Introduction

Despite increased funding on malarial control programs, malaria remains a major public health problem, with approximately 781,000 deaths in 2009 [1]. Due to its geographical situation in sub-Saharan Africa, Côte d’Ivoire is facing many cases of malaria, especially among children under five years of age, in whom the prevalence of symptomatic and asymptomatic carriers has been found to be approximately 43% [2]. Many African countries have changed their treatment regimen for malaria after learning of chloroquine resistance in Plasmodium falciparum. In 2005, Côte d’Ivoire adopted Artemisinin-based combination therapy (ACT) for first-line treatment of uncomplicated malaria and sulphadoxine-pyrimethamine in preventive treatment for pregnant women. In Abidjan in 2002, chloroquine and sulphadoxine showed were found to have a treatment failure rate...
of 34% among children aged 1 to 5 years [3].

Several molecular markers have been proposed to act as key contributors to antimalarial drug resistance in *P. falciparum*. The K76T mutation in the *Pfcr* gene confers resistance to chloroquine [4]. This marker has been clinically correlated with resistance in Africa, at least in areas where the strain sensitivity is not negligible. *In vitro* tests have found the S108N mutation in the *Pfdhfr* gene to be associated with resistance to pyrimethamine, and the presence of other mutations at codons 51, 59 and 164 is known to increase the level of resistance [5]. Molecular monitoring is now included in the *P. falciparum* sensitivity surveillance strategy promoted by the WHO, in association with *in vivo* tests. Resistance to chloroquine has been linked to a *Pfcr* gene mutation located on chromosome 7, [6] and clinical assays have confirmed this association [7–8].

The *dhfr* S108N mutation, carried by chromosome 4, is the key mutation site for resistance to pyrimethamine, and this is modulated by mutations of SNPs 51 and 59. The triple mutation *dhfr*S108N, *dhfr*C59R, *dhfr*N51I has been shown to be selected in failures of the sulphadoxine–pyrimethamine association (SP) [9–10]. The *dhps* gene on chromosome 8 codes for dihydropteroate synthetase (*dhps*), and mutations of this gene are linked to sulphamamide resistance. Mutations *dhps*S436A, *dhps*A437G, *dhps*K540E and *dhps*A581G have been described [11]. The *dhps*A437G, *dhps*K540E double mutation or the *dhps*S436A, *dhps*A437G, *dhps*K540E triple mutation are known to be linked to higher resistance levels [10].

Apart from these genes, the sarco/endoplasmic reticulum Ca²⁺-ATPase orthologue of *P. falciparum* (*pfatpase6*) was suggested to be involved in the mechanism of action and resistance of the parasite to artemisinins. L263E, S769N, E431K and A623E single nucleotide polymorphisms (SNPs) in *pfatpase6* have been proposed to be associated with reduced parasite sensitivity to artemisinins [12–13].

It should be noted that little research has been performed on *pfatpase* 6 in Côte d’Ivoire. *In vivo, in vitro* and molecular analyses have been performed over the last few years to help prevent the development of resistance to each component drug and reduce the overall transmission of malaria [14]. The aim of this study was to determine the association between the presence of the gene mutation and amplification polymorphisms *pfatpase6* and *Pfcr*, and the *in vitro* sensitivity of clinical *P. falciparum* isolates to CQ, PYR and DHA in Abobo (Abidjan), Côte d’Ivoire, an area with multidrug-resistant *P. falciparum*.

**Materials and Methods**

**Drugs.** Dihydroartemisinin (DHA), chloroquine (CQ) and pyronaridine (PYR) were obtained from Sigma Aldrich®. A stock solution of dihydroartemisinin was prepared in 70% methanol while stock solutions of chloroquine and pyronaridine were prepared in sterile distilled water. Two-fold serial dilutions of the stock solutions were prepared in RPMI 1640 medium. The final concentrations of the drugs tested ranged from 12.5 to 1,600 nM for chloroquine, 1.25 to 160 nM for pyronaridine and 0.5 to 64 nM for dihydroartemisinin.

**Maturation of *P. falciparum* isolates.** Clinical isolates of *P. falciparum* were obtained from patients before they were treated. Venous blood samples (5mL) were collected in EDTA-coated tubes (Terumo Europe N.V., Leuven, Belgium) from patients who gave their informed consent. Giemsa-stained thin and thick blood smears were examined to check for mono-infection with *P. falciparum* and to determine parasite density. Maturation assays were performed in 96-well tissue culture plates. Each well contained 200 μL of parasite suspension at 1.5% hematocrit and different drug concentrations. The plates were maintained for 42 h at 37°C in a candle jar as previously described [15]. Parasite growth was stopped by freezing at −20°C for at least 3 hours. Patients were treated by artemether-lumefantrine (AL) according to the recommendations of the national program for malaria control. The study was reviewed and approved by the Ivorian National Ethics Committee. All patients gave verbal consent before blood collection.

**In vitro assay.** Venous blood samples were washed three times in RPMI 1640 medium. The erythrocytes were resuspended in complete RPMI 1640 medium (RPMI 1640 supplemented with 10% type O+ human serum), 25 mM HEPES buffer, and 25 mM sodium bicarbonate) at a haematocrit of 1.5% and initial parasitaemia of 0.1–0.5%. The sensitivity of isolates to antimalarial drugs was assessed using the World Health Organization (WHO) microtest technique, and the inhibition of schizont maturation...
Polymorphisms of Pfatpase 6

was measured microscopically. A suspension of infected erythrocytes (200 μL) was distributed in each well of the 96-well tissue culture plates containing the antimalarial drug solutions. After incubation, parasites were harvested and Giemsa-stained thick blood films were prepared. The number of mature schizonts (defined as parasites with > 3 nuclei) was counted per 200 asexual parasites. Isolates with less than 20% of mature schizonts in the control well were excluded.

Sample collection and DNA extraction. Blood samples were collected at the El Rapha and Anokoua Kouté health centers from patients with uncomplicated P. falciparum infection prior to drug treatment. All patients were from the Abobo health district in the period 2006-2007. The initial diagnosis was made by microscopic examination of Giemsa-stained thick blood films or a rapid diagnostic test. For the molecular test, distilled water in a 0.5 mL micro-tube and incubated at 99°C for 30 min. For each PCR, 10 μL of the supernatant was used. The secondary PCR was performed with primer pairs TCRD-1 (5’-TGT-GCT-CATGTG-TTT-AAA-CTT-3’) and TCRD-2 (5’-CAA-AAC-TAT-AGTTAC-CAA-TTT-TG-3’) for Pfcr (200 bp fragment) and PFATP-3 (5’GGT-TTG-AAT-GAA-TTA-GAA-GTA-GAA-AAG-AAG) for region A and (5’ ACA-GAA-TAC-CAA-CTA-TGT-CAA-AAA-GGG-GAT) for region B for Pfatpase 6. The Pfcr PCR products were digested by Apo I (PCR-RFLP) (50°C, Tris-HCl 50 mM, MgCl2 10 mM, NaCl 100 mM, DTT 1 mM), while the Pfatpase 6 amplification products were sequenced. Electro-pherograms were analyzed using Edit view sequence analysis software (PerkinElmers, Les Ulis, France). The wild-type Pfcr was defined by the allele K76.

Test analysis. IC50 values with 95% confidence intervals (CI) were calculated by using an Emox model (available at http://www.antimalarial-icestimator.net) as RE=100 − [(100% Cγ)/(Cγ + IC50γ)], where IC50 is the Drug Inhibiting Concentration at 50% of parasite activity, γ is a sigmoidicity factor which expresses the steepness of the curve, RE is the relative effect of the drug (as a percentage, Y-axis), and C is the drug concentration (X-axis). The IC50 cut off values for resistance to chloroquine and pyronaridine were 100 nM and 15 nM, respectively, and the cut-off for diminished susceptibility to DHA was 10 nM. The associations between drug sensitivity and parasite gene polymorphisms were evaluated with Cohen’s kappa test [16]. The degree of agreement was scored as follows: 0–0.20, slight agreement; 0.21–0.4, fair agreement; 0.41–0.60, moderate agreement; 0.61–0.80, good agreement; and > 0.81, very good agreement.

Results

In vitro drug sensitivity of P. falciparum isolates

A total of 45 P. falciparum isolates were
collected from the Anonkoua Kouté and El Rapha health centers. Only 32 (78%) had a parasite density > 0.1% and a total of 128 in vitro tests have been performed. A total of 112 assays (87.5%) were successful, and Table 1 shows the corresponding geometric mean IC_50 (GMIC_50) values determined from these assays for each drug tested.

Dihydroartemisinin (DHA) was the most active drug tested against *P. falciparum* isolates: Only 3.6% (1/28) of the parasites had reduced susceptibility to this drug against 97.4% (27/28) of sensitivity *P. falciparum* isolates. The following proportions of resistance observed for the other drugs were 56.25% and 48% for chloroquine and pyronaridine respectively (Fig. 1). The geometric mean CQ IC_50 value was 145.5 (65–226) nM, and 56.2% (n=32) of isolates were CQ-resistant. The geometric mean PYR IC_50 value was 17.7 (9.1–26.3) nM, and 48% (n=27) of isolates were PYR-resistant. The geometric mean DHA IC_50 value was 2.7 (1.45–3.99) nM, and 3.6% (n=28) of isolates had reduced DHA susceptibility. Eleven isolates were sensitive to all three drugs. A moderate level of cross-resistance was observed. As can be seen in Table 1, a positive correlation was found between the activities of DHA and PYR (r^2=0.40, p<0.05), as well as between the activities of CQ and PYR (r^2=0.45, p<0.05). DHA activity did not correlate with CQ activity (r^2=0.29, p=0.08) (Table 1).

**Drug sensitivity and *P. falciparum* gene polymorphism**

PCR-RFLP analysis (enzymatic digestion) of DNA fragments from the *P. falciparum* isolates (n=18) indicated that 55.6% were K76T mutants (n=10) and 44.4% were wild-type K76 (wt K76) (n=8). Of 18 chloroquine resistant (CQ-R) isolates, 88.9% were PfcrT mutant T76 (n=16) against 11.1% wild K76 (n=2).

<table>
<thead>
<tr>
<th>Drug 1</th>
<th>Drug 2</th>
<th>Coefficient of determination (r^2)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>CQ</td>
<td>PYR</td>
<td>0.45</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>DHA</td>
<td>CQ</td>
<td>0.29</td>
<td>0.08</td>
</tr>
<tr>
<td>PYR</td>
<td>DHA</td>
<td>0.40</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>
On the other hand, of 14 chloroquine-sensitive isolates, two were Pfcrt mutant K76T (14.3%) and 12 were wt K76 (85.7%), kappa coefficient k=0.76. This reflects a good agreement between the mutation at position 76 Pfcrt and sensitivity of P. falciparum to chloroquine.

Thirteen isolates were found to be resistant to pyronaridine (PYR-R) of which 4 Pfcrt were mutant K76T (30.8%) and 9 were wt K76 (69.2%), while 14 were susceptible isolates (PYR-S), comprising 6 mutant K76T (42.9%) and 8 wt K76 (57.1%). There is a slight correlation between the sensitivity of P. falciparum to PYR and Pfcrt mutation at position 76 (k=0.21).

Of 28 isolates tested against DHA, only one isolate (wt K76) was found to have reduced Polymorphisms of Pfatpase 6 263

Table 2. Distribution of pfcrt polymorphism according to drugs sensitivities

<table>
<thead>
<tr>
<th>Antimalarial drugs profile</th>
<th>Number (%) of isolates with pfcrt K76T</th>
<th>( \kappa^* )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K</td>
<td>T</td>
</tr>
<tr>
<td>CQ Resistant (IC(_{50} \geq 100) nM)</td>
<td>2 (11.1%)</td>
<td>16 (88.9%)</td>
</tr>
<tr>
<td>CQ Sensitive (IC(_{50} \leq 100) nM)</td>
<td>12 (85.7%)</td>
<td>2 (14.3%)</td>
</tr>
<tr>
<td>PYR Resistant (IC(_{50} \geq 15) nM)</td>
<td>9 (69.2%)</td>
<td>4 (30.8%)</td>
</tr>
<tr>
<td>PYR Sensitive (IC(_{50} \leq 15) nM)</td>
<td>8 (57.1%)</td>
<td>6 (42.9%)</td>
</tr>
<tr>
<td>DHA Reduced susceptibility (IC(_{50} \geq 10) nM)</td>
<td>1 (100%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>DHA Sensitive (IC(_{50} \leq 10) nM)</td>
<td>8 (29.6%)</td>
<td>19 (70.4%)</td>
</tr>
</tbody>
</table>

* Kappa test of Cohen: The degree of agreement was scored as follows: 0-0.20, slight agreement; 0.21-0.4, fair agreement; 0.41-0.60, moderate agreement; 0.61-0.80, good agreement; and > 0.81, very good agreement

On the other hand, of 14 chloroquine-sensitive isolates, two were Pfcrt mutant K76T (14.3%) and 12 were wt K76 (85.7%), kappa coefficient k=0.76. This reflects a good agreement between the mutation at position 76 Pfcrt and sensitivity of P. falciparum to chloroquine.

Thirteen isolates were found to be resistant to pyronaridine (PYR-R) of which 4 Pfcrt were mutant K76T (30.8%) and 9 were wt K76 (69.2%), while 14 were susceptible isolates (PYR-S), comprising 6 mutant K76T (42.9%) and 8 wt K76 (57.1%). There is a slight correlation between the sensitivity of P. falciparum to PYR and Pfcrt mutation at position 76 (k=0.21).

Of 28 isolates tested against DHA, only one isolate (wt K76) was found to have reduced

Table 3. Distribution of Pfatpase 6 polymorphisms according to antimalarial drugs sensitivities

<table>
<thead>
<tr>
<th>Antimalarial drugs profile</th>
<th>Polymorphisms of Pfatpase 6 (number of isolates)</th>
<th>( \kappa^* )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N** Y</td>
<td>D Y</td>
</tr>
<tr>
<td>CQ(^1) Resistant (IC(_{50} \geq 100) nM)</td>
<td>17 (94.4%)</td>
<td>1 (5.6%)</td>
</tr>
<tr>
<td>CQ(^1) Sensitive (IC(_{50} \leq 100) nM)</td>
<td>13 (92.8%)</td>
<td>1 (7.2%)</td>
</tr>
<tr>
<td>PYR Resistant (IC(_{50} \geq 15) nM)</td>
<td>12 (92.3%)</td>
<td>1 (7.7%)</td>
</tr>
<tr>
<td>PYR Sensitive (IC(_{50} \leq 15) nM)</td>
<td>13 (92.8%)</td>
<td>1 (7.2%)</td>
</tr>
<tr>
<td>DHA Reduced susceptibility (IC(_{50} \geq 10) nM)</td>
<td>1 (100%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>DHA Sensitive (IC(_{50} \leq 10) nM)</td>
<td>19 (70.4%)</td>
<td>8 (29.6%)</td>
</tr>
</tbody>
</table>

\(^{1}\) CQ: chloroquine; PYR: pyronaridine; DHA: dihydroartemisinin

* Kappa test of Cohen: The degree of agreement was scored as follows: 0-0.20, slight agreement; 0.21-0.4, fair agreement; 0.41-0.60, moderate agreement; 0.61-0.80, good agreement; and > 0.81, very good agreement

** N: Asparagine; Y: Tyrosine; D: Aspartic acid; H: Histidine
sensitivity. Of the 27 isolates sensitive to DHA, 8 isolates were wt K76 (29.6%) and 19 were mutant K76T (70.4%) (Table 2).

Regarding the Pfatpase 6 gene polymorphism, N669Y (16.1%) is relatively high as 734 haplotypes (30.4%) with D734Y (28.6%) and D734H (1.8%). The codons of the polymorphism and their involvement with malaria are given in more detail in Table 3.

Discussion

The high level of chloroquine resistance observed since 2003 has prompted the Malaria Control Program to develop their strategy for reducing malaria prevalence. Drug resistance arises rarely but spreads relatively quickly. The most spectacular activity of chloroquine is its ability to increase from nanomolar levels outside the parasite to millimolar levels in the digestive vacuole of the erythrocyte trophozoite [17]. All CQ-R isolates demonstrated an alteration in the accumulation of chloroquine in the food vacuole, which is thought to be due to an alteration of the pH gradients and/or membrane permeability as a result of an efflux mechanism. It now appears that CQ resistance is associated with a decreased uptake of the molecule. Drug accumulation has been observed to be highly structurally specific, which means either a carrier / specific permease is involved, or a molecule associated with hematin in the food vacuole [18].

The results of the present study confirm that the K76T mutation plays a significant role in the occurrence of Pfert CQ-R (κ=0.76) as reported by Djimde et al. (2001) [7]. A similar study conducted in Côte d’Ivoire in 2007 showed a relationship between the presence of the Pfert K76T mutation and therapeutic failure of chloroquine [4]. Moreover, the present findings indicate that mutations Pfert K76T/Pfatpase N669Y or Pfatpase D734H could exert a synergistic effect. All isolates of P. falciparum Pfert / Pfatpase double mutants are CQ-R.

All isolates of P. falciparum apart from the twofold N669Y and D734H mutant are sensitive to DHA. In accordance with information for isolates from Cambodia, French Guiana, and Senegal, none of the isolates observed in our study carried single nucleotide polymorphisms of pfatpase6 at codon 769 or at codons 37, 693 or 898 [13]. These results are consistent with those of Zhang et al. (2008) from isolates of Chinese origin which note that codon 769 is not associated with reduced sensitivity of P. falciparum to artemisinin derivatives [19]. However, an important genetic polymorphism in the Pfatpase6 gene has 23 point mutations throughout the entire gene but has no direct impact on the susceptibility of isolates to CQ or DHA. The kappa coefficient obtained is not significant. This lack of correlation, despite the large number of point mutations, may sometimes exist because they did not rise to a characteristic location of the gene. Within codon 734, the substitutions D734Y (28.6%) and D734H (1.8%) were observed. This could significantly alter the codons involved in the changes of molecular target [20] which are enzyme ATPase-calcium dependent.

Although the mechanism of action of artemisinins remains unclear, one proposal is based on its interaction with the sarcoplasmic reticulum Ca$^{2+}$ ATPase 6 (Pfatpase 6). This enzyme, when expressed in Xenopus oocytes, was specifically inhibited by artemisinin derivatives containing an endoperoxide bridge [21]. In addition, the activity of the enzyme was greatly influenced by the introduction of several gene mutations. An analysis of naturally occurring polymorphisms in Pfatpase 6 in field isolates from French Guyana suggests that a polymorphism at codon 769 may be associated with reduced susceptibility of these isolates to artemether in vitro [12]. However, other studies have failed to detect any codon 769 polymorphism in field isolates [10,22]. The results of our study do not indicate any link between either amplification or mutation of Pfatpase6 and the in vitro response to DHA. Both 669 and 734 are haplotypes identified in this study, and considering the frequency of mutation and physico-chemical changes of the amino acids, it can be assumed that there are two new allelic sites in the Pfatp6 gene. This gene has been identified as the genetic basis of the reduction of resistance of P. falciparum to artemisinin derivatives, as the presence of new point mutations in Pfatp6 genes has been associated with reduced effectiveness of artemisinin derivatives [23,24].

Conclusions

These data showed that the resistance of P. falciparum is indeed a reality in Cote d’Ivoire. Till 2003, Chloroquine was the first-line therapy, despite its removal, continues to have a high level of resistance. It was shown in this study that CQ-R is bonded to mutation of codon Pfert K76T, but no
mutation linked with artemisinin resistance in \textit{PfATPase} 6. Despite the decrease in efficiency, DHA is the best alternative for the treatment of uncomplicated malaria. In addition, molecular epidemiology should be part of routine surveillance to produce complementary information to assess the appropriateness of the current national anti-malarial drug policy.

Acknowledgements

The authors thank sincerely the director of the health center of Anokoua-Kouté and EL Rapha for having permitted this study. We are also grateful to the laboratory technicians for their assistance.

References


Received 19 February 2014
Accepted 15 September 2014