Ex vivo susceptibility and molecular signature assessment of antimalarial-based combination therapies (ACT) partner drugs resistance in Senegal

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Abstract

Background

Over the last decades, *Plasmodium falciparum* – the main causative agent of malaria – has constantly developed resistance to antimalarial drugs such as chloroquine, sulfadoxine-pyrimethamine or artemisinin derivatives. Therefore, active surveillance in the *ex vivo* susceptibility to the antimalarial drugs used as partner drugs in artemisinin-based combination therapies (the current frontline antimalarial) such as amodiaquine, lumefantrine or piperaquine, is essential.

Methods

Here, we evaluated the *ex vivo* susceptibility, expressed with the *ex vivo* SYBR™ Green, to six antimalarial drugs (amodiaquine, chloroquine, lumefantrine, meoquine, piperaquine and quinine) from 34 *P. falciparum* isolates collected in 2018 in Thiès (Senegal). Whole-genome sequencing (WGS) was used to search for mutations in *P. falciparum* genes known to be associated with drug resistance.

Results

*P. falciparum* isolates showed reduced *ex vivo* susceptibility only to chloroquine (16% of the isolates). Mutations in pfcr* K76T (21%) and pfmdr1 Y184F (53%) were the most prevalent. A significant correlation was observed between the mutants pfcr* 76T and pfmdr1 184F and IC$_{50}$ values for chloroquine. A significant decrease in *ex vivo* susceptibility to chloroquine and quinine associated with the pfcr* R371I was also detected ($P < 0.001$).

Conclusion

Our results suggest that the *ex vivo* susceptibility of *P. falciparum* isolates to amodiaquine, lumefantrine, mefloquine, piperaquine and quinine remains high in Thiès. Directly measuring *ex vivo* parasite drug response and sequencing resistance mutations overtime are both useful tools for monitoring parasite drug response in field samples.

Introduction

In recent decades, *Plasmodium falciparum* parasites have developed resistance to antimalarial drugs such as chloroquine, sulfadoxine-pyrimethamine, amodiaquine, mefloquine and quinine$^1$. Recently, *P. falciparum* parasites in Southeast Asia$^{2,3}$, Guyana, Papua New Guinea, and East Africa$^{1,4-6}$ also developed partial resistance to artemisinin (ART) and its derivatives, the main components of current frontline curative treatments, posing a serious threat for controlling and eliminating malaria. Given the
high level of use of ART-based combination therapies (ACT) throughout Africa, there is speculation that ART partial resistance (ART-R) could either emerge locally in the western and central regions of Africa, or could spread from East Africa, as it was observed with chloroquine\textsuperscript{7}. ART-R has not been yet reported in Senegal, which is progressing towards malaria elimination. However, previous studies in the country showed a decrease in \textit{ex vivo} parasite susceptibility to amodiaquine and chloroquine overtime, and rarer to ART\textsuperscript{8}. Reduced \textit{in vitro} drug susceptibilities have been associated with non-synonymous mutations in \textit{pfcrt} (chloroquine, amodiaquine, piperaquine and quinine), \textit{pfmdr1} (chloroquine, amodiaquine lumefantrine, and mefloquine), \textit{pfdrhfr} (pyrimethamine, proguanil), \textit{pfdrhps} (sulfonamide, sulfadoxine, sulfone, and dapsone) and \textit{pkelch13} (ART and its derivatives)\textsuperscript{9-12}. This situation could therefore jeopardize the successes that have led to a reduction in the proportion of malaria morbidity – the proportion of malaria morbidity fell from 35.72\% of clinical cases in 2001 to 3.29\% of confirmed cases in Senegal in 2016\textsuperscript{13}. In 2003, in response to the rapid spread and high rate of chloroquine resistance\textsuperscript{14,15}, the Senegal National Malaria Control Programme (NMCP) introduced sulfadoxine-pyrimethamine (SP). In 2006, the NMCP adopted ACT as first-line treatment for uncomplicated malaria\textsuperscript{16}, according to the World Health Organization (WHO) recommendations. In 2008, the Senegalese government and its partners made artesunate-amodiaquine (ASAQ) more accessible by reducing its price in public health facilities and private pharmacies\textsuperscript{17}. In 2010, ACT became free in the public sector\textsuperscript{18}. As a result, active surveillance of \textit{ex vivo} susceptibility to the antimalarial drugs used as partner drugs in ACT, such as amodiaquine, lumefantrine or piperaquine, is essential. Antimalarial drug susceptibility testing provides useful complementary data from clinical drug efficacy studies\textsuperscript{19}. Therefore, it is important to assess the decrease in the susceptibility of \textit{P. falciparum} populations from the Thiès region (Senegal) to amodiaquine several years after the withdrawal of chloroquine in 2003. This was done by combining \textit{ex vivo} SYBR\textsuperscript{™} Green and whole-genome sequencing (WGS) approaches on \textit{P. falciparum} isolates collected in 2018.

**Materials and methods**

**Ethics**

The National Ethics Committee for Health Research and the Ministry of Health of Senegal approved the protocol used for this study under number 0000081/MSAS/DPRS/CNERS (July 14, 2017). Written and informed consent was obtained from all participants, before participant recruitment and sample collection.

**Study site**

Patient recruitment took place during the seasonal malaria period (from September to December) in 2018 in Thiès, a region located at 75 km from Dakar where malaria incidence ranges between five and 15 per 1000 inhabitants\textsuperscript{20,21}. Thiès is characterized as Sahelian facies, defined by a short malaria seasonal transmission (generally fewer than 4 months after the rainy season ends). In this region, \textit{Anopheles arabiensis} and \textit{Anopheles gambiae} are the main malaria mosquito vectors and the entomological
inoculation rate (EIR) estimated to be < 5, varies from one year to another (0–20 infectious bites/person/year). Malaria transmission is perennial and hypoendemic, with an increase in cases at the end of the rainy season from September to December.

**Study design**

Individuals who presented at the Service de Lutte Antipaludique (SLAP) – a health facility specializing in malaria case management – and tested positive for *P. falciparum* by microscopy were enrolled. This site was chosen due to its close proximity to our laboratory situated in Dakar. This allowed us to transport the samples for *ex vivo* testing on a daily basis. Other criteria for inclusion were uncomplicated malaria due to *P. falciparum* with a parasite density between 1000 and 200,000 asexual forms/µL of blood. Exclusion criteria were a presence of general danger signs or other signs of severe and complicated *P. falciparum* malaria as defined by the World Health Organization (WHO) or individual who had a history of taking anti-malarial treatment prior to the visit. Informed consent or assent of the patient and their guardian (for children) was obtained before collecting the blood. Patients with clinical malaria were treated with artemether–lumefantrine (AL, Coartem®), according to the treatment guideline of the NMCP. For each patient, 5 mL vacutainer tubes of venous blood were collected for *ex vivo* SYBR™ Green, prior ACT treatment.

**Antimalarial drugs preparation**

The following drugs were reconstituted with dimethyl sulfoxide (DMSO; Sigma, D8418) and tested for the *ex vivo* assay: amodiaquine hydrochloride (AM, USP, reference standard [500 mg]), chloroquine diphosphate salt (CQ, C6628-25G), lumefantrine (LUM, USP reference standard [100 mg]), mefloquine hydrochloride (MQ, M2319-100MG), piperaquine tetraphosphate tetrahydrate (PPQ, C7874-25MG) and quinine hydrochloride dihydrate (QN, Q1125-10G). Two-fold serial dilutions were performed with the Roswell Park Memorial Institute (RPMI 1640 medium, Gibco, Life technologies) medium. Drug concentrations plated were 100 nM for amodiaquine, 750 nM for chloroquine, 2000 nM for lumefantrine, 500 nM for mefloquine, 500 nM for piperaquine, and 1,500 nM for quinine. Drugs were plated in duplicate and distributed in 96-wells plates at 20 µL per well. Plates were frozen at −20°C until needed.

**Culture and parasite DNA quantification**

*Ex vivo* assays were performed as follows: venous blood from patients was first centrifuged (2,500g for 10 min) to remove plasma, then the pellet was washed twice with RPMI 1640 by centrifugation at 2,500g for 5 min. Parasitaemia was calculated and corrected if greater than 1%. Samples with parasitaemia lower than 0.3% were discarded. Supplemented RPMI 1640 media was added to the sample to adjust the haematocrit to 2% before loading on plates already filled with drugs. The plates were incubated at 37°C with gas mixture (1% O₂, 5% CO₂ and 94% N₂) for 48 or 72 h, until parasite re-invasion as assessed by microscopy. Plates were frozen at -20°C until parasite DNA quantification. The *P. falciparum* 3D7 laboratory reference strain was used as internal control. Quantification was performed using SYBR™ Green dye. One hundred µL of buffer solution containing SYBR™ Green was added to each well, gently mixed and incubated for an hour in the dark. DNA quantification was performed using a Fluoroskan™
instrument (Fluoroskan ascent, Thermo Fisher). The *in vitro* analysis and reporting tool (IVART) was used to analyze the *ex vivo* data\(^{19,25}\).

**DNA extraction**

Total DNA was extracted from the same whole blood samples used for the *ex vivo* SYBR™ Green using the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA, USA) according to manufacturer’s instructions. The final DNA volume of the eluates was 100 µL and stored at -20°C until use.

**Whole-genome sequencing**

A total of 250 ng of each gDNA sample based on Qubit quantification was mechanically fragmented on a Covaris S220 focused ultrasonicator (Covaris, Woburn, MA, USA). Sheared gDNA was used to perform end-repair, A-tailing and adapter ligation and finally amplified using the KAPA library HyperPrep kit (Kapa Biosystems Inc. Wilmington, MA, USA) according to the manufacturer’s instructions. After quality control and quantification using High Sensitivity DNA chip (Agilent Technologies) and Qubit fluorometer (Thermofisher), the libraries were sequenced on an Illumina Nextseq 500 system (Illumina Inc, San Diego, CA, USA) using paired-end mode (2X151 cycles) with V2 chemistry at the Cochin Institute (Paris, France).

**Statistical analysis**

Analysis of variance (ANOVA) software was used to calculate the IC\(_{50}\) values for all drugs for each parasite isolate tested. The correlation between the IC\(_{50}\) values for different drugs was assessed by using the Spearman’s rank order correlation coefficient (\(r\)). The test was considered as significant if the \(P\)-value was less than 0.05. Bioinformatics analyses were performed using the CLC Genomics Workbench 22 software (Qiagen).

**Results**

**Baseline characteristics.** A total of 34 patients with uncomplicated *P. falciparum* malaria meeting the inclusion criteria were enrolled. The sex ratio (M/F) was largely dominated by males (30 males/4 females). The age of the participants ranged from 7 to 73 years (mean of 27.06 years). Mean weight was 55.74 kg and mean body temperature was 39.15°C. The mean parasitaemia was 0.79%, ranging from 0.43–1.15% (Table 1).
Table 1
Baseline characteristics of the study participants and the *P. falciparum* isolates, Thiès, Senegal, 2018.

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>CI95%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>27.06</td>
<td>21.94–32.18</td>
</tr>
<tr>
<td>Weight (kilogram)</td>
<td>55.74</td>
<td>48.90-62.58</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>39.15</td>
<td>38.82–39.48</td>
</tr>
<tr>
<td>Glycaemia</td>
<td>1.01</td>
<td>0.94–1.08</td>
</tr>
<tr>
<td>Haemoglobin</td>
<td>11.5</td>
<td>10.76–12.24</td>
</tr>
<tr>
<td>Parasitaemia (%)</td>
<td>0.79</td>
<td>0.43–1.15</td>
</tr>
</tbody>
</table>

**Ex vivo susceptibility to chloroquine, amodiaquine, lumefantrine, mefloquine, piperaquine, and quinine.** Of the 34 clinical *P. falciparum* isolates, 13 (38%) were successfully tested for *ex vivo* susceptibility to antimalarial drugs. Amodiaquine (AQ) and mefloquine (MQ) had the lowest IC<sub>50</sub> geometric mean (GM) against *P. falciparum* isolates (Fig. 1 and Table 2). Parasites showed reduced *ex vivo* susceptibility only to chloroquine (16%, 2/12).

Table 2
*Ex vivo* drug susceptibility of *Plasmodium falciparum* isolates collected from Thiès, Senegal

<table>
<thead>
<tr>
<th>Drug</th>
<th>Number of isolates</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; geometric mean (nM)</th>
<th>CI95% for the Geometric mean</th>
<th>Range</th>
<th>Proportion of resistant isolates§</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroquine</td>
<td>12</td>
<td>26.9</td>
<td>12.6–57.3</td>
<td>7.0–296.2</td>
<td>16.6% (2/12)</td>
</tr>
<tr>
<td>Amodiaquine</td>
<td>11</td>
<td>4.6</td>
<td>2.5–8.3</td>
<td>2.0–26.0</td>
<td>0% (0/11)</td>
</tr>
<tr>
<td>Lumefantrine</td>
<td>12</td>
<td>87.2</td>
<td>49.8–162.3</td>
<td>14.5–412.9</td>
<td>NA</td>
</tr>
<tr>
<td>Mefloquine</td>
<td>13</td>
<td>7.7</td>
<td>5.7–10.6</td>
<td>2.9–18.3</td>
<td>0% (0/13)</td>
</tr>
<tr>
<td>Piperaquine</td>
<td>12</td>
<td>19.4</td>
<td>10.5–35.8</td>
<td>4.5–73.3</td>
<td>NA</td>
</tr>
<tr>
<td>Quinine</td>
<td>13</td>
<td>41.5</td>
<td>28.3–60.8</td>
<td>13.3–111.6</td>
<td>0% (0/13)</td>
</tr>
</tbody>
</table>

§Threshold IC<sub>50</sub> value (nM) for resistance: chloroquine = 100 nM, amodiaquine = 80 nM, quinine = 300–500 nM, mefloquine = 20–30 nM, NA: Not applicable (i.e., no threshold IC<sub>50</sub> reported to date for lumefantrine or piperaquine), CI95%: 95% confidence interval.
Association between antimalarial drugs IC\textsubscript{50}. The association of the \textit{ex vivo} susceptibility of the paired drugs was then assessed. The only positive and significant correlation ($r = 0.72$ $P = 0.007$) was observed between piperaquine and amodiaquine (Table 3).

Table 3
Associations between the \textit{ex vivo} responses of \textit{Plasmodium falciparum} isolates from Thiès (Senegal) to antimalarial drugs

<table>
<thead>
<tr>
<th>Drug pair</th>
<th>$r$</th>
<th>$P$ value$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroquine-Quinine</td>
<td>0.44</td>
<td>0.125</td>
</tr>
<tr>
<td>Chloroquine-Amodiaquine</td>
<td>0.09</td>
<td>0.786</td>
</tr>
<tr>
<td>Chloroquine-Lumefantrine</td>
<td>-0.20</td>
<td>0.529</td>
</tr>
<tr>
<td>Chloroquine-Mefloquine</td>
<td>-0.31</td>
<td>0.307</td>
</tr>
<tr>
<td>Chloroquine-Piperaquine</td>
<td>0.03</td>
<td>0.926</td>
</tr>
<tr>
<td>Quinine-Amodiaquine</td>
<td>-0.07</td>
<td>0.813</td>
</tr>
<tr>
<td>Quinine-Lumefantrine</td>
<td>0.26</td>
<td>0.380</td>
</tr>
<tr>
<td>Quinine-Mefloquine</td>
<td>-0.23</td>
<td>0.418</td>
</tr>
<tr>
<td>Quinine-Piperaquine</td>
<td>0.32</td>
<td>0.276</td>
</tr>
<tr>
<td>Amodiaquine-Lumefantrine</td>
<td>0.01</td>
<td>0.972</td>
</tr>
<tr>
<td>Amodiaquine-Mefloquine</td>
<td>0.39</td>
<td>0.198</td>
</tr>
<tr>
<td>Amodiaquine-Piperaquine</td>
<td>0.72</td>
<td>$0.007$</td>
</tr>
<tr>
<td>Lumefantrine-Mefloquine</td>
<td>0.08</td>
<td>0.789</td>
</tr>
<tr>
<td>Lumefantrine-Piperaquine</td>
<td>0.44</td>
<td>0.150</td>
</tr>
<tr>
<td>Mefloquine-Piperaquine</td>
<td>0.25</td>
<td>0.395</td>
</tr>
</tbody>
</table>

$r = \text{Spearman's rank order correlation coefficient}$; $^a\text{Value in bold is significant } P \text{ value for pairwise comparisons}$

Prevalence of mutations in the \textit{pfcr}, \textit{pfmdr1}, \textit{pfdhfr} and \textit{pfdhps} genes. The proportion of mutations at codons $74$ (M $>$ I), $75$ (N $>$ E) and $76$ (K $>$ T) of \textit{pfcr} were intermediate with a frequency of $21\%$. The most common mutation in the \textit{pfmdr1} gene was Y184F ($53\%$), followed by N86Y, I740M, V798I and A891T found in $3\%$ of the isolates that have been successfully tested. A high frequency of mutations in the \textit{pf dhfr} gene was observed at codons $51$ (60%; N51I), $59$ (64%; C59R) and $108$ (75%; S108N). Fourty-two and fifty percents of the isolates harboured the \textit{pf dhps} mutations S436A and A437G, respectively. The \textit{pf dhps} I431V, K540E, A581G and A613S/T mutations were not detected (Table 4). Triple and quadruple inferred \textit{pf dhfr/ pf dhps} mutants were observed with a prevalence of $50\%$ and $28\%$, respectively (Table 5).
Table 4
Prevalence of point mutations found in genes associated with antimalarial drug resistance in isolates from Thiès, Senegal.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Codon</th>
<th>Reference allele</th>
<th>Mutant allele</th>
<th>Prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>pfcrt</td>
<td>74</td>
<td>M</td>
<td>I</td>
<td>21% (6/28)</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>N</td>
<td>E</td>
<td>21% (6/28)</td>
</tr>
<tr>
<td></td>
<td>76</td>
<td>K</td>
<td>T</td>
<td>21% (6/28)</td>
</tr>
<tr>
<td></td>
<td>356</td>
<td>I</td>
<td>T</td>
<td>7% (2/28)</td>
</tr>
<tr>
<td></td>
<td>371</td>
<td>R</td>
<td>I</td>
<td>3% (1/28)</td>
</tr>
<tr>
<td>pfmdr1</td>
<td>86</td>
<td>N</td>
<td>Y</td>
<td>3% (1/28)</td>
</tr>
<tr>
<td></td>
<td>184</td>
<td>Y</td>
<td>F</td>
<td>53% (15/28)</td>
</tr>
<tr>
<td></td>
<td>740</td>
<td>I</td>
<td>M</td>
<td>3% (1/28)</td>
</tr>
<tr>
<td></td>
<td>798</td>
<td>V</td>
<td>I</td>
<td>3% (1/28)</td>
</tr>
<tr>
<td></td>
<td>891</td>
<td>A</td>
<td>T</td>
<td>3% (1/28)</td>
</tr>
<tr>
<td>pfdhfr</td>
<td>51</td>
<td>N</td>
<td>I</td>
<td>60% (17/28)</td>
</tr>
<tr>
<td></td>
<td>59</td>
<td>C</td>
<td>R</td>
<td>64% (18/28)</td>
</tr>
<tr>
<td></td>
<td>108</td>
<td>S</td>
<td>N</td>
<td>75% (21/28)</td>
</tr>
<tr>
<td>pfdhps</td>
<td>436</td>
<td>S</td>
<td>A</td>
<td>42% (12/28)</td>
</tr>
<tr>
<td></td>
<td>437</td>
<td>A</td>
<td>G</td>
<td>50% (14/28)</td>
</tr>
</tbody>
</table>

Table 5
Prevalence of the inferred mutant haplotype in the pfdhps and the pfdhfr genes.

<table>
<thead>
<tr>
<th>Inferred haplotype</th>
<th>Proportion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triple mutation</td>
<td>50% (14/28)</td>
</tr>
<tr>
<td>Quadruple mutation</td>
<td>28% (8/28)</td>
</tr>
</tbody>
</table>

Association between genotype and phenotype

We looked for associations between *ex vivo* susceptibility, as expressed by IC$_{50}$, and molecular signatures detected by WGS. Despite our small sample size, we observed that *pfcrt* mutations M74I, N75E, K76T, R371I and *pfmdr1* Y184F were significantly associated with decreased *ex vivo* susceptibility to chloroquine (Table 6). Similar trends were observed for quinine in the isolate carrying the *pfcrt* R371I mutation, for amodiaquine in the isolate carrying the *pfmdr1* V798I mutation and for mefloquine in the
isolate carrying the \textit{pfmdr1} I740M mutation. For lumefantrine and piperaquine, no change in \textit{ex vivo} susceptibility according to the genotype was observed.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Codon</th>
<th>GM of IC$_{50}$ (nM) for wild type</th>
<th>GM of IC$_{50}$ (nM) for mutant</th>
<th>\textit{P} value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroquine</td>
<td>\textit{pfcrt} M74I</td>
<td>27</td>
<td>147</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>\textit{pfcrt} N75E</td>
<td>27</td>
<td>147</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>\textit{pfcrt} K76T</td>
<td>27</td>
<td>147</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>\textit{pfcrt} R371I</td>
<td>35</td>
<td>296</td>
<td>\textit{&lt; 0.001}</td>
</tr>
<tr>
<td></td>
<td>\textit{pfmdr1} Y184F</td>
<td>21</td>
<td>107</td>
<td>0.02</td>
</tr>
<tr>
<td>Amodiaquine</td>
<td>\textit{pfmdr1} V798I</td>
<td>5.2</td>
<td>25.9</td>
<td>0.01</td>
</tr>
<tr>
<td>Mefloquine</td>
<td>\textit{pfmdr1} I740M</td>
<td>7.9</td>
<td>18.3</td>
<td>0.01</td>
</tr>
<tr>
<td>Quinine</td>
<td>\textit{pfcrt} R371I</td>
<td>44.3</td>
<td>111.6</td>
<td>0.03</td>
</tr>
</tbody>
</table>

GM: geometric mean, IC$_{50}$: half maximal inhibitory concentration

\textbf{Discussion}

In 2006, Senegal introduced ACT (artemether-lumefantrine, AL, and artesunate-amodiaquine, ASAQ) as first-line and dihydroartemisinin-piperaquine (DHA-PQ) as second-line in the treatment of uncomplicated \textit{P. falciparum} malaria. To date, prompt management of the malaria cases using these antimalarial drugs have contributed significantly to a reduction in malaria-related morbidity and mortality\textsuperscript{26}, confirming that AL, ASAQ and DHA-PQ remain effective and well tolerated in Senegal\textsuperscript{27–30}. However, 1.2% of AL and 2.5% of DHA-PQ treatment failures have been reported in Senegal in 2020\textsuperscript{31}. These results suggest that monitoring the efficacy of these antimalarial drugs is essential for malaria control, as there is no other alternative drug treatment available\textsuperscript{32}. An important tool for monitoring antimalarial drug resistance in field isolates is the use of \textit{ex vivo} assays. They complement clinical drug studies, allowing researchers to measure parasite responses to different drugs individually, without patient-related factors. Importantly, \textit{ex vivo} monitoring of malaria parasite drug responses plays an important role in early detection of reduced parasite drug susceptibility and treatment failure\textsuperscript{8}. In this study, the \textit{ex vivo} SYBR™ Green assay was used to test samples collected during the seasonal period of 2018 in Thiès, Senegal. Six drugs (amodiaquine, chloroquine, lumefantrine, mefloquine, piperaquine and quinine), combined as partner drug in ACT with the exception of chloroquine and quinine, were evaluated.
Our results showed that the IC₅₀ values of chloroquine were slightly higher (GM = 26.9 nM) than those previously reported in Thiès in 2013 (GM = 18.29 nM)⁴³, but lower than those reported in Dakar in 2014–2015 (GM = 62.2 nM)⁴³⁴, suggesting a decrease in parasite susceptibility to chloroquine and consistent with the high chloroquine resistance rate found in Dakar in 2015 (46.9%). The higher level of chloroquine susceptibility in Thiès compared to Dakar is not surprising, as Dakar is a known centre of chloroquine resistance⁴⁵. No cross-resistance between chloroquine and amodiaquine was observed, but association between the pfcr⁴ K76T and pfmdr¹ Y184F mutations ex vivo susceptibility to chloroquine was detected.

Conversely, we found increased susceptibility to amodiaquine in Thiès (GM = 4.6 nM) with an IC₅₀ < 60 nM according to the WHO guideline. The GM varied from 13.84 nM in 2012 to 6.48 nM in 2013⁴³. Here, no ex vivo resistant parasite was found to amodiaquine, contrasting with data from Dakar in 2015, where 28.1% of isolates were associated with in vitro monodesethylamodiaquine resistance⁴⁴. We noticed that the GM of isolates with the pfmdr¹ V798I mutation was higher than that of isolates with the wild-type allele.

Similarly to amodiaquine, we observed an increase in ex vivo susceptibility to lumefantrine in Thiès (from 173.4 nM in 2012, to 113.2 nM in 2013 and 87.2 nM in 2018)⁴³. These values were very high in comparison with Dakar in 2015 (mean = 3.5 nM) and Ghana in 2016–2018 (mean = 2.7 nM)⁴⁷. However, no AL treatment failure was reported in a clinical trial conducted in Thiès from 2012 to 2014⁴⁸.

The GM for piperaquine was estimated to 19.4nM. This is slightly higher than that reported in Pikine in 2014 (15.28 nM)⁴⁹, but lower than that reported in Dakar in 2014 (36.5 nM). A mean of 4.6 nM was reported in a study conducted in Ghana⁴⁷. Further studies are needed to better estimate piperaquine ex vivo susceptibility, as the lack of resistance cut-offs makes it impossible to estimate the resistance rates.

Mutations in pfcr M74I, N75E, K76T, R37I are associated with chloroquine resistance in P. falciparum. The mutations at codons 76 of the pfcr gene and pfmdr¹ 86 were found to be highly associated with resistance to chloroquine⁴⁰–⁴², and again observed in this study. We detected that 16% of the tested isolates were resistant to chloroquine (i.e., IC50 > 100 nM), a value similar to the one observed from a study performed in Thiès in 2010 (23% of isolates exhibited chloroquine resistance)⁴³. Similar trends are also observed in other regions of Senegal⁴⁴.

The study presented here has several limitations. First, our work included only 34 samples. Second, despite this small sample size, only 13 isolates from the ex vivo SYBR™ Green test were successfully assayed. Third, only 28 out of the 34 samples were successfully sequenced. Fourth, the study was conducted at only one site in Senegal (Thiès).

Conclusion

The pfcr K76T and pfmdr¹ Y184F mutations were the most common among the pfcr and pfmdr¹ genes reported in this study, accounting for 21% and 53%, respectively. The high occurrence of pfcr K76T and
pfmdr1 Y184F mutants likely indicate the reduced susceptibility of P. falciparum parasites to chloroquine in Thiès. The results of this study show high susceptibility of P. falciparum populations to amodiaquine, lumefantrine, mefloquine, piperaquine and quinine, except for chloroquine with reduced in vitro susceptibility.

Declarations

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Transparency declarations

The remaining authors have no financial conflicts of interest to disclose.

References


**Figures**

**Figure 1**

![Graphs showing susceptibility and genotyping of *Plasmodium falciparum* isolates](image-url)
Distribution of $IC_{50}$ values among parasites collected in Thiès (Senegal) in 2018 and tested against (a) amodiaquine, (b) chloroquine, (c) lumefantrine, (d) mefloquine, (e) piperaquine and (f) quinine. Horizontal lines indicate the geometric mean of $IC_{50}$ value in red with the 95% CI.