Surveillance of plasmepsin 2 copy number gene in Plasmodium falciparum isolates from Senegal

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The development of resistance to antimalarial drugs is a major challenge for global malaria control. Full Plasmodium falciparum resistance to dihydroartemisinin–piperaquine treatment has been reported recently in Cambodia. These events were directly associated with increased copy number variations (CNVs) in the plasmepsin system, including the PfPlasmepsin 2 gene. Pfplasmepsin 2 copy number was the most significant molecular signature associated with dihydroartemisinin–piperaquine treatment failure. Even though the piperaquine resistance has not been observed in regions in which artemisinin resistance has not been documented, it is possible to find an amplification of the Pfplasmepsin 2 gene in these regions. In this present study, we investigate to do a surveillance of Pfplasmepsin 2 copy number variations in Senegal by qPCR. Pfplasmepsin 2 copy number was assessed in 120 P. falciparum positive patients, 60 from Dakar and 60 from Kedougou by qPCR and an amplification of the Pfplasmepsin 2 genes was measured by using five standards of mixed synthetic gene fragments. Using a copy number threshold of 1.7 and 1.73% carried a multiple copies of Pfplasmepsin 2, whilst one copy of the gene was found in 98.26% of the isolates. Our results show that the CNVs associated with resistance to piperaquine are probably already frequent in Senegal. Paradoxically, Pfplasmepsin 2 multi-copy is generally found in parts of Africa where dihydroartemisinin–piperaquine failures are rare and resistance to piperaquine has not yet been described. However, it is no evidence to confirm piperaquine resistance in Senegal.

Key words: PfPlasmepsin 2 copy number, Senegal, Piperaquine, resistance, drug resistance.

INTRODUCTION

Artemisinin combination therapy (ACT), the use of a short acting artemisinin derivative and a long-acting partner drug, is recommended worldwide for the treatment of Plasmodium falciparum malaria (World Health

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Organization, 2018). In 2006, in line with World Health Organization (WHO) recommendation, the Senegalese National Malaria Control Programme (NMCP) introduced the ACT regimen with Arthmeter-Lumefantrine (AL) as first-line treatment of uncomplicated *P. falciparum* malaria and dihydroartemisinin–piperaquine (DHA-PPQ) combination was then recommended as a second-line treatment. Although DHA-PPQ, is not currently used as the first-line treatment against uncomplicated malaria, this antimalarial drug has been widely used to compensate for antimalarial drugs shortages in 2010 and 2011 (Thiam et al., 2012).

In addition, the NMCP recommended the intermittent preventive treatment IPT of pregnant women with sulfadoxine–pyrimethamine (SP) and seasonal malaria chemoprevention (SMC) for children with SP-amodiaquine (Bamba et al., 2013; Mbaye et al., 2017). Unfortunately, the recent emergence of *P. falciparum* resistance to artemisinin derivatives in Southeast Asia challenges malaria control and elimination efforts. This situation is increasingly compromised by concurrent resistance to partner drugs in combination therapies, such as the piperaquine (Witkowski et al., 2017).

In 2014, the first report of DHA–PPQ treatment failures was published (Saunders et al., 2014). Therapeutic failures were estimated to reach 60% indicating a dramatic expansion of piperaquine resistance. Moreover, full *P. falciparum* resistance to DHA/PPQ treatment has been reported recently in Cambodia (Amato et al., 2017). These events were directly associated with increased copy number variations (CNVs) in the plasmepsin system, including the *pfPM2* gene (PF3D7_1408000) coding for the food vacuole enzyme plasmepsin II (Sanogo et al., 2018). CNV is generally considered as emerging at relatively rapid mutation rates (Cheeseman et al., 2009).

The relevance of the *Pfplasmepsin 2* (*PfPIM2*) gene amplification is conferring any survival or fitness advantages in response to PPQ pressure. Piperaquine resistance is poorly characterized. It is currently identified by late clinical failures with amplification of the plasmepsin 2 copy number gene. Although several genetic variations have been associated with decreased piperaquine susceptibility: a single copy of the mdr1 gene has been associated with dihydroartemisinin–piperaquine treatment failures in Cambodian patients (Witkowski et al., 2017).

However, *PfPIM2* copy number was the most significant molecular signature associated with dihydroartemisinin–piperaquine treatment failure (Witkowski et al., 2017). The piperaquine resistance has not yet been observed in area where artemisinin resistance has not also been documented as Senegal. However, it is possible that *plasmepsin 2* amplifications will be found in other regions where piperaquine has been used as a partner drug (Bopp et al., 2018). Thus the assessment of *PfPIM2* gene copy number to areas where piperaquine is being used in artemisinin-based combination therapies is important. In line with the NMCP strategy for the surveillance of antimalarial drug efficacy, the aim of this study was to do a surveillance of multiple copies of *PfPIM2* gene, potentially involved in piperaquine resistance.

**MATERIALS AND METHODS**

**Study site and sample collection**

In total, 120 patient samples from two regions of Senegal were selected: Dakar (60), Kedougou (60), which present different levels of malaria transmission intensity (Figure 1). In Dakar malaria transmission is low and parasite prevalence is estimated at 1.3%. In the southeastern Kedougou region, the level of malaria transmission is high with an incidence higher than fifteen malaria cases per 1000 habitants (PNLP-Senegal, 2018). In Dakar samples were collected between October and November 2015 while in Kedougou the samples were collected in September 2016. These Samples were collected in the some months because these months correspond to periods of high transmission in Senegal. For routine surveillance in a region of low transmission such as Dakar, we estimate that a maximum sample size would be required to obtain a positive case compared to Kedougou with high transmission. Venous blood samples were collected in 5 ml vacutainer tubes and filter paper was made for molecular testing. All individuals in this study presented with uncomplicated malaria and parasite presence and species was confirmed by microscopy.

**Ethical approval**

The study protocol was approved by the National Ethics Committee for Health Research of Senegal (CNERS). Before participant recruitment and sample collections were initiated, written and informed consent was obtained from all participants.

**Copy number variation assays**

Parasite DNA was extracted from 120 samples using the QIAamp DNA Blood Mini kit (Qiagen) according to manufacturer instructions (QIAamp DNA Blood Mini Kit, Qiagen, Valencia, CA). The relative *PfPIM2* copy number were assessed by qPCR using real-time PCR machine (ABI 7500) as described by Witkowski with minor modifications in the Sequences of fragments synthetic genes (Witkowski et al., 2017). As a single copy endogenous gene control, we used the single copy *β-tubulin* gene and *P. falciparum* genomic DNA from strain 3D7 which has one copy of *PfPIM2* was included in each run as controls.

Briefly, quantitative PCR (qPCR) was carried out in 20 μl volumes in a 96-well plate containing 10 μl qPCR EvaGreen dye Supermix, 1 μl of each forward and reverse primer (Table 1), 3 μl H₂O and 5 μl of template DNA. Amplifications were performed under the following conditions: 98°C for 3 min, followed by 40 cycles of 95°C for 10s and 58°C for 20 s and amplifications were run in triplicates.

**Determination of the copy number**

Amplification of the *PfPIM2* genes was measured by using five standards of mixed synthetic gene fragments (Table 2). The number of copies for each gene is determined relative to a standard curve. The standard curve is obtained from a mixture of fragments synthetic genes of *Pfplasmepsin2* and *Pfβtubulin* by making five
Table 1. Primer sequences of PfPM2 and Pf β-tubulin.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primers</th>
<th>Reverse primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pf PM2-CN</td>
<td>5'-TGGTGATGCAAGTTGGAG-3'</td>
<td>5'-TGGGACCCATAAATTAGCAGA-3'</td>
</tr>
<tr>
<td>Pfβ-tubulin-CN</td>
<td>5'-TGATGTGCGCAAGTGATCC-3'</td>
<td>5'-TCCTTTGTGGACATTCTTCCTC-3'</td>
</tr>
</tbody>
</table>


RESULTS

Using a copy number threshold of 1.7 the relative PfPM2 copy number was assessed in 120 P. falciparum positive patients, 60 in Dakar and 60 in Kedougou. From the samples with a valid copy number estimate (n = 115), the standard curve.

Once the $2^{-\Delta Ct}$ is obtained for the five standards, we used it to make a standard curve with a line equation $y = ax + b$, it will be used to determine the number of copies of the PfPM2 gene present in each of our samples (Figure 2). In our study a copy number of PfPM2 $\geq 1.7$ was defined as an amplification of this gene. Our CVN for the control (3D7) was 0.7.

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RESULT

Using a copy number threshold of 1.7 the relative PfPM2 copy number was assessed in 120 P. falciparum positive patients, 60 in Dakar and 60 in Kedougou. From the samples with a valid copy number estimate (n = 115),...
Table 2. Sequences of fragments synthetic genes of PfPM2 and Pf β-tubulin.

<table>
<thead>
<tr>
<th>Synthetic genes</th>
<th>Sequences</th>
<th>Molecular weight</th>
<th>GC content</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pf PM2-CN</td>
<td>TAT CTG GTG ATA CAT GAA CAG ATC CGT GCA CCG TCA CGT ATT TCA AAT GAT ATC GAA TTA GTA GAT TTC AAA CAT CTA TAT GAT TCA TCT AAA TCA CAC TTA GAC CAG ATG TCC GTG ACG TCT AGC TTG A</td>
<td>80872.5 g/mole</td>
<td>35.1 %</td>
<td>262</td>
</tr>
<tr>
<td>Pf β-tubulin-CN</td>
<td>TAT CTG GTG ATA CAT GAA CAG ATC CGT GCA CCG TCT TCA ACT ACA GAG CCT TGA CTG TGC CGG AGT TAA CAC AAC AAA TGT AAA AAT ATG ATG TGC GCA AGT GAT CCA AGA CAT GGA AGA TAT TTA ACG GCA TGT GCT ATG TTT AGA GGA AGA ATG TCC ACA AAG GAA GTT GAC GAA CAA ATG TTA AAC GTT AAA ATA AAA ACT CAT GTT ATT TTG TCG AAA GGA CAC ACT TAG ACC AGA TGT CCG TGA CGT CTA GCT TGA</td>
<td>80873.5 g/mole</td>
<td>39.8%</td>
<td>261</td>
</tr>
</tbody>
</table>

Source: Witkowski et al. (2017)

Figure 2. PfPM2 (Plasmodium falciparum plasmepsin 2) gene standard curve. The number of copies of PfPlasmepsin2 will be calculated according to the fold change (2^-ΔCt) method. ΔCt = Ct PfPlasmepsin2 - Ct Pfβ-tubulin. A line equation y = 1.2556x - 0.7443 will be used to determine the number of copies of the PfPM2 gene present in each of our samples. In our study a copy number of PfPM2 > 1.7 was defined as an amplification of this gene.

Source: Excel Microsoft 2013

1.73% carried a multiple copies of PfPM2, whilst one copy of the gene were found in 98.26% of the isolates (Figure 3). A multiple copies were noted only in Kedougou with 3.33%. In Dakar all the samples presented...
Figure 3. Dot plot of copy numbers of the PIPM2 (P. falciparum plasmepsin 2) gene by locality. Each point represents an isolate. The black band corresponds to $2^{-\text{dct}} = 1.7$ which represents an amplification of the PIPM2 gene. KG= Kedougou, DK= Dakar.

Source: GraphPad Prism 5

DISCUSSION

DHA/PPQ has shown near-perfect efficacy levels in clinical trials conducted in Africa, the combination also has been proposed as second-line treatment of Plasmodium falciparum malaria in Senegal. Unfortunately, full P. falciparum resistance to DHA/PPQ treatment has been reported recently in Cambodia. These events were directly associated with increased copy number variations in the PIPM2 gene. Therefore, preexisting PIPM2 duplications in Cambodia might have been rapidly selected by DHA/PPQ, aided by a less effective protective action of the artemisinin derivative (Hastings et al., 2016). Such a scenario suggests that this copy number variation may already be present in Africa. Accurate and timely surveillance of drug resistance markers aids in maintaining and prolonging the efficacy of the limited selection of anti-malarial drugs available. Thus, it becomes opportune to follow this emergence and it is for this reason that we have chosen to study the number of copies of the PIPM2 gene in order to determine the number of copies circulating in each individual of P. falciparum in Dakar and in Kedougou. 1.73% carried a multiple copies of PIPM2, whilst one copy of the gene were found in 98.26% of the isolates from Kedougou (Figure 2). The copy number of PIPM2 genes was differ between isolates from different sites. An amplification of this gene was only reported in Kedougou. Kedougou is a particular site with presence of gold mining, more than 10 nationalities are represented in this region, with a strong representation of Malians, Burkinabés, and Guineans. This relative affluence favors the informal sale of antimalarial drugs and promotes abusive self-medication. This is one of the key factors in the emergence of drug resistance (Ministere de l’économie et des finances, 2018). This situation can explain the presence of multiple copies of PIPM2 gene in this area.

Our results show that the multiplications associated with resistance to piperaquine are probably already frequent in Senegal, which is of concern given the use of DHA / PPQ as a second line of treatment in Senegal. Recently complete resistance of P. falciparum following DHA / PPQ treatment has been reported in Cambodia and this resistance has been directly associated with an increase in the number of copies of the PIPM2 2 gene (Amato et al., 2017).

In Mali, in 65 out of the 96 samples it was confirmed the presence of 7 infections carrying 2 copies of PIPM2 (Sanogo et al., 2018). Previously a multiple copies of pfpm2 was also found in Mozambique with a frequency of...
isolates across endemic regions. In this current study we found a presence of multiple copies of \textit{PfPM2} gene but the association between these amplifications and a clinical resistance to PPQ was not verifiable. However, an additional functional work is needed to better understand the mechanisms of PPQ resistance and to identify the association of increased piperazine IC50 values with the copy-number variation. Also a continuous Surveillance for increased \textit{PfPM2} copy number could aid malaria control efforts by pinpointing areas where these drugs may be failing like in Senegal.

### CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

### ACKNOWLEDGMENTS

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### ABBREVIATIONS


### REFERENCES


