Molecular surveillance of the Pfmdr1 N86Y allele in P. falciparum isolates from Brazzaville, Republic of Congo

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Abstract

Background: Regular surveillance of artemisinin–based combination therapy tolerance or resistance molecular makers is vital for effective malaria treatment, control and eradication programmes. *P. falciparum* multiple drug resistance-1 gene (Pfmdr1) N86Y mutation is associated with reduced susceptibility to lumefantrine. This study assessed the prevalence of Pfmdr1 N86Y in Brazzaville, Republic of Congo.

Methods: A total 1001 of *P. falciparum*–infected blood samples obtained from asymptomatic malaria pregnant women having a normal child delivery at the Madibou Integrated Health Center were analysed. Pfmdr1 N86Y genotyping was conducted using PCR-Restriction fragment length polymorphism (RFLP).

Results: The wild type Pfmdr1 N86 allele was predominant (>68 %) in this study whereas a few isolates carrying the either the mutant allele (Pfmdr1 86Y) alone or both alleles (mixed genotype). The dominance of the wildtype allele (pfmdr1 N86) indicates the plausible decline *P. falciparum* susceptibility to lumefantrine.

Conclusion: This study gives an update on the prevalence of Pfmdr1 N86Y alleles in Brazzaville, Republic of Congo. It also raises concern on the imminent emergence of resistance against artemether–lumefantrine in this setting. Our study underscores the importance to regular artemether–lumefantrine efficacy monitoring to inform malaria control programme of the Republic of Congo.

introduction

Anti-malarial drug resistance is a major impediment to malaria reduction/eradication globally. Resistance against chloroquine (CQ) and its successor, sulfadoxine-
pyrimethamine (SP), had devastating consequences in sub-Saharan Africa in 1990s and 2000, particularly among children below 5 years[1]. Currently, artemisinin-based combined therapy (ACT) is the first-line treatment for *P. falciparum* uncomplicated malaria alongside SP for intermittent preventive treatment-SP of pregnant women (ITPp) and infants (ITPi)[2]. After the introduction of ACTs, malaria mortalities and morbidities global declined drastically until 2015. Even though ACTs are still highly efficacious, there is heightened concern on the potential spread of ACT resistance from Southeast Asia to sub-Saharan Africa reminiscent of the spread of CQ- and SP resistance[3-7]. The WHO recommends routine surveillance of antimalarial drug efficacy once every two years. However, high cost of clinical trials limit efforts to monitor the emergence and spread of antimalarial drug resistance in resource limited settings. Molecular surveillance of distinct point mutation(s) in *P. falciparum* genes linked to antimalaria treatment failure offers a cost-effective tool to monitor spatiotemporal emergence and spread of resistant parasites. High prevalence of gene mutations associated with *P. falciparum* CQ (*P. falciparum* chloroquine transporter gene *Pfcrt*) and SP (*P. falciparum* dihydrofolate reductase gene; *Pfdhfr* and dihydropteroate synthase gene, *Pfdhps*) resistance informed, in part, the decision to replace these antimalarial drugs with ACTs including the Republic of Congo [8-11]. *P. falciparum* multidrug resistance1 (*Pfmdr1*), also known as P-glycoprotein homologue, is a transmembrane protein of the *P. falciparum* digestive vacuole (DV)[12]. It is involved transportation substrates into digestive vacuole of *P. falciparum* including antimalaria drugs[13]. Distinct changes in the sequence and/or amplification of the copy number of *Pfmdr1* gene alters *P. falciparum* susceptibility to several antimalarial drugs [14]. In particular, the *Pfmdr1* N86Y single nucleotide polymorphism (SNP) has been implicated in *P. falciparum*
resistance to chloroquine and amodiaquine [15]. Pfmdr1 N86Y is mostly found in Africa. High prevalence of Pfmdr186N and 86Y alleles is currently being driven by ACT-linked P. falciparum opposite selection. Previous studies shown that parasites carrying Pfmdr1 N86 are less susceptible lumefantrine[16, 17], artemisinin-lumefantrine, AL, select Pfmdr 186N whereas artesunate-amodiaquine, ASAQ, and piperaquine select Pfmdr1 86Y [16, 18-20]. Since this phenomenon indicates potential decline of malaria parasite sensitivity or increased tolerance to ACT partner drugs,Pfmdr1 N86Y genotyping has been proposed as a useful marker to guide rotation of ACTs in a given geographical area[20, 21]. The present study aimed to genotype and to determine the prevalence of Pfmdr1 N89Y in Brazzaville, Republic of Congo. Data for this study is useful for the refinement and adaption of the current malaria treatment policy with the long-term goal of reducing malaria in the Republic of Congo.

Methods

Sample collection

This study analysed a total of 101 marched blood samples (maternal peripheral, placenta, and cord blood) collected from asymptomatic malaria pregnant women having a normal child delivery at the Madibou Integrated Health Center, Brazzaville, between March 2014andApril 2015(21). Brazzaville is the capital of the Republic of Congo. It is situated along the Congo River and has about 1.8 million inhabitants (22). Malaria transmission in this area is perennial with P. falciparum being the predominant Plasmodium specie [22, 23].AL and ASAQ are the frontline and second-line antimalarial drugs for uncomplicated P. falciparum malaria in the Republic of Congo, respectively [24].
**Pfmdr1 genotyping**

Total genomic DNA was isolated using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Amplification of *P. falciparum* merozoite surface protein 2 gene (*Pfmsp2*) was used to determine *P. falciparum* multiplicity of infection (MOI) as described previously [25]. Nested-PCR and restriction fragment length polymorphism (PCR-RFLP) were used to genotype *Pfmd1* N86Y as described previously [26]. In brief, *Pfmdr1* primary and nested PCRs were conducted by adding 2µl DNA template into a PCR master mix (50µl) containing 1X PCR buffer, 2.8mM MgCl₂, 200µM dNTPs, 5pM of each primer, 1UTaq DNA polymerase. The primers for the primary PCR were A1 (5’- CGGAGTGACAAATCTGGGA-3’) and A3 (5’-GGGAATCTGGTGGTAACAGC-3’) and for the secondary PCR were A2 (5’-TTGAAGGAACAGAAATTACATGATGA-3’) and A4 (5’- AAAGATGGAACCTCAGTATCAAAAGAAGAG-3’). The thermal cycler conditions were as follows: initial denaturation at 94°C for 2 min, followed by 40 cycles at 94°C for 1 min, 45°C for 1 min, 72°C for 1 min and a final extension at 72°C for 5 min. The secondary reaction was conducted using the product of the primary reaction as a template. DNA extracted *P. falciparum* laboratory strains (3D7 and Dd2) and PCR grade water were used as positive and negative controls, respectively.

The *Pfmdr1*N86Y mutation was identified by digesting *Pfmdr1*A2/A4secondary PCR products (10µl) using Apol (New England Biolabs Inc. MA, USA) restriction enzyme for 15 minutes at 50°C according to manufacturer’s instructions. The resulting DNA fragments were separated in 2% agarose gel stained with SYBR green at 100V for 45 mins electrophoresis. Apol digests *Pfmdr1*PCR product when *Pfmdr1*N86 (wild type allele) is present. **Data analyses** Chi-square and Fisher exact tests were applied to
compare the proportions of Pfmdr1 N86Y alleles in this study. The statistical significance was set at $p\text{-value} < 0.05$. Ethical considerations

This study was approved by the Institutional Ethics Committee of Fondation Congolaise pour la Recherche Médicale, FCRM, Brazzaville, Republic of Congo. Written informed consent was obtained from all participants before samples collection. The objectives of the study including the study procedures, sample to be taken, study benefits, potential risks and discomforts were explained. Newly opened needle and syringe were used for each subject.

results

Table 1 shows that baseline characteristics of participants recruited in this study. The mean age of participants was 23.7 $\pm$ 5.75 years. Overall, 24% of the pregnant women did not take intermittent preventive treatment during pregnancy (IPTp) during pregnancy and most of the participants (70%) had $>1$ parity.

Of the 101 marched sample analysed in this study, Pfmdr1 was successfully amplified in 59 (58.4%), 38 (37.6%) and 21 (20.8%) maternal peripheral blood, placental blood and cord blood samples, respectively. Pfmsp2 genotyping showed mean MOI was 1.06±0.24. High prevalence of Pfmdr1 wild type allele (N86) was observed among the different sample types. Pfmdr1 N86 was present in 69.5% (41/59), 57.9% (22/38) and 85.7% (18/21) of the peripheral blood, placenta blood and cord blood samples, respectively. The remaining samples had the mutant allele (Pfmdr186Y) with few samples having both wild type and mutant alleles. Three peripheral blood samples, two placental blood samples and one cord blood sample had both the wild type and mutant Pfmdr1 N86Y alleles. The Pfmdr1 N86 allele prevalence was similar ($p\text{-value}>0.05$) among different sample types, parity and the
number of Intermittent preventive treatment during pregnancy (Table 2).

Discussion

This study set out to determine the prevalence of *Pfmdr1* N86Y mutation among pregnant women having a normal child delivery at the Madibou Integrated Health Center, Brazzaville Republic of Congo. The frequency of *Pfmdr1* 86Y(mutated allele) in this study was lower than previously estimated in this setting in 2010 (73%) and in 2015 (27%) [27, 28]. Our findings are also comparable to *Pfmdr1* 86Y allele (23%) global frequency and most parts of Africa (17% –24%) except Central Africa, where higher resistant allele frequency (44%) has been observed [29]. In Southeast Asia, however, the frequency of *Pfmdr* 186Y is much lower than observed in this study whereas it has almost reached fixation in Papua New Guinea[29]. *Pfmdr1* N86Y mutation is known to modulate *P. falciparum* susceptibility to various antimalarial drugs by regulating the influx of the drugs into the parasite’s digestive vacuole. Previous studies have shown that parasite carrying this mutation are less susceptible to 4-aminoquinolines, namely chloroquine, amodiaquine and piperaquine, in vitro [30] and increase the risk of chloroquine or amodiaquine therapeutic failure [15]. On the other hand, the *Pfmdr1* 86Y mutation enhance malaria parasite susceptibility to lumefantrine, mefloquine and the active derivative of artemisinin, dihydroartemisinin [30]. The converse impact of *Pfmdr1* N86Y on *P. falciparum* response to longer–acting partner drugs of ACTs implies that wide spread use of artemether–lumefantrine (AL) and artesunate–amodiaquine (AS –AQ), particularly in Africa, exert opposite selection pressure on *P. falciparum* populations and allele frequency [31, 32]. Changes in malaria treatment policies greatly influence the frequency of mutations that modulate *P. falciparum*
susceptibility to antimalarial drugs including Pfmdr1 N86Y[31]. The introduction of ACTs in the early 2000s and cessation of chloroquine use in the 1990s led to drastic changes in Pfmdr1 N86Y allele frequency in various malaria–endemic settings[27, 33, 34]. For instance, the frequency of Pfmdr1 N86Y has declined dramatically, in favour of Pfmdr1 N86, in countries where AL is used as the first-line treatment for malaria. The increase in Pfmdr1 N86 allele frequency is faster when AL is used compared to AS–AQ usage [31]. In areas where AS–AQ is the primary treatment for malaria, the decline of Pfmdr1 86Y allele frequency is slow owing to reduced susceptibility of parasites carrying this mutation to amodiaquine. Previous studies demonstrate that parasites carrying Pfmdr1 N86 tolerate higher lumefantrine levels and have short-time to reinfection or recrudescence in patients with high lumefantrine concentration following AL treatment[16, 17]. Even though there is no evidence directly linking Pfmdr1 N86 to AL treatment failure and AL is still highly efficacious, parasite tolerance to lumefantrine is a clear warning sign for plausible emergence of resistance against AL. In this context, Pfmdr1 86N can be used to track lumefantrine selective pressure in a given area[17]. Our findings show that Pfmdr1N 86 allele is approaching fixation in the Republic of Congo and could provide the genetic background needed for the emergence of resistance against lumefantrine threatening AL usefulness in this setting. However, this possibility could be averted by concurrent use of AL and ASAQ as first-line treatment for uncomplicated P. falciparum malaria. Such a strategy is supported by evidence showing the opposite effect of both Pfmdr1 N86Y alleles on P. falciparum susceptibility to AL and ASAQ [18].

conclusions
This study offers an update on the frequency of Pfmdr1 N86Y alleles in Brazzaville, Republic of Congo and provides evidence supporting the concomitant deployment or rotation of AL and ASAQ as the primary treatment for uncomplicated P. falciparum malaria. This will be helpful to halt any further selection of Pfmdr1 alleles that dampen parasite susceptibility and safeguard AL efficacy.

declarations

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Disclaimer

The findings and conclusions in this paper are those of the authors and do not necessarily represent the views of the funding agency.

Author’s contributions

LR D-Y performed all the lab analyses, FKK supervised the lab work, JCV was responsible of statistical analysis, AA and AL participated in the interpretation of the results, DN and TPV reviewed and edited the manuscript. FN supervised the overall work. All authors approved the final manuscript. All the authors contributed in drafting the paper.
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Availability of data and material

All raw data provided in this work are available upon request to the corresponding author.

Ethical approval and consent to participate

This study was approved by the Institutional Ethics Committee of Fondation Congolaise pour la Recherche Médicale, FCRM, Brazzaville, Republic of Congo. Written informed consent was obtained from all participants before samples collection.

Consent for publication

Not applicable

Competing interests

Authors declare they have no competing interests.

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**Table 1:** Baseline characteristics of participants.

| Age group (years) | No. of participants (n = 101) | Percentage (%) |
|-------------------|-------------------------------|----------------|
| 15-30             | 89                            | 88.1           |
| >30               | 12                            | 11.9           |

**Dose of IPTp**

| Dose of IPTp | No. of participants (n = 101) | Percentage (%) |
|--------------|-------------------------------|----------------|
| 0            | 24                            | 23.8           |
| 1            | 28                            | 27.7           |
| ≥2           | 49                            | 48.5           |

**Parity**

| Parity       | No. of participants (n = 101) | Percentage (%) |
|--------------|-------------------------------|----------------|
| Primipare    | 30                            | 29.7           |
| Secondipare  | 33                            | 32.7           |
| Multiparous  | 38                            | 37.6           |

IPTp: Intermittent preventive treatment in pregnancy.

**Table 2:** Distribution of the Pfmdr1N86 allele among peripheral blood, placenta blood and cord blood samples from the Republic of Congo.

| Dose IPTp | Peripheral blood n (%) | Placental blood n (%) | Cord blood n (%) |
|-----------|------------------------|-----------------------|------------------|
| 0         | 8/14 (57.1)            | 6/9 (66.7)            | 5/6 (83.3)       |
| 1         | 13/18 (72.2)           | 4/13 (33.3)           | 8/10 (80)        |
| ≥2        | 20/27 (74.1)           | 12/16 (71.4)          | 5/5 (100)        |

| Parity     | Peripheral blood n (%) | Placental blood n (%) | Cord blood n (%) |
|------------|------------------------|-----------------------|------------------|
| Primipare  | 13/18 (72.2)           | 4/10 (40)             | 7/9 (77.8)       |
| Secondipare| 12/18 (66.7)           | 10/10 (100)           | 6/6 (100)        |
| Multiparous| 16/23 (69.6)           | 8/18 (44.4)           | 5/6 (83.3)       |

| p-value    | 0.512                  | 0.251                 | 0.569            |

| Parity     | Peripheral blood n (%) | Placental blood n (%) | Cord blood n (%) |
|------------|------------------------|-----------------------|------------------|
| Primipare  | 13/18 (72.2)           | 4/10 (40)             | 7/9 (77.8)       |
| Secondipare| 12/18 (66.7)           | 10/10 (100)           | 6/6 (100)        |
| Multiparous| 16/23 (69.6)           | 8/18 (44.4)           | 5/6 (83.3)       |

| p-value    | 0.936                  | 0.007                 | 0.475            |

IPTp: Intermittent preventive treatment in pregnancy.