Molecular surveillance of the Pfmdr1 N86Y allele among Congolese pregnant women with asymptomatic malaria

Louis Regis Dossou-Yovo
  Universite Marien Ngouabi

Francine Ntoumi
  Fondation Congolaise pour la Recherche Médicale

Felix Koukouikila-Koussounda
  Universite Marien Ngouabi

Jeannhey Christevy Vouvoungui
  Fondation Congolaise pour la Recherche Medicale

Ayodele Adedoja
  Fondation Congolaise pour la recherche medicale

David Nderu
  Eberhard Karls Universitat Tubingen

Thirumalaisamy P Velavan
  Eberhard Karls Universitat Tubingen

Arsene Lenga
  Universite Marien Ngouabi

Research

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Abstract

Background: Malaria in pregnancy is associated with considerable morbidity and mortality 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.

Background

Plasmodium falciparum malaria among pregnant women is a major public health concern in sub-Saharan Africa. Pregnant women have substantial risks and malaria in pregnant women are related to preterm delivery, intrauterine growth restriction, low birth weight and maternal anaemia. World Health Organization (WHO) recommends the use of intermittent preventive treatment (IPTp) with sulfadoxine-pyrimethamine (SP) for pregnant women and also for infants (IPTi) [2]. Currently, artemisinin-based combined therapy (ACT) is the first-line treatment for *P. falciparum* uncomplicated malaria. Resistance against chloroquine (CQ) and its successor, sulfadoxine-pyrimethamine (SP), had devasting consequences in sub-Saharan Africa in 1990s and 2000, particularly among children below 5 years [1]. After the introduction of ACTs, malaria mortalities and morbidities has globally declined until 2015. Even though ACTs are still efficacious, there is sensitive concern on the potential spread of artemisinin-resistant *P. falciparum* parasites 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, the efforts to monitor the emergence and spread of antimalarial drug resistance in resource limited settings are hampered due to high clinical trial costs. Molecular surveillance of distinct point mutation(s) in *P. falciparum* genes linked to antimalaria treatment failure offers a cost-effective tool to monitor spatial and temporal emergence and spread of resistant parasites. High prevalence of gene mutations associated with *P. falciparum* CQ (*P. falciparum* chloroquine transporter *Pfcrt*) and SP (*P. falciparum* dihydrofolate reductase gene; *Pfdhfr* and *Plasmodium falciparum* dihydropteroate synthase; *Pfdhps*) resistance informed, in part, the decision to replace these antimalarial

drugs with ACTs including the Republic of Congo [8-11]. *The Plasmodium falciparum multidrug resistance 1 (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 abundant in African settings. High prevalence of *Pfmdr1* 86N and 86Y alleles are currently being driven by ACT-linked *P. falciparum* selection. Previous studies have shown that parasites carrying *Pfmdr1* N86 are less susceptible to lumefantrine [16, 17], AL selects for *Pfmdr1* 86N whereas artesunate-amodiaquine, ASAQ, and piperaquine is selective for *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* N86Y in Brazzaville, Republic of Congo among pregnant women using maternal peripheral, placental, and cord blood. The study aims to provide factual data as a useful measure 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 matched blood samples (maternal peripheral, placenta, and cord blood) collected from pregnant women with asymptomatic malaria who had a normal child delivery at the Madibou Integrated Health Center, Brazzaville, between March 2014 and April 2015 (21). The study was conducted in Brazzaville, the capital of the Republic of Congo with 1.8 million inhabitants (22). Malaria transmission in this area is perennial with *P. falciparum* being the predominant *Plasmodium* species [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 followed by a Restriction Fragment Length Polymorphism (PCR-RFLP) were used to genotype *Pfmd1* N86Y as described earlier [26]. In brief, *Pfmdr1* primary and nested PCRs were amplified by adding 2µl DNA template into a PCR master mix (50µl) containing 1X PCR buffer, 2.8mM $MgCl_2$, 200µM dNTPs, 5pM of each primer, 1UTaq DNA polymerases (Qiagen, Hilden, Germany). The primer pairs for the primary PCR were A1 (5'-CGGAGTGACCAAATCTGGGA-3') and A3 (5'-GGGAATCTGGTGGTAACAGC-3') and for the secondary PCR were A2 (5'-TTGAAGAACAGAAATTACATGATGA-3') and A4 (5'-

AAAGATGGTAACCTCAGTATCAAAGAAGAG-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 5min. The secondary reaction was amplified 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/A4 secondary PCR products (10µl) using ApoI (New England Biolabs Inc., Ipswich, Massachusetts, USA) restriction enzyme for 15 minutes at 50°C following manufacturer's instructions. The resulting DNA fragments were separated and resolved by gel electrophoresis on a 2% agarose gel stained with SYBR green at 100V for 45 mins. ApoI digests *Pfmdr1*PCR product when *Pfmdr1*N86 (wild type allele) is present. The PCR amplification was performed three consecutive times for a given sample in order to get a successful amplification. Also, the nested PCR products were subjected to RFLP using ApoI twice (with independent PCR products) to reconfirm the *Pfmdr1* N86Y alleles. Additionally, few random samples were chosen and were subjected to sanger sequencing.

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-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

The baseline characteristics of participants recruited in this study is summarized in Table 1. The mean age of participants was 23.7 ± 5.75 years. Overall, 24% of the pregnant women did not take intermittent preventive treatment during pregnancy and most of the participants (70%) had >1 parity. Of the 101 matched samples analysed in this study, *Pfmdr1* was successfully amplified in 59 (58%), 38 (38%) and 21 (21%) maternal peripheral blood, placental blood and cord blood samples, respectively. Figure 1 shows an electrophoresis gel of PCR products before and after digestion with specific enzyme restriction. *Pfmsp2* genotyping showed mean multiplicity of infection (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 70% (41/59), 58% (22/38) and 86% (18/21) of the peripheral blood, placenta blood and cord blood samples, respectively. The remaining samples had the mutant allele (*Pfmdr1*86Y). 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-value*>0.05) among different sample types, parity and the number of Intermittent preventive treatment during pregnancy (Table 2).

Discussion

Anti-malarial drug resistance is a major obstacle in malaria reduction/eradication globally. Molecular surveillance is important to identify resistant phenotypes and to constantly monitor for any anti-malarial drug resistance. This study was 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 pfmsp2 gene used to determine MOI showed mean multiplicity of infection as 1.06±0.24. In areas with constant transmission of malaria, MOI may increase as immunity develops. MOI in pregnant women is a factor for the acquisition and maintenance of immunity against malaria. In this study, only using msp2 genotyping, we could show only one set of parasite clones were predominantly present among the pregnant women investigated. However, there are possibilities that these individuals may harbour more than one parasite, and this could be explained only by an additional msp1 genotyping for K1, MAD20, and RO33 alleles. 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 high resistant allele frequency (44%) has been observed [29]. In Southeast Asia, however, the frequency of *Pfmdr 1*86Y 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 (ASAQ),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*86Y 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 the 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 areas[17].Our findings show that *Pfmdr1*N 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.

List Of Abbreviations

Artemisinin-based combined therapy (ACT)

Artemether–lumefantrine (AL)

Artesunate–amodiaquine (ASAQ)

Chloroquine (CQ)

Fondation Congolaise pour la Recherche Médicale (FCRM)

Intermittent preventive treatment for pregnant women (IPTp)

 Intermittent preventive treatment for infants (IPTi)

Single nucleotide polymorphism (SNP)

Sulfadoxine-pyrimethamine (SP)

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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Tables

Table 1: Baseline characteristics of pregnant women with asymptomatic malaria.

	No. of participants (n =101)	Percentage (%)
Age group (years)		
15-30	89	88
>30	12	12
Dose of IPTp		
0	24	24
1	28	28
≥2	49	49
Parity		
Primipare	30	30
Secondipare	33	33
Multiparous	38	38

IPTp: Intermittent preventive treatment in pregnancy.

Table 2: Distribution of the *Pfmdr1*N86 allele among peripheral blood, placenta blood and cord blood samples from the Republic of Congo.

	Peripheral blood n (%)	Placental blood n (%)	Cord blood n (%)
Dose IPTp			
0	8/14 (57)	6/9 (67)	5/6 (83)
1	13/18 (72)	4/13 (33)	8/10 (80)
> 2	20/27 (74)	12/16 (71)	5/5 (100)
p-value	0.512	0.251	0.569
Parity			
Primipare	13/18 (72)	4/10 (40)	7/9 (78)
Secondipare	12/18 (67)	10/10 (100)	6/6 (100)
Multiparous	16/23 (70)	8/18 (44)	5/6 (83)
p-value	0.936	0.007	0.475

IPTp: Intermittent preventive treatment in pregnancy.

Figures

NC : negative control
MPC : muted type positive control
WPC : wild type positive control
S1 : sample 1 wild profile
S2 : sample 2 negative
S3 : sample 3 mixed
S4 : sample4 negative
S5 : sample 5 mutant profile
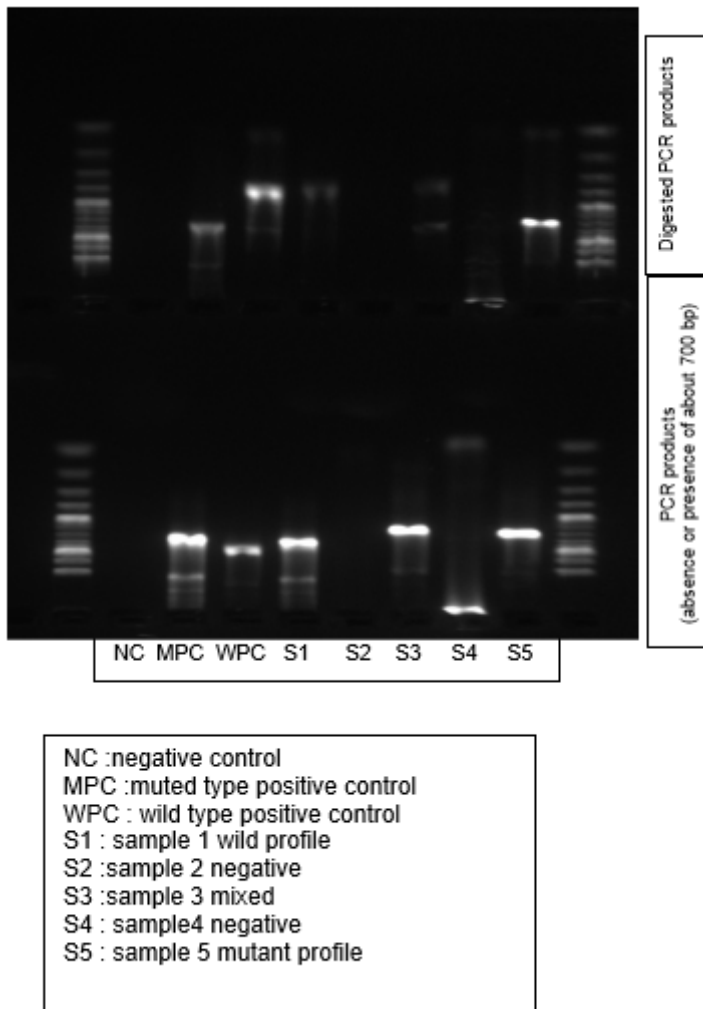
Figure 1

Electrophoresis gel before and after digestion by enzymes of restriction.