Prevalence of anti-malarial resistance genes in Dakar, Senegal from 2013 to 2014

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Abstract

Background: To determine the impact of the introduction of artemisinin-based combination therapy (ACT) on parasite susceptibility, a molecular surveillance for antimalarial drug resistance was conducted on local isolates from the Hôpital Principal de Dakar between November 2013 and January 2014 and between August 2014 and December 2014.

Methods: The prevalence of genetic polymorphisms in antimalarial resistance genes (pfcrt, pfmdr1, pfdhfr and pfdhps) was evaluated in 103 isolates.

Results: The chloroquine-resistant haplotypes CVIET and CVMET were identified in 31.4 and 3.9% of the isolates, respectively. The frequency of the pfcrt K76T mutation was increased from 29.3% in 2013–2014 to 43.2% in 2014. The pfmdr1 N86Y and Y184F mutations were identified in 6.1 and 53.5% of the isolates, respectively. The pfdhfr triple mutant (S108N, N51I and C59R) was detected in the majority of the isolates (82.3%). The prevalence of quadruple mutants (pfdhfr S108N, N51I, C59R and pfdhps A437G) was 40.4%. One isolate (1.1%) harboured the pfdhps mutations A437G and K540E and the pfdhfr mutations S108N, N51I and C59R.

Conclusions: Despite a decline in the prevalence of chloroquine resistance due to the official withdrawal of the drug and to the introduction of ACT, the spread of resistance to chloroquine has continued. Furthermore, susceptibility to amodiaquine may be decreased as a result of cross-resistance. The frequency of the pfmdr1 mutation N86Y declined while the Y184F mutation increased in prevalence, suggesting that selective pressure is acting on pfmdr1, leading to a high prevalence of mutations in these isolates and the lack of specific mutations. The 50.5% prevalence of the pfmdr1 polymorphisms N86Y and Y184F suggests a decrease in lumefantrine susceptibility. Based on these results, intensive surveillance of ACT partner drugs must be conducted regularly in Senegal.

Keywords: Malaria, Plasmodium falciparum, Anti-malarial, In vitro, Resistance, Senegal, Molecular marker

Background

Due to increasing chloroquine resistance, the first-line malaria treatment in Senegal was switched to sulfadoxine-pyrimethamine with amodiaquine in 2004. In 2006, the Senegalese National Malaria Control Programme recommended artemisinin-based combination therapy (ACT) as the first-line treatment for uncomplicated malaria. Therefore, the first-line therapy for uncomplicated malaria became artemether-lumefantrine or artesunate-amodiaquine. The dihydroartemisinin-piperaquine combination was then recommended as a second-line treatment for uncomplicated Plasmodium falciparum malaria in Senegal.

Intermittent preventive treatment (IPT) consists of administering sulfadoxine-pyrimethamine and one dose of artesunate during the transmission season and resulted in a 90% reduction in incidence of clinical malaria in Senegal [1]. Administered once a month to all children...
or pregnant women, this seasonal IPT can provide a high degree of protection against malaria.

The combination of sulfadoxine–pyrimethamine and amodiaquine was more effective than sulfadoxine–pyrimethamine and artesunate or amodiaquine and artesunate in malaria preventive treatment [2]. During IPT with sulfadoxine–pyrimethamine and piperaquine, only 3.4 % of the treated children had malaria [3].

Since the introduction of ACT and IPT trials in Senegal, very few studies have examined P. falciparum resistance to antimalarial drugs. To determine the impact of the introduction of new anti-malarial drugs on parasite susceptibility, a molecular study of anti-malarial drug resistance was conducted on local isolates from the Hôpital Principal de Dakar between November 2013 and January 2014 and between August 2014 and December 2014. The prevalence of genetic polymorphisms in antimalarial resistance genes, such as the P. falciparum chloroquine resistance transporter (pfCRT) for chloroquine [4], P. falciparum multidrug resistance 1 (pfMDR1), which is involved in mefloquine resistance [5] and potentially in quinoline resistance [6, 7], P. falciparum dihydrofolate reductase (pfDHFR) for pyrimethamine [8] and P. falciparum dihydropteroate synthase (pfDHPS) for sulfadoxine, were evaluated [9].

Methods

Plasmodium falciparum isolates

In total, 103 symptomatic patients were recruited at the Hôpital Principal de Dakar. Fifty-nine P. falciparum isolates were collected between November 2013 and January 2014 and 44 between August 2014 and December 2014. The majority of patients (64 %) were recruited from the emergency department. The other patients were recruited from the intensive care unit (12 %), paediatric department (7 %), infectious diseases department (5 %), maternity department (3 %), and other units (9 %). Antimalarial treatment prior to admission was not recorded. Despite the WHO’s recommendations, the P. falciparum treatment administered at the Hôpital Principal de Dakar until November 2014 was quinine followed by artesunate or artemether–lumefantrine. All the patients or their parents/guardians provided their verbal consent before blood collection. The ethical committee of the Hôpital Principal de Dakar approved the study.

Peripheral venous blood samples were collected in Vacutainer® ACD tubes (Becton–Dickinson, Rutherford, NJ, USA) prior to patient treatment. The diagnosis was performed on thin blood smears stained using a RAL® kit (Réactifs RAL, Paris, France) to determine P. falciparum density and to confirm species-specific monoinfection. The level of parasitaemia ranged from 0.001 to 3.3 % in 2013–2014 and 0.06 to 14.1 % in 2014.

Nucleic acid extraction

Total genomic DNA was extracted from blood sample using a QIAamp DNA Blood Mini Kit according to the manufacturer’s recommendations (Qiagen, Germany).

Anti-malarial resistance gene single-nucleotide polymorphisms (SNPs)

Four genes, pfCRT, pfMDR1, pfDHFR and pfDHPS, were amplified by PCR using the reaction conditions described in Table 1 [10–12]. The reaction mixture included 2.5 µL of genomic DNA, 1X reaction buffer (Euromedex), 200 µM of deoxynucleoside triphosphate mixture (dGTP, dATP, dTTP and dCTP) (Euromedex, Souffelweyersheim, France), variable concentration of MgCl2 (Table 1), 0.32 µM of forward and reverse primers and one unit of Red Diamond Taq® DNA polymerase (Euromedex) in a final volume of 25 µL. The thermal cycler (T3 Biometra, Archamps, France) was programmed as follows: an initial denaturation at 94 °C for 5 min followed by 40 cycles of 94 °C for 30 s, specific hybridization temperature for variable elongation times (Table 1) and 72 °C for extension at 1 min per 1000 bp, and a final 5 min extension step at 72 °C. Purified genomic DNA from P. falciparum clone D7 was used as a positive control, and water and human DNA were used as negative controls. The reaction products were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) with the primers described in Table 1. The sequencing reaction products were purified using the BigDye XTerminator Purification Kit (Applied Biosystems) in accordance with the manufacturer’s instructions. Sanger sequencing of PCR products was performed using an ABI Prism 3100 analyser (Applied Biosystems). The sequence data were analysed using Vector NTI Advance™ software (version 11, Invitrogen, Cergy Pontoise, France).

Results

Of 103 P. falciparum isolates collected at the Hôpital Principal de Dakar, 59 isolates were obtained between November 2013 and January 2014 and 44 between August 2014 and December 2014.

The pfCRT gene was successfully sequenced in 102 samples. The frequency of the haplotype CVIET was 31.4 % (n = 32 isolates), the haplotype CVMET was 3.9 % (n = 4 isolates) and the haplotype CVMNK was 64.7 % (n = 66 isolates). A molecular resistance profile was identified in 35.3 % of cases (n = 36 isolates), including 29.3 % (n = 17 isolates) in 2013–2014 and 43.2 % (n = 19 isolates) in 2014. The difference is not significant (P value = 0.146, Pearson’s Chi squared test). The CVEMT haplotype was first identified in the 2014 malaria season.

The results for pfMDR1 polymorphisms are shown in Table 2. The frequency of the 86Y mutation was 5.1 %
(n = 5 isolates), and one mixed sample (1 %) harboured both N86 and 86Y alleles. The 184F mutation frequency was 53.5 % (n = 53 isolates), and five of the six isolates harboured both the 86Y and 184F codons. Parasites with the N86 allele and the 184F mutation represented 50.5 % of the isolates. No new SNPs were detected in the pfmdr1 gene.

The results for pfmdr1 polymorphisms are presented in Table 3. The mutation frequencies were 87.9 % for the S108N mutation and 85.9 % for both the N51I and C59R polymorphisms. The triple mutant (S108N, N51I and C59R) was detected in 82.3 % of samples.

The results for the pfmdhps polymorphisms are presented in Table 4. The isolates harboured A437G in 47.2 % of the cases, S436A in 20.2 % of the cases, A613S in 3.2 % of the cases, K540E in 2.1 % of the cases and A581G in 1.1 % of the cases.

The prevalence of the quadruple mutant (pfdfhr 108N, 511, 59R and pfdhps 437G) was 40.4 %. One isolate (1.1 %) simultaneously harboured the two pfdhps mutations 437G and 540E and the three pfdfhr mutations 108N, 511 and 59R.

**Discussion**

In total, isolates from only 103 malaria patients were collected in 2013 and 2014 at the Hôpital Principal de Dakar, 55 during the 2013–2014 malaria season and 44 during the 2014–2015 season. This is due to the decreased prevalence of malaria in Senegal (reduction of 27.6 % from 2013 to 2014) [13]. Chloroquine resistance is principally mediated by pfcr gene mutations in different parts of the world [14]. In this study, the pfcr gene was mutated in 35.3 % of the patients recruited at the Hôpital Principal de Dakar in 2013 and 2014, including 29.3 % of the
cases in the 2013–2014 season and 43.2 % of the cases in the 2014–2015 season. This increase in chloroquine resistance has been observed in recent years following a decrease due to the withdrawal of chloroquine and the introduction of ACT in 2002 in Senegal (Table 5). Before the introduction of ACT in Senegal, the prevalence of isolates harbouring the \textit{pfcrt} K76T and in vitro chloroquine resistance was above 50 and 40 %, respectively, in Dakar and its suburb Pikine and in south areas (Dielmo and Ndiop) [15–21]. During the beginning of the ACT implementation (2004–2009), the prevalence of K76T mutant parasites maintained around 50 % [19, 22]. From 2009 to 2011, the prevalence of K76T mutant parasites and in vitro chloroquine resistance decreased to 40 and 25 %, respectively in Dakar [23–25]. Since 2013, the level of chloroquine resistance has increased again to that of 2002 in Dakar [26, 27].

While chloroquine is no longer used in Senegal, the prevalence of in vitro chloroquine resistance and of the \textit{pfcrt} K76T mutation has increased. Two hypotheses could explain the observed increase: (i) the use of artesunate–amodiaquine in Senegal led to the emergence of resistant parasites to amodiaquine; (ii) the development of cross-resistance to chloroquine and monodesethylamodiaquine increased the resistance [23, 28]. This study describes the first detection of the CVMET haplotype in Senegal. In Senegal, the 76T mutation has previously been associated with the CVIET haplotype.

The isolates harbouring the \textit{pfmdr1} mutations 86Y, 184F, 1034C and 1042D were identified in 6.1, 53.5, 0 and 0 % of the patients, respectively. The prevalence of 86Y has decreased over the past few years in Senegal from >30 % in 2000 to 6 % in 2013–2014 (Table 6) [18–20, 22, 24, 25]. Since 2010, the prevalence of parasites harbouring the 184F mutation has remained stable and above 50 % in Dakar [24, 25]. This prevalence has more than doubled from 30 % in 2008 to greater than 70 % in 2011 in Thiès [22]. The frequency of the \textit{pfmdr1} mutation N86Y declined, while the frequency of the Y184F mutation increased, suggesting that selective pressure is acting on \textit{pfmdr1}, leading to a high prevalence in these isolates and the lack of specific mutations. The role of polymorphisms in \textit{pfmdr1} is still debated. The 86Y mutation was associated with increased in vitro susceptibility of \textit{P. falciparum} parasites to dihydroartemisinin, lumefantrine, monodesethylamodiaquine and mefloquine [29]. In contrast, this \textit{pfmdr1} 86Y mutation was associated with a decrease of in vitro susceptibility to dihydroartemisinin, lumefantrine, and mefloquine in \textit{P. falciparum} isolates from Asia [30], Kenya [31] and Benin [32]. Field studies in east Africa have also shown selection of the N86 allele.

### Table 4 Frequency (%) and number (no) of the pfdhps mutations

| Codon  | No | Wild type % (no) | Mutated % (no) |
|--------|----|-----------------|----------------|
| S436A  | 89 | 79.8 (71)       | 20.2 (18)      |
| A437G  | 89 | 52.8 (47)       | 47.2 (42)      |
| K540E  | 94 | 14.1 (97.9)     | 2.1 (2)        |
| A581G  | 94 | 98.9 (93)       | 1.1 (1)        |
| A613S  | 94 | 96.8 (91)       | 3.2 (3)        |

### Table 5 Molecular (pfcrt K76T) and in vitro studies on evaluation of \textit{P. falciparum} susceptibility to chloroquine in Senegal

| Year of collection | Site of collection | In vitro chloroquine resistance (%) | \textit{pfcrt} 76T | References |
|--------------------|-------------------|------------------------------------|-------------------|------------|
| 1996               | Dielmo/Ndiop      | 49                                 |                   | [15]       |
| 1997–1998          | Dielmo/Ndiop      | 43.5                               |                   | [16]       |
| 1999               | Dielmo/Ndiop      | 55                                 |                   | [17]       |
| 2000               | Pikine            | 31                                 | 79 %              | [18]       |
| 2000–2003          | Pikine            | 52                                 | 72.4 %            | [19]       |
| 2001               | Pikine            | 52                                 | 64 %              | [20]       |
| 2002               | Dakar             | 52                                 | 54 %              | [21]       |
| 2004–2005          | Pikine            | 52                                 | 47.2 %            | [19]       |
| 2006–2009          | Pikine            | 59.5                               |                   | [19]       |
| 2008–2011          | Thies             | >50 %                              |                   | [22]       |
| 2009–2010          | Dakar             | 52                                 | 37.2 %            | [23]       |
| 2009–2010          | Dakar             | 22                                 | 43.6 %            | [24]       |
| 2010–2011          | Dakar             | 24.2                               | 50                | [25]       |
| 2013–2014          | Dakar             | 29.3                               | Present data      | [27]       |
| 2014               | Dakar             | 52.8                               | 43.2 %            | Present data |
The emergence of parasites resistant to amodiaquine in Dakar, which can generate resistance between in vitro susceptibility to chloroquine strains resistant to chloroquine appears to be due to cross-

- The N86Y mutation in P. falciparum strains resistant to amodiaquine increased significantly from 5.6% in 2013 to 30.6% in 2014 with an increase in IC_{50} values from 9.8 to 25.3 nM [26, 27];
- The re-increase of P. falciparum strains resistant to chloroquine appears to be due to cross-
- The prevalences of isolates harboring the N86Y mutation in Dakar increased significantly from 5.6% in 2013 to 30.6% in 2014. Additional mutations—51I (odds ratio of 1.7) or 59R (odds ratio of 1.9)—increase the level of in vitro resistance to anti-folate drugs and sulfadoxine–pyrimethamine [40]. The risk of in vivo resistance to sulfadoxine–pyrimethamine increased by 4.3 with the triple mutation (108N, 51I and 59R) [40]. In 2013–2014, the prevalence of the pfmdr1 108N mutation was 87.9% in malaria patients who were treated at the Hôpital Principal de Dakar. dhfr triple mutants at codons 51I, 59R and 108N were associated with high-level pyrimethamine resistance and represented 82.3% of the isolates. Since 2002, the prevalence of triple mutants has increased from 50 to 82.3% in 2013–2014 (Table 7). This increase was observed in different areas of Senegal [3, 42, 43].

The pfldhps 437G mutation has been shown to be correlated with in vitro and in vivo resistance to sulfadoxine [6]. The risk of therapeutic failure with sulfadoxine–pyrimethamine increased by 1.5 and 3.9 with the single mutation A437G and the double mutation A437G and K540E, respectively [40]. In 2013–2014, 47.2% of the isolates harboured the 437G mutation in Dakar. This prevalence increased after 2002 and was then stable from 2009 to 2014 with 40–50% of the isolates harbouring the 437G mutation in Senegal (Table 8). Several studies from 2006 to 2008 in Senegal showed that the prevalence of pfldhps 437G significantly increased after IPT of infants with sulfadoxine–pyrimethamine [3, 44]. Only two isolates (2.1%) carried the double mutation (437G and 540E) that is associated with high-level sulfadoxine resistance. The pfldhps mutation of codon 613 (A613S) (3.2%) is very rare in Africa.

In Dakar, the prevalence of isolates harbouring the quadruple mutants (dhfr 108N, 51I, 59R and dhps 437G) was stable from 2009 to 2014: 36.5% in 2009, 36.7% in 2010 and 40.4% in 2014 [24, 25]. In Thiès, the prevalence of quadruple mutants increased from 20 to 66% between 2003 and 2011 and then dropped to 44% in 2013 [43]. In 2010, the quadruple mutants were identified in 79.4% of the isolates from areas of Senegal where sulfadoxine–pyrimethamine plus amodiaquine were administered

### Table 6 Evolution of pfmdr1 N86Y mutation in P. falciparum parasites in Senegal

| Year of collection | Site of collection | N86Y | References |
|--------------------|-------------------|------|------------|
| 2000               | Pikine            | 31%  | [18]       |
| 2001               | Pikine            | 30.6%| [20]       |
| 2002–2003          | Pikine            | 40%  | (about) [19] |
| 2005–2009          | Thiès             | 20%  | (about) [19] |
| 2009               | Thiès             | 20%  | (about) [22] |
| 2009–2010          | Dakar             | 17.2%| [24]       |
| 2010–2011          | Dakar             | 16.1 | [25]       |
| 2011               | Thiès             | <5%  | [22]       |
| 2013–2014          | Dakar             | 6.1% | Present data |

### Table 7 Evolution of pfldhps 51I, 59R and 108N triple mutation in P. falciparum parasites in Senegal

| Year of collection | Site of collection | 51I, 59R, 108N (%) | References |
|--------------------|-------------------|-------------------|------------|
| 2002               | Dakar             | 50                | [21]       |
| 2003               | Pikine            | 61                | [42]       |
| 2003               | Thiès             | 40                | [43]       |
| 2007               | Keur Soce         | 67                | [3]        |
| 2009–2010          | Dakar             | 75.3              | [24]       |
| 2010–2011          | Dakar             | 73.6              | [25]       |
| 2011               | Thiès             | 93                | [43]       |
| 2013–2014          | Dakar             | 82.3              | Present data |
to children during seasonal malaria chemoprevention versus 67.1% in areas where they were not treated [45]. In 2014, only one isolate harboured the pfdhps mutations 437G and 540E and the pfdhfr mutations 108N, 51I and 59R; these mutations are associated with high-level sulfadoxine–pyrimethamine resistance. These findings suggest that regular surveillance of molecular markers should be performed in areas where IPT with sulfadoxine–pyrimethamine is used.

In summary, the prevalence of chloroquine resistance continues to increase after a decline due to the official withdrawal of the drug and the introduction of ACT. Furthermore, amodiaquine susceptibility may be decreased as a result of cross-resistance. The frequency of the pfmdr1 mutation N86Y declined while the frequency of the Y184F mutation increased, suggesting that selective pressure is acting on pfmdr1, leading to a high prevalence in these isolates and the lack of specific mutations. The 50.5% prevalence of the pfmdr1 polymorphisms N86 and 184F suggests a decrease in lumefantrine susceptibility. Based on these results, intensive surveillance of ACT partner drugs must be conducted regularly in Senegal.

Furthermore, molecular surveillance in Dakar has demonstrated the emergence of polymorphisms in the K13 propeller domain gene, which is associated with in vitro and in vivo resistance to artemisinin in Asia [46–48]. However, these mutations detected in Dakar and more generally in Africa have not yet been associated with artemisinin resistance.

Abbreviations
ACT: artemisinin-based combination therapy; pfcrt: Plasmodium falciparum chloroquine resistance transporter gene; pfmdr1: Plasmodium falciparum multidrug resistance 1 gene; pfdhfr: Plasmodium falciparum dihydrofolate reductase gene; pfdhps: Plasmodium falciparum dihydropteroate synthase; IPT: intermittent preventive treatment; WHO: World Health Organization; SNPs: single-nucleotide polymorphisms; DNA: deoxyribonucleic acid.

Authors’ contributions
AB, NB and HM carried out the molecular genetic studies. BF, KAW, MF, AN, KKF, PD, BD, YD and BW carried out the diagnostic tests, monitored the patients, collected clinical and epidemiological data. BP conceived of and coordinated the study. AB, MM and BP analysed the data and 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 Ndeye Fatou Diop and Maurice Gomis from the Hôpital Principal de Dakar for technical support.

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

Availability of data and material
We didn’t wish to share our data. There is no recommended repository for the kind of data showed in this publication. All the data have not been yet valued.

Ethics approval and consent to participate
All the patients or their parents/guardians provided their verbal consent before blood collection. Bio-banking and secondary use for scientific purposes of human clinical samples used for malaria diagnostic are possible as long as the corresponding patients are informed and have not indicated any objections. This requirement was fulfilled here since oral information is given to every patient and no immediate or delayed patient opposition was reported to the hospital clinicians. The ethical committee of the Hôpital Principal de Dakar approved the study.

Funding
This study was supported by the Schéma directeur Paludisme, Etat Major des Armées Françaises (Grant LR 607A), by the Délégation Générale pour l’Armement (Grant PDH-2-NRBC-4-B1-402) and by the Ministère des Affaires Etrangères.

Received: 19 March 2016 Accepted: 8 June 2016
Published online: 07 July 2016

Table 8 Evolution of pfdhps A437G mutation in P. falciparum parasites in Senegal

| Year of collection | Site of collection | A437G (%) | References |
|--------------------|--------------------|-----------|------------|
| 2002               | Dakar              | 20        | [21]       |
| 2003               | Pikine             | 40        | [42]       |
| 2009–2010          | Dakar              | 40.4      | [24]       |
| 2010–2011          | Dakar              | 47.5      | [25]       |
| 2013–2014          | Dakar              | 47.2      | Present data |

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