URBAN MALARIA IN DAKAR, SENEGAL: CHEMOSUSCEPTIBILITY AND GENETIC DIVERSITY OF PLASMODIUM FALCIPARUM ISOLATES

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Abstract. The chemosusceptibility and genetic polymorphism of Plasmodium falciparum populations from 48 patients hospitalized for malaria at the Hospital Principal in Dakar, Senegal were investigated during the 2002 malaria transmission season. Sixty-two percent of the isolates collected were from patients with severe malaria and 38% were from patients with mild malaria. In vitro activities of chloroquine, quinine, cycloguanil, atovaquone, mefloquine, halofantrine, and artesunate were evaluated. The prevalence of mutations in the Plasmodium falciparum dihydrololate reductase (dhfr) and dihyropteroate synthetase (dhps) genes and the P. falciparum chloroquine resistance transporter (Pfcr) gene associated with cycloguanil, pyrimethamine, sulfadoxine, and chloroquine resistance were estimated. The genetic polymorphism of the parasite populations was evaluated by analysis of the highly polymorphic regions of merozoite surface protein 1 (msp1) block 2 and msp2. Seventy percent of the isolates were assessed by an in vitro assay. Fifty-two percent of the isolates were chloroquine resistant, 45% were cycloguanil resistant, and 24% were atovaquone resistant. Four percent had low susceptibility to quinine. The Pfcr and dhfr mutations were associated with in vitro chloroquine- and antimetabolic drug-resistant isolates, respectively. Approximately 70% of the isolates contained two or more clones. Genetic diversity of P. falciparum was high. The prevalence of allelic family K1 of msp1 was 68%. Isolates of P. falciparum were highly resistant to chloroquine, cycloguanil and atovaquone. The transmission rate of malaria in Dakar is low but a high degree of genetic polymorphism can increase severe malaria, as shown by persons coming to Dakar from areas highly endemic for malaria. Areas with urban malaria should use vector control measures and efficient chemoprophylaxis for non-immune populations.

INTRODUCTION

Malaria is the most prevalent parasitic disease worldwide. Although young African children from rural areas have the greatest risk for acquiring malaria, pregnant women, non-immune travelers, refugees, and displaced persons are also at high risk.1 In Africa, different populations are not exposed to the malaria in same epidemiologic patterns. These patterns are related to climatic, phytogeographic, and transmission (entomologic, inoculation rates and periodicity of transmission) characteristics.2 In west Africa, e.g., Senegal, four epidemiologic patterns of malaria transmission have been described: stable transmission throughout the year, stable transmission with a long seasonal outbreak (more than six months), transmission over a short period, and epidemic transmission.

Genetic diversity of Plasmodium falciparum is common in high transmission areas. The diversity is associated with the transmission intensity3 and can induce a high level of severe malaria, especially in cities where the pressure against malaria is lower than other disease-endemic areas.4,5 Dakar, the capital of Senegal, has an urban population of approximately 1.6 million and a suburban population of 2.3 million. A study has shown that the level of malaria transmission in Dakar is generally low.6 Two distinct areas can be distinguished in this city, the central area with many buildings and an infrastructure, and the suburbs with many gardens and perennial water pools.6 The density of Anopheles arabiensis and malaria transmission decrease as one travels from the suburbs to central Dakar, but this situation can be modified urban development in the suburbs or the presence of marshlands where transmission is the same as in rural areas. A relatively low entomologic inoculation rate is observed in Dakar (< 1 infected bites/person/year), but Senegal has high rates of malaria transmission in rural areas (89–350 infected bites/person/year).2 Dakar is a site of considerable mixing of populations from towns, rural areas, and other countries with different rates of malaria transmission. Thus, we conducted this study to estimate antimalarial drug susceptibilities of P. falciparum isolates and their genetic diversity in this city.

MATERIALS AND METHODS

Patients and samples. Patients were recruited at the Hôpital Principal in Dakar, Senegal, between September and November 2002. We included in the study all patients who came to the hospital for treatment of malaria who had not used chemoprophylaxis or chemotherapy. All age, sex, and ethnic groups were included. Malaria was diagnosed by detection of P. falciparum on thick blood films by light microscopy. We included 48 Senegalese patients between 3 and 65 years of age (mean = 22 years). Venous blood was collected into Vacutainer® tubes (Becton Dickinson, Franklin Lakes, NJ) before treatment and transported at 4°C to our laboratory in Marseille. Informed oral consent was obtained from patients and/or their parents before collection of blood. All protocols were reviewed and approved by the ethical commission of Hôpital Principal de Dakar. Upon arrival of the isolates in Marseille, blood smears were prepared and stained using an RAL® kit (Réactifs RAL, Paris, France). They were examined to determine parasitemias, which ranged from 0.01% to 6.0%. Two 500-μL samples were used for extraction of DNA to determine genetic polymorphisms. The 3D7 and W2 strains

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of *P. falciparum* were used as standards for determination of chloroquine and proguanil susceptibility.

**In vitro chemosusceptibility assay.** Thirty-two samples with parasitemias greater than 0.05% were used to test drug susceptibility (only 16 samples were tested for artesunate susceptibility because of insufficient amounts of blood). Parasitized erythrocytes were washed three times in RPMI 1640 medium (Invitrogen, Paisley, United Kingdom). If parasitemias exceeded 0.8%, infected erythrocytes were diluted to 0.5–0.8% with uninfected erythrocytes and resuspended in culture medium to a hematocrit of 1.5%. Susceptibilities to chloroquine, quinine, mefloquine, halofantrine, artesunate, and atovaquone were determined after suspension in RPMI 1640 medium and to cycloguanil after suspension in RPMI 1640 SP823 medium with less p-aminobenzoic acid (0.5 μg/mL) and folates (10 μg/L) (Invitrogen). The two suspensions were supplemented with 10% human serum and buffered with 25 mM HEPES and 25 mM NaHCO₃.

Chloroquine diphosphate and quinine hydrochloride were obtained from Sigma (St. Louis, MO), atovaquone and halofantrine from Glaxo Beecham Wellcome (Uxbridge, United Kingdom), artesunate from Sanofi Winthrop (Trentilly, France), cycloguanil from Zeneca Pharma (Reims, France), and mefloquine from Roche (Paris, France). Stock solutions were prepared in methanol for quinine, mefloquine, halofantrine, artesunate and atovaquone, and in sterile water for chloroquine and cycloguanil. Two-fold serial dilutions were prepared in sterile water. The eight final concentrations, which ranged from 5 to 3,200 nM for chloroquine, 25 to 3,200 nM for quinine, 10 to 20,000 for cycloguanil, 0.1 to 100 nM for artesunate, 0.25 to 32 nM for halofantrine, 3.2 to 400 nM for quinine, and 0.32 to 100 nM for atovaquone, were distributed in triplicate into Falcon 96-well flat-bottomed plates (Becton, Dickinson). The chloroquine-resistant 3D7 *P. falciparum* clone (Africa) and the chloroquine-resistant W2 clone (Indochina) were used as references to test each batch of plates. For *in vitro* isotopic microtests, 200 μL/well of the suspension of parasitized erythrocytes was distributed in 96-well plates preloaded with antimalarial agents. Parasite growth was assessed by adding 1 μCi of 3H-hypoxanthine with a specific activity of 14.1 Ci/mmol (New England Nuclear, Dreieich, Germany) to each well. Plates were incubated for 42 hours at 37°C in an atmosphere of 10% O₂, 6% CO₂, and 84% N₂, and a humidity of 95%. Immediately after incubation, the plates were frozen and thawed to lyse the erythrocytes. The contents of each well were collected on standard filter microplates (Unifilter™ GF/B; Perkin Elmer, Wellesley, MA) and washed using a cell harvester (FilterMate™ Cell Harvester; Perkin Elmer). Filter microplates were dried and 25 μL of scintillation cocktail (Microscint™ O; Packard, Meriden, CT) was placed in each well. Radioactivity incorporated by the parasites was measured using a scintillation counter (Top Counter™; Perkin Elmer). The 50% inhibitory concentration (IC₅₀), i.e., the drug concentration corresponding to 50% of the uptake of 3H-hypoxanthine by the parasites in drug-free control wells, was determined by non-linear regression analysis of log-dose/response curves. Data were analyzed after logarithmic transformation and expressed as the geometric mean IC₅₀ and 95% confidence intervals (95% CIs) were calculated.

**Genetic polymorphisms.** Total genomic DNA of each isolate was extracted using the E.Z.N.A.® Blood DNA kit (Omega Bio-tek, Doraville, GA). The dihydrofolate reductase gene (*dhfr*) sequence (862 basepairs) was amplified using primers DHFR/D-9/40, 5′-CAT TTT GCT GCC GGT CAC TCC TTG -TGA TGG AAC AAG T-3′ and DHFR/C-853/27, 5′-AAA ATA AAC AAA ATC TTC TTC TTC-3′. The dihydropteroate synthase gene (*dhps*) sequence (1,136 basepairs) was amplified using primers DHPS/8004/D-1772/30, 5′-TGC TTA AAT GAT ATG ATA CCC GAA TAT AAG-3′ and DHPS/D-2908/25, 5′-TCC ACC TGA AAA GAA ATA CAT AAA T-3′. The *P. falciparum* chloroquine resistance transporter (*Pfcr*) gene allele-specific (*Pfcr*-76T) sequence (537 basepairs) was amplified using primers TCRP1, 5′-CGG TTA ATA ATA ATA CAC GCA G-3′ and TCRP2, 5′-CGG ATG TTA CAA AAC TAT AGT TAC C-3′.

**Genetic diversity of *P. falciparum.** The genomic DNA of the *P. falciparum* isolates was investigated for genetic diversity at highly polymorphic loci (merozoite surface protein 1 [MSP1] and MSP2). The *msp1* and *msp2* loci have been genotyped using the nested polymerase chain reaction (PCR) strategy and conditions previously described with the following modification. Fluorescent-tagged primers were used for the nested allele family-specific PCRs, i.e., for families K1, Mad20 and Ro33 at the *msp1* locus and for families 3D7 and FC27 at the *msp2* locus. Size length was analyzed with GeneScan (Metairie, LA) technology. Positive samples for K1 *msp1* and 3D7 *msp2* were obtained from the 3D7 strain of *P. falciparum* and positive samples for Mad20 *msp1* and FC27 *msp2* were obtained from the W2 strain of *P. falciparum*.

**RESULTS**

Forty-eight Senegalese patients (30 males and 18 females) with parasitemias ranging from 0.01% to 6.0% were enrolled in this study. They had a mean age was 22 years (range = 3–65 years). Seventy-four percent had remained in Dakar in the 30-day period before hospitalization and 16% lived in the suburbs of Dakar. Eighty had mild cases of malaria and 30 had severe cases, based on one or more World Health Organization criteria. The group with severe malaria had at least one of the following manifestations: 45% with confusion or drowsiness (Blantryre coma scores > 3 and < 5, Glasgow coma scores > 9 and < 14), 24% had cerebral malaria with prostration (Blantryre coma score < 3 and Glasgow coma score < 10), 105 had metabolic acidosis with respiratory distress or respiratory distress syndrome, 34% had hyperparasitemia (> 4% of red blood cells), 3% had hypothermia, and 3% had acute renal failure. Thrombocytopenia differed significantly (P = 0.0076) between severe cases (mean ± SD = 88,107 ± 15,000 platelets/mL) and mild cases (175,000 ± 30,000 platelets/mL). Two patients in this group died.

Thirty-three isolates had a growth ratio (maximum cpm/minimum cpm) that ranged from 4.6 to 17.7. Nine isolates with a ratio < 4 were considered failures of *in vitro* culture. The following proportions of isolates were successfully cultured for each drug tested: 23 of 32 for chloroquine, quinine, mefloquine and halofantrine, 22 of 32 for cycloguanil and atovaquone, and 11 of 16 for artesunate. Average parameter estimates for seven drugs against all isolates are shown given in Table 1. The IC₅₀ values for chloroquine ranged from 70 to 148 nM with a geometric mean of 102 nM. The IC₅₀ values for...
In vitro activity of chloroquine, quinine, mefloquine, halofantrine, cycloguanil, artemunate, and atovaquone assessed on 23 Plasmodium falciparum isolates collected in Dakar, Senegal in 2002

<table>
<thead>
<tr>
<th>Drug</th>
<th>No. of isolates</th>
<th>Mean IC₅₀ (nM)</th>
<th>95% confidence interval (nM)</th>
<th>Cut-off value (nM)</th>
<th>Percent of resistant isolates</th>
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<tr>
<td>Chloroquine</td>
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<td>Quinine</td>
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<td>Halofantrine</td>
<td>23</td>
<td>1.95</td>
<td>1.39–2.74</td>
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<td>Cycloguanil</td>
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* Values are mean 50% inhibitory concentration (IC₅₀) i.e., the drug concentration corresponding to 50% of the uptake of 3⁵⁷-hypoxanthine by the parasites in drug-free control wells.
† Values are proportion of resistant isolates according to cut-off values.

The frequency of merozoite surface protein 1 (msp1) and merozoite surface protein 2 in 37 isolates from the study sample.

**DISCUSSION**

Malaria transmission rates are usually lower in urban areas than in rural areas because of a lower density of anopheles vectors. Thus, malaria can be contracted throughout the year at the same intensity or seasonally, which is dependent on climate in an area. In addition, variations in transmission have been observed in different parts of the same region (i.e., city versus suburbs). Chloroquine resistance in P. falciparum is established in many part of Africa and is become more prevalent in areas of west Africa such as Senegal. As far back as 1988, in vitro resistance to chloroquine was reported in Dakar and in other regions of the country shortly afterward. From 1991 to 1995, parasitologic failures were observed in 21% of patients in Pikine and in 23% in another region of Senegal.

**Table 1**

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**Table 2**

Frequency of Plasmodium falciparum (codons 51, 59, 108), dhfr (codons 436, 437, 540), and Pfcrt (codon 76) mutations among 48 blood samples from Dakar

<table>
<thead>
<tr>
<th>Codon</th>
<th>Wild (%)</th>
<th>Mixed (%)</th>
<th>Mutant (%)</th>
<th>No.</th>
</tr>
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<tr>
<td>dhfr</td>
<td>Codon 51</td>
<td>12 (34%)</td>
<td>1 (3%)</td>
<td>22 (63%)</td>
</tr>
<tr>
<td></td>
<td>Codon 59</td>
<td>13 (37%)</td>
<td>2 (6%)</td>
<td>20 (57%)</td>
</tr>
<tr>
<td></td>
<td>Codon 108</td>
<td>11 (30%)</td>
<td>2 (5%)</td>
<td>24 (65%)</td>
</tr>
<tr>
<td>dhps</td>
<td>Codon 436</td>
<td>27 (90%)</td>
<td>0</td>
<td>3 (10%)</td>
</tr>
<tr>
<td></td>
<td>Codon 437</td>
<td>23 (77%)</td>
<td>1 (3%)</td>
<td>6 (20%)</td>
</tr>
<tr>
<td></td>
<td>Codon 540</td>
<td>33 (100%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pfcrt</td>
<td>Codon 76</td>
<td>13 (33%)</td>
<td>1 (2%)</td>
<td>26 (65%)</td>
</tr>
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* dhfr = dihydrofolate reductase; dhps = dihydropteroate synthetase; Pfcrt = chloroquine resistance transporter.
between 1999 and 2001 showed that 29 (55%) of 53 isolates obtained 280 km southeast of Dakar and 19 (53%) of 36 isolates from Pikine were resistant in vitro to chloroquine.\textsuperscript{16,17} The K76T mutation in \textit{Pfert} was present in 23 (64%) of 36 isolates.\textsuperscript{17} In this report, we determined that 12 (52%) of 23 isolates were resistant in \textit{vitro} to chloroquine and examination of molecular markers of chloroquine resistance showed that 26 (65%) of 40 had the T76 resistant allele. This mutation in \textit{Pfert} was associated with in \textit{vitro} chloroquine-resistant isolates; this mutation was also prevalent in three chloroquine-susceptible isolates in \textit{vitro}. This suggests involvement of additional genetic loci in modulating chloroquine resistance: the \textit{Pfert} T76 allele is required but not a sufficient predictor for in \textit{vitro} chloroquine-resistance. These data are consistent with previous analysis in Senegal.\textsuperscript{17,18}

The same prevalence of chloroquine resistance found in different areas of Senegal, which suggests that there is a relationship between resistance and drug pressure and population migration.\textsuperscript{2} The World Health Organization refers to “weekend malaria” in Africa. It occurs when inhabitants return to their rural settings and this phenomenon seems to be an increasing problem.

In West Africa, failure of antimalarial prophylaxis with mefloquine in non-immune populations associated with in \textit{vitro} resistance has been observed.\textsuperscript{19} Prophylaxis failures with mefloquine were previously described in Senegal.\textsuperscript{20} In our study, we have observed a reduced susceptibility of 13% to mefloquine. This level has not increased since 1996,\textsuperscript{16,21} which suggests a stability of this efficiency. Similarly, a reduced susceptibility of 9% to halofantrine has been observed in Dakar, as in other areas of Senegal.\textsuperscript{16} In the last 10 years, the susceptibility to these two antimalarial drugs has not changed because of less use in prophylaxis regimens by local populations.

The prevalence of \textit{dhfr} S108N, S51I, and C59R mutations was 65%, 63%, and 57%, respectively, in the genotyped samples. The overall prevalence of the triple mutation associated with high-level pyrimethamine resistance was 57%. In 2004, Senegal switched to sulfadoxine-pyrimethamine with amodiaquine as first-line therapy for malaria in response to increasing chloroquine resistance. The combination of \textit{dhfr} C59R and \textit{dhps} K540E mutations, which predict clinical failure of sulfadoxine-pyrimethamine,\textsuperscript{22,23} has not been found. These data suggest that pyrimethamine-sulfadoxine is still an efficient for malaria in Dakar. The high rate of cycloguanil resistance (65%) and chloroquine resistance suggest that chloroquine and proguanil in antimalarial prophylaxis can still be used in Dakar and in Senegal.\textsuperscript{16,21} These data are consistent with previous analysis in Senegal, which showed areas with a high prevalence of chloroquine resistance and multiresistant isolates in malaria prophylaxis.\textsuperscript{24,25}

The activity of atovaquone against \textit{P. falciparum} was demonstrated in 1990s.\textsuperscript{26} The combination of atovaquone and proguanil hydrochloride has recently been approved for treatment and prophylaxis of malaria. When atovaquone was used alone as an antimalarial drug, resistance appeared rapidly in southeast Asia, Central America, and west Africa\textsuperscript{27,28} and recently in other malaria-endemic areas.\textsuperscript{29} Approximately 32% of our isolates in Dakar showed decreased susceptibility to atovaquone, with an \textit{IC}_{50} > 6 nM.\textsuperscript{13} However, the validity of this cut-off is still debated. A new threshold has been proposed and is currently being tested (Muset L, unpublished data). If this novel cut-off (>) is used, none of the isolates is resistant to atovaquone. The atovaquone resistance is associated with a Y268S mutation in the cytochrome B gene.\textsuperscript{30} The atovaquone-proguanil combination has shown treatment and prophylaxis failure in \textit{P. falciparum} infections in malaria-endemic areas.\textsuperscript{31} However, this association seems to be efficient against hypnozoite stages of \textit{P. vivax}.\textsuperscript{32}

We have observed high genetic diversity in \textit{P. falciparum} in Dakar with a mean of 2.2 parasite populations (maximum = 5). The \textit{msp1} gene shows a higher polymorphism than the \textit{msp2} gene. The \textit{msp1} K1 (68%) family has with many (24) allelic fragments. Conversely, the RO33 family has only one allelic fragment. The \textit{msp2} 3D7 family has a higher allelic polymorphism than \textit{msp2} FC27 (18 and 6 fragments, respectively). No allele was specifically associated with mild or severe cases of malaria. Our results are consistent with those of other studies\textsuperscript{4,5} that reported extensive genetic diversity analyzed by antigenic markers in isolates collected in Dakar. However, the allelic distribution of \textit{msp1} is different from the data of Robert and others\textsuperscript{2} (\textit{msp1} K1 = 17% and RO33 = 54%). These differences are associated with genetic markers \textit{msp1} and \textit{msp2} that are subject to selective pressure. Studies of microsatellite markers could be an alternative in analyzing genetic diversity of \textit{P. falciparum} isolates; these markers are may not be subject to selective pressure. Thus, the high genetic diversity in Dakar is identical to that found in other mesoendemic areas such as Dielmo or Ndiop\textsuperscript{30,33,34} in Senegal, Gambia,\textsuperscript{35} and Gabon.\textsuperscript{36}

In conclusion, urban transmission of malaria depends on the epidemiologic pattern of the geographic area where a town is located (i.e., stable, unstable, or intermediary) and on human population structure in the town.\textsuperscript{37} Consequently, urban malaria displays a great heterogeneity of population structures, which explains the present results. Therefore, it is necessary to consider urban malaria as a new transmission zone with the potential to induce clinical forms of severe malaria in non-immune populations.

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