour Fall, Aminata Nakoulima, Pierre Dionne, et al.. Plasmodium falciparum susceptibility to standard and potential anti-malarial drugs in Dakar, Senegal, during the 2013–2014 malaria season. Malaria Journal, BioMed Central, 2015, 14 (60), 10.1186/s12936-015-0589-3. hal-01220028

HAL Id: hal-01220028
https://hal-amu.archives-ouvertes.fr/hal-01220028
Submitted on 23 Oct 2015

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Plasmodium falciparum susceptibility to standard and potential anti-malarial drugs in Dakar, Senegal, during the 2013–2014 malaria season

Bécaye Fall¹, Cheikhou Camara², Mansour Fall³, Aminata Nakoulima⁴, Pierre Dionne⁵, Bakary Diatta³, Yaya Diemé¹, Boubacar Wade⁶ and Bruno Pradines¹,⁷,⁸,⁹*

Abstract

Background: In 2006, the Senegalese National Malaria Control Programme recommended artemisinin-based combination therapy (ACT) as the first-line treatment for uncomplicated malaria. Since the introduction of ACT, there have been very few reports on the level of Plasmodium falciparum resistance to anti-malarial drugs. An ex vivo susceptibility study was conducted on local isolates obtained from the Hôpital Principal de Dakar (Dakar, Senegal) from November 2013 to January 2014.

Methods: Eighteen P. falciparum isolates were successfully assessed for ex vivo susceptibility to chloroquine (CQ), quinine (QN), monodesethylamodiaquine (MDAQ), the active metabolite of amodiaquine, mefloquine (MQ), lumefantrine (LMF), artesunate (AS), dihydroartemisinin (DHA), the active metabolite of artemisinin derivatives, pyronaridine (PND), piperaquine (PPQ), and Proveblue (PVB), a methylene blue preparation, using the HRP2-based ELISA test.

Results: The prevalence of isolates with reduced susceptibility was 55.6% for MQ, 50% for CQ, 5.6% for QN and MDAQ, and 0% for DHA, AS and LMF. The mean IC₅₀ for PND, PPQ and PVB were 5.8 nM, 32.2 nM and 5.3 nM, respectively.

Conclusions: The prevalence of isolates with a reduced susceptibility to MQ remains high and stable in Dakar. Since 2004, the prevalence of CQ resistance decreased, but rebounded in 2013 in Dakar. PND, PPQ and PVB showed high in vitro activity in P. falciparum parasites from Dakar.

Keywords: Malaria, Plasmodium falciparum, Anti-malarial, In vitro, Resistance, Senegal, Proveblue, Methylene blue
susceptibility has been affected by the new anti-malarial policies, an *ex vivo* susceptibility study was conducted on local isolates from Dakar obtained from the Hôpital Principal de Dakar between November 2013 and January 2014. The malaria isolates were assessed for susceptibility to standard drugs such as chloroquine (CQ), quinine (QN), monodesethylamodiaquine (MDAQ), the active metabolite of amodiaquine, mefloquine (MQ), lumefantrine (LMF), artesunate (AS), dihydroartemisinin (DHA), the active metabolite of artemisinin derivatives, and new anti-malarial drugs, such as pyronaridine (PND), piperaquine (PPQ) and Proveblue (PVB), a methylene blue preparation that complies with the European Pharmacopoeia and contains limited organic impurities and heavy metals of recognised toxicity.

**Methods**

**Plasmodium falciparum isolates**

In total, 24 patients (seven females and 17 males) with malaria were recruited from 19 November 2013 to 7 January 2014 at the Hôpital Principal de Dakar. Venous blood samples were collected in Vacutainer® ACD tubes (Becton Dickinson, Rutherford, NJ, USA) prior to patient treatment. Parasitaemia ranged from 0.001 to 0.33% in the male group and from 0.001 to 3.3% in the female group. Of the 24 patients, 67% were recruited from the emergency department and the remainder were recruited from the intensive care unit (21%), paediatric department (8%) and maternity department (4%). Informed verbal consent was obtained from patients and/or their parents before blood collection. Assessments of *P. falciparum* susceptibility to anti-malarial drugs were performed with the same venous blood sample used for diagnostic purposes. The study was reviewed and approved by the ethical committee of the Hôpital Principal de Dakar. Patients were successfully treated by QN.

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 monoinfection. Parasitized erythrocytes were washed three times in RPMI 1640 medium (Invitrogen, Paisley, UK) buffered with 25 mM HEPES and 25 mM NaHCO₃. If parasitaemia exceeded 0.5%, infected erythrocytes were diluted to 0.5% with uninfected erythrocytes (human blood type A+) and resuspended in RPMI 1640 medium supplemented with 10% human serum (Abcys S.A. Paris, France), for a final haematocrit of 1.5%.

**Drugs**

CQ, QN and DHA were purchased from Sigma (Saint Louis, MO, USA). MDAQ was obtained from the World Health Organization (Geneva, Switzerland), MQ was purchased from Roche (Paris, France), and LMF was purchased from Novartis Pharma (Basel, Switzerland). AS, PPQ and PND were obtained from Shin Poong Pharm Co (Seoul, Korea) and PVB from Provapharm SAS (Marseille, France). QN, MDAQ, MQ, DHA, AS, and PPQ were first dissolved in methanol and then diluted in water to final concentrations ranging from 6 nM to 3,149 nM for QN, 1.9 to 1,988 nM for MDAQ, 1.5 to 392 nM for MQ, 0.1 to 107 nM for DHA, 0.1 to 98 nM for AS and 1.9 to 998 nM for PPQ. CQ, PND and PVB were resuspended and diluted in water to final concentrations ranging from 6 nM to 3,231 nM, 0.4 to 199 nM and 0.5 to 500 nM, respectively. LMF was resuspended and diluted in ethanol to obtain final concentrations ranging from 0.6 nM to 310 nM.

The batches of plates were tested and validated on the CQ-susceptible 3D7 strain (West-Africa) and the CQ-resistant W2 strain (Indochina) (MR4, Virginia, USA) in three to six independent experiments using the same conditions described in the paragraph below. The two strains were synchronized twice with sorbitol before use [5], and clonality was verified every 15 days using PCR genotyping of the polymorphic genetic markers *msp1* and *msp2* and microsatellite loci [6,7] and annually by an independent laboratory from the Worldwide Anti-malarial Resistance Network (WWARN).

**Ex vivo assay**

For the *in vitro* isotopic microtests, 100 µl of synchronous parasitized red blood cells (final parasitaemia, 0.5%; final haematocrit, 1.5%) was aliquoted into 96-well plates pre-dosed with anti-malarial drugs. The plates were incubated in a sealed bag for 72 hrs at 37°C with the atmospheric generators for capnophilic bacteria, Genbag CO₂ at 5% CO₂ and 15% O₂ (BioMérieux, Marcy l’Etoile, France) [8]. After thawing the plates, haemolysed cultures were homogenized by vortexing the plates. Both the success of the drug susceptibility assay and the appropriate volume of haemolysed culture to use for each assay were determined for each clinical isolate during a preliminary HRP2 ELISA. Both the pre-test and subsequent ELISAs were performed using a commercial kit (Malaria Ag Celisa, ref KM2159, Cellabs PTY LTD, Brookvale, Australia) according to the manufacturer’s recommendations. The optical density (OD) of each sample was measured with a spectrophotometer (Multiskan EX, Thermo Scientific, Vantaa, Finland).

The concentration at which the drugs were able to inhibit 50% of parasite growth (*IC₅₀*) was calculated with the inhibitory sigmoid *E*ₘ₅₀ model, with estimation of the *IC₅₀* through non-linear regression using a standard function of the R software (ICEstimator version 1.2) [9]. *IC₅₀* values were validated only if the OD ratio (OD at concentration 0/OD at concentration max) was greater than 1.6 and the confidence interval ratio (upper 95%
confidence interval of the IC50 estimation/lower 95% confidence interval of the IC50 estimation) was less than 2.0 [9].

**Data and statistical analysis**

IC50 values were analysed after logarithmic transformation and expressed as the geometric mean of the IC50 and a confidence interval of 95% (CI95%).

Using the Genbag conditions, the cut-off values for in vitro resistance, or reduced susceptibility, were 77 nM, 61 nM, 115 nM, 12 nM, 12 nM, 611 nM, and 30 nM for CQ, MDAQ, LMF, DHA, AS, QN, and MQ, respectively [10].

**Results**

Of the 24 patients recruited at the Hôpital Principal de Dakar, 19 were tested ex vivo, and 18 isolates were successfully cultured. The average parameter estimates for the ten anti-malarial drugs used against the *P. falciparum* isolates are given in Table 1. The prevalence of *P. falciparum* isolates with decreased susceptibility to MQ in vitro reached 55.6%. Fifty per cent of the isolates were resistant in vitro to CQ.

**Discussion**

This report describes the evaluation of the ex vivo susceptibility of *P. falciparum* isolates, taken from patients in Dakar, to ten standard or potential anti-malarial drugs. The patients, recruited at the Hôpital Principal de Dakar from November 2013 to January 2014, said that they did not leave Dakar and its surrounding suburbs during the month preceding their malaria attack.

One limitation of this study was the low number of recruited patients (24) during those two months, due to the diminution of malaria prevalence in Senegal. The malaria prevalence in public health facilities decreased from 17.9% in 2007 to 2.6% in 2008 in Dakar [11]. In Dielmo, a village located at 280 km southeast of Dakar and approximately 15 km north of The Gambia border, the prevalence of malaria decreased from 87.2 to 0.3% in children and 58.3 to 0.3% from 1990 to 2012 [12].

The prevalence of isolates with reduced susceptibility to MQ remained high (55.6%) in Dakar, but was relatively stable compared with the previous year (55 to 62%) [3,10]. The level of in vitro MQ resistance increased since previous studies conducted in Dakar. In Dakar, the per cent of isolates with decreased susceptibility was 17% in 2001 [13] and 13% in 2002 [7]. MQ prophylaxis failure has been previously described in Senegal [14], and MQ is one of the three anti-malarial drugs recommended for travellers as an anti-malarial prophylaxis in Senegal. Clinical trials are in progress to evaluate the efficacy of MQ for intermittent preventive treatment of infants and pregnant women, whereas MQ is still used for the treatment of uncomplicated malaria in infants in Dakar. Nevertheless, MQ has been employed relatively infrequently in Africa compared to Asia. The combination of artesunate-mefloquine, which is administered to patients in Asia, is not yet used in Senegal. However, scientific data are not available for MQ monotherapy, and very little data are available on the in vitro decreased susceptibility to MQ and its clinical implications in Africa. It is important to monitor the evolution of *P. falciparum* susceptibility to MQ, to archive suspicious isolates and to correlate clinical outcomes with pharmacokinetic and phenotypic responses and with molecular markers.

As far back as 1988, in vitro *P. falciparum* resistance to CQ was reported in Dakar, and reports of resistance

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**Table 1** Ex vivo susceptibility of 18 *Plasmodium falciparum* isolates from Dakar to chloroquine (CQ), monodesethylamodiaquine (MDAQ), lumefantrine (LMF), dihydroartemisinin (DHA), quinine (QN), mefloquine (MQ), artesunate (AS), pyronardine (PND), piperaquine (PPQ) and Proveblue (PVB)

| Drug  | Isolate IC50 | Resistance or reduced susceptibility |
|-------|--------------|-------------------------------------|
| CQ    | Mean 52.2 nM | Min 6.1 Max 346.4 Cut-off 77 nM    |
| MDAQ  | Mean 8.8 nM  | Min 1.9 Max 117.1 Cut-off 61 nM    |
| LMF   | Mean 4.1 nM  | Min 0.61 Max 82.9 Cut-off 115 nM   |
| DHA   | Mean 2.72 nM | Min 0.1 Max 5.08 Cut-off 12 nM     |
| AS    | Mean 1.35 nM | Min 0.12 Max 5.53 Cut-off 12 nM    |
| QN    | Mean 63.1 nM | Min 6.2 Max 1430 Cut-off 611 nM    |
| MQ    | Mean 30.1 nM | Min 7.1 Max 63.4 Cut-off 30 nM     |
| PND   | Mean 5.8 nM  | Min 0.4 Max 19.7 ND                |
| PPQ   | Mean 3.22 nM | Min 2.5 Max 168.0 ND               |
| PVB   | Mean 5.3 nM  | Min 0.9 Max 40.2 ND                |

Mean: geometric mean.
CI 95%: 95% confidence interval.
ND: not determined.
in other regions of the country followed shortly thereafter [15]. From 1991 to 1995, parasitological failures were observed in 21% of patients in Pikine [16]. The prevalence of in vitro CQ resistance then decreased in from 52% in 2002 [7] to approximately 20-25% in Dakar in 2009–2011 [3,10]. In 2013–2014, the prevalence of in vitro resistance to CQ in Dakar increased again to 50%. A limitation of these results is the very small number of studied samples. However, this phenomenon was already described in Thies and Pikine. Parasites also became less susceptible to CQ from 2008 (median IC$_{50}$ = 30.7 nM) to 2011 (median IC$_{50}$ = 76.1 nM) in Thies [4]. In Pikine, after a decrease of the prevalence of the pfcrt 76 T mutation, involved in CQ resistance, from 64–79% before CQ withdrawal (2000 to 2003) [17–19] to 47–60% [20,21] when amodiaquine plus pyrimethamine-sulphadoxine was the first-line treatment (2004–2005), this prevalence has increased slightly to 59% since ACT has been implemented (2006 to 2009) [19]. It is important to monitor the evolution of P. falciparum susceptibility to CQ.

The decrease in CQ resistance parallels the withdrawal of CQ treatment and the introduction of ACT in 2002 in Senegal. However, in 2006, CQ was still being administered to patients. In Dakar in 2006, CQ represented 5.1% of the anti-malarial drugs used in children [22] and 3.5% in 2009 [23]. The rapid dissemination of CQ resistance in Dielmo, despite strictly controlled anti-malarial drug use, argues against the re-introduction of CQ, at least in monotherapy, in places where the resistance allele has dropped to very low levels following the discontinuation of CQ treatment [24]. Despite the re-aquisition of CQ susceptibility, any re-introduction would likely result in a rapid re-emergence of resistant strains. Additionally, the increase of CQ resistance in the hypothetical absence of CQ pressure leads to an avoidance of re-introducing CQ in Senegal. There is an urgent need to evaluate the presence and use of CQ in Dakar and to evaluate the capacity of drug pressure on CQ resistance of the different partners combined with artemisinin derivatives in ACT.

The prevalence of isolates with in vitro-reduced susceptibility to MDAQ remains low and stable in 2013 (5.6 versus 6% in 2009 and 11.8% in 2010) [3,10]. The resistance to amodiaquine has remained low even after the introduction of artesunate-amodiaquine in 2006 in Senegal. A study in Dakar and Momp from 1996 to 1998 demonstrated that monotherapy with amodiaquine remained effective for treating uncomplicated malaria in areas where CQ resistance was prevalent [25]. In 2011–2012, the efficacy of ASAQ was evaluated at 99.3% [26]. However, ACT efficacy and resistance must be monitored because clinical failures, or at least extended parasite clearance times, have been described in Southeast Asia [27–30]. In this context, it is important to implement in vitro and in vivo surveillance programmes.

No isolate exhibited reduced in vitro susceptibility to DHA or to AS. This result is consistent with previous studies that did not show any parasites resistant to AS [7,31,32]. However, high IC$_{50}$ values can be found for artemisinin, with an IC$_{50}$ > 30 nM in Dakar [33] and AS with an IC$_{50}$ > 45 nM [13]. The median IC$_{50}$ values increased from 2008 to 2011 (3.2 to 10.1 nM) with a highest IC$_{50}$ value of 73.1 nM in Thies [4]. In the present study, IC$_{50}$ ranged from 0.1 to 5.08 nM for DHA and 0.12 to 8.53 nM for AS. However, the standard in vitro test was not adapted to follow resistance to artemisinin derivatives. The clinical resistance to artemisinin correlated with in vitro resistance, manifested by an increase in the ring-stage survival rate after contact with artemisinin (ring survival test) [34,35]. In addition, mutations in the P. falciparum K13 gene (PF3D7_1343700) that encodes the kelel propeller domain were associated with in vitro resistance to artemisinin and with delayed clearance after artemisinin treatment in southern Asia [30,36,37]. It will be a priority to introduce this new in vitro test in Senegal.

The other ACT first-line treatment for uncomplicated P. falciparum malaria in Senegal is the combination of artemether-lumefantrine. No isolate presented reduced susceptibility to LMF, and this prevalence was remains under 3% in Dakar since the introduction of ACT [3,10]. In 2011–2012, the efficacy of artemether-lumefantrine was evaluated at 100% in Senegal [26].

A new ACT second-line treatment for uncomplicated P. falciparum malaria in Senegal is the combination of dihydroartemisinin-piperazine (DP). DP (Artekina®, Duo-Cotexin®, Eurartesim®) is administered as single daily dose for three days. It has been demonstrated to be well tolerated and highly effective for the treatment of uncomplicated P. falciparum malaria in Africa [38–40]. In 2011–2012, the efficacy of DP was evaluated at 100% in Senegal [26]. The PPQ IC$_{50}$ values (geometric mean IC$_{50}$ = 32.2 nM) observed in Dakar in 2013 were slightly lower than those found in other ex vivo studies in Africa (geometric mean IC$_{50}$ = 81.3 nM and 66.8 nM) [41,42].

The pyronaridine-artesunate combination (Pyramax®) is one of the most recent ACT combinations and is currently under development by the not-for-profit organisation Medicines for Malaria Venture (Geneva, Switzerland) and the pharmaceutical company Shin Poong Pharmaceuticals (Seoul, Republic of Korea) for the treatment of uncomplicated P. falciparum malaria and for the blood stages of Plasmodium vivax malaria. The efficacy of PND-artesunate was not inferior to that of artemether-lumefantrine in the treatment of uncomplicated P. falciparum malaria in Africa [43]. The PND IC$_{50}$ values (geometric mean IC$_{50}$ = 5.8 nM) observed in Dakar...
in 2013 were comparable to those obtained in Dielmo in 1996 and 1997 (3.8 nM and 4.52 nM) [44,45].

In 2013, 7% of isolates showed low reduced susceptibility to QN, which is in accordance with previous studies in Dakar [3,7,10,13]. Even in areas where QN efficacy remains good, such as sub-Saharan Africa, the susceptibility of individual \textit{P. falciparum} isolates to QN has varied widely. The IC\textsubscript{50} for isolates collected in Dakar were 6 to 1,291 nM in 2009 [10] and 5 to 1,195 nM in 2010 [3]. The wide range in QN susceptibility and recent evidence for QN treatment failure observed across Africa [46,47] or in Senegal in a patient who spent two months in Dielmo in 2007 [48] suggest that the evolution of parasites with reduced susceptibility may contribute to QN decreased efficacy. However, the 24 patients in this study were successfully treated with QN.

Proveblue (PVB), which is a methylene blue preparation that complies with the European Pharmacopoeia and contains limited organic impurities and heavy metals of recognized toxicity, has previously been demonstrated to possess \textit{in vitro} anti-malarial activity against 23 \textit{P. falciparum} strains that were resistant to various anti-malarial drugs [49]. PVB exhibited noticeable synergistic effects in combination with MQ and QN and high synergistic effects associated with DHA [50]. Treatment with 1 to 10 mg/kg of weight of PVB for five days significantly reduced or prevented cerebral malaria in mice [51-53]. The IC\textsubscript{50} for PVB ranged from 0.88 nM to 40.2 nM with a mean of 5.3 nM. These data show that PVB is active \textit{in vitro}, in line with previous studies with methylene blue with organic as well as inorganic impurities in parasites from Nigeria, Kenya and Thailande [54-56]. Another advantage of the use of PVB is that methylene blue has gametocytocidal properties and can reduce the transmission of \textit{P. falciparum} [57,58].

Limitations must be taken into account such as the very small number of samples which are not representative of susceptibility in Senegal but only from a facility in Dakar, the Hôpital Principal de Dakar, which certainly selects most severe malaria than in neighborhood clinics.

The introduction of ACT in 2002 in Senegal did not induce a decrease in \textit{P. falciparum} susceptibility to individual drug components, such as DHA, AS, MDAQ, and LMF. The prevalence of \textit{P. falciparum} isolates with reduced drug susceptibility to MQ remains high and stable in Dakar. Since 2004, the prevalence of CQ resistance has decreased, but then rebounded in 2013 in Dakar. PND, PPQ and PVB showed high \textit{in vitro} activity against \textit{P. falciparum} parasites from Dakar. Maximizing the efficacy and longevity of ACT as a tool to control malaria will critically depend on pursuing intensive research into identifying \textit{in vitro} markers as well as implementing \textit{ex vivo} and \textit{in vivo} surveillance programmes.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
BF, YD and BP carried out the \textit{in vitro} testing of drug susceptibility. CC, MF, PD, and BD supervised, carried out and coordinated the field collection of isolates from patients. BW and BP conceived and coordinated the study. BP analysed the data. BF and BP drafted the manuscript. All authors read and approved the final manuscript.

Acknowledgements
The authors thank the patients and the staff of the Hôpital Principal de Dakar. The authors thank Ndèye Fatou Diop and Maurice Gomis from the Hôpital Principal de Dakar for technical support. This study was supported by the Schéma directeur Paludisme, Etat Major des Armées Françaises (grant LR 607a) and by the Ministère des Affaires Étrangères.

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Received: 9 October 2014 Accepted: 27 January 2015
Published online: 06 February 2015

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