Journal of Global Antimicrobial Resistance 14 (2018) 277–283

Contents lists available at ScienceDirect

Journal of Global Antimicrobial Resistance

journal homepage: www.elsevier.com/locate/jgar

High prevalence of *Plasmodium falciparum* antimalarial drug resistance markers in isolates from asymptomatic patients from the Republic of the Congo between 2010 and 2015

Dominique Fatima Voumbo-Matoumona[a,b,c], Jean Akiana[d,e], Marylin Madamet[b,f,g], Lady Charlène Kouna[a], Jean Bernard Lekana-Douki[a,h], Bruno Pradines[b,f,g,*]

[a] *Unité d'Evolution, Epidémiologie et Résistances Parasitaires (UNEEREP), Centre International de Recherche Médicales de Franceville, BP 769 Franceville, Gabon*
[b] *Unité Parasitologie et Entomologie, Département des Maladies Infectieuses, Institut de Recherche Biomédicale des Armées, Marseille, France*
[c] *Ecole Doctorale Régionale d'Afrique Centrale en Infectiologie Tropicale, BP 876 Franceville, Gabon*
[d] *Départements des Masters/Licences, Parcours-Types des Sciences Biologiques, Faculté des Sciences et Techniques, Université Marien Ngouabi, BP 69, Brazzaville, Congo*
[e] *Direction de la Médecine Préventive et des Essais Cliniques, Laboratoire National de Santé Publique, BP 120 Brazzaville, Congo*
[f] *Aix-Marseille Univ., IRD, AP-HM, SSA, VITROME, IHU Méditerranée Infection, Marseille, France*
[g] *Centre National de Référence du Paludisme, Marseille, France*
[h] *Département de Parasitologie Mycologie et de Médecine Tropicale, Université des Science de la Santé, BP 4005 Libreville, Gabon*

ARTICLE INFO

Article history:
Received 5 May 2018
Received in revised form 1 August 2018
Accepted 2 August 2018
Available online 16 August 2018

Keywords:
Asymptomatic malaria
Molecular resistance marker
Haplotype
Antimalarial drugs
Republic of Congo

ABSTRACT

Objectives: This study investigated the prevalence of haplotypes of the *Pfdhps*, *Pfdhfr*, *Pfcrt*, *Pfmdr1* and *PfK13* resistance markers in isolates from asymptomatic patients from the Republic of the Congo following implementation of artemisinin based-combination therapy (ACT).
Methods: Peripheral blood was collected from asymptomatic children in 2010 and 2015 from Brazzaville in the south and in 2013 in the north of the Congo. Genotypes of *Pfmdr1*, *Pfcrt*, *Pfdhps*, *Pfdhfr* and *PfK13* were assessed by PCR.
Results: Children from 2010 were younger than those from 2015 (mean age 5.38 years vs. 8.67 years; $P = 0.003$). The main *Pfcrt* haplotype was the wild-type CVMNK (84.85%) in 2010, whereas the mutant CVIET (61.64%) predominated in 2015 ($P < 0.001$). In the north, 45.00% of samples were CVMNK and 10.00% were CVIET. Other samples harboured new haplotypes in the country or mixed alleles. No significant difference in *Pfmdr1* haplotypes was observed in 2010 and 2015 and the main haplotypes were NYD and NFD (30.56% vs. 28.57% and 61.11% vs. 42.86% for 2010 and 2015, respectively). In the south, the *Pfdhps* haplotypes observed were AAKAA, AGKAA, SGKAA and SGEGA (87.50% vs. 0%, 12.50% vs. 33.33%, 0% vs. 33.33% and 0% vs. 33.33% for 2010 and 2015, respectively). For *Pfdhfr*, the IRNI haplotype was most prevalent (85.71% for 2010, 87.50% for 2013 and 100% for 2015). No *PfK13* mutations were found.
Conclusions: Monitoring the efficacy of ACT and intermittent preventive treatment with sulfadoxine–pyrimethamine is necessary to ensure an epidemiological survey of asymptomatic malaria.

1. Introduction

Malaria is the most lethal parasitic disease and constitutes one of the major issues of public health in the world, especially in sub-Saharan Africa where this disease is endemic and the transmission perennial [1]. Asymptomatic malaria infection is characterised by the presence (microscopic or submicroscopic) of *Plasmodium falciparum* in the human body without symptoms of uncomplicated or complicated malaria disease. People with asymptomatic infection are a great reservoir for malaria parasites and are an important factor of multiplication and spread. Moreover, malaria contracted during pregnancy has many consequences both for the mother and the unborn child. Furthermore, most of the time malaria during pregnancy is submicroscopic and therefore asymptomatic [2–4]. To fight against this disease, the World

* Corresponding author. Present address: Unité Parasitologie et Entomologie, Institut de Recherche Biomédicale des Armées, IHU Méditerranée Infection, 19–21 Bd. Jean Moulin, 13005 Marseille, France.
E-mail address: bruno.pradines@gmail.com (B. Pradines).

https://doi.org/10.1016/j.jgar.2018.08.003
2213-7165/

Health Organization (WHO) recommends the use of intermittent preventive treatment (IPT) with sulfadoxine–pyrimethamine (SP) for pregnant women, insecticide-treated bed nets for prevention, and artemisinin based-combination therapy (ACT) for treating uncomplicated cases [5]. Consequently, since 2000 the incidence of malaria cases has decreased by 41% and mortality rates have fallen by 62% among all age groups and by 69% among children under-5 [1]. Among the obstacles facing the fight against malaria, there are asymptomatic infections as well as the emergence and spread of drug resistance despite the change in treatment for uncomplicated malaria from chloroquine (CQ) and others monotherapies to ACT. *Plasmodium falciparum* has adapted its metabolism to survive despite the presence of these drugs in the human body by substituting amino acids in the site of action of their enzymes [6]. Widespread parasite resistance therefore remains a global challenge.

CQ and others monotherapies were replaced because of the high level of *P. falciparum* resistance [5]. Since its implementation, many studies have reported that ACT has had an influence on molecular markers of resistance. Several studies have reported a significant return to the susceptible haplotype of *Pfcrt* (CVMNK) and selection of the NFD haplotype of *Pfmdr1* following the introduction of ACT in parts of Africa [7–12].

Following the introduction of IPT, a significant increase in quintuple mutations in the *Pfdhfr* (N51I, C59R and S108N) and *Pfdhps* (A437G and K540E) genes associated with SP resistance is well described in Africa [13–17]. In Central Africa, the distribution of *Pfdhps* K540E, A581G or A613S is variable according to the country. In Gabon and Angola, only K540E has been observed, whereas K540E and A581G are observed in the Democratic Republic of the Congo [16–19].

In sub-Saharan Africa, a delay in clearance of parasitaemia following treatment with ACT has been reported, but no Asian mutations of the K13-propeller sequence associated with artemisinin resistance were found [20–23]. Although A578S was often identified in Bangladesh and some African countries [24–27], this mutation was not associated with resistance or treatment failure [23,24,28,29]. Recently, the emergence of an indigenous artemisinin-resistant *P. falciparum* strain originating from Equatorial Guinea has been observed [30].

In the Republic of the Congo, before the implementation of ACT the prevalence of the *Pfcrt* 76T single nucleotide polymorphism (SNP) associated with CQ resistance was highest, with the mutation present in 100% of isolates in febrile patients with CQ treatment failure [31]. Before the implementation of IPT, in parasites collected from febrile children with uncomplicated malaria, the rates of *Pfdhfr* and *Pfdhps* mutations were >97% for *Pfdhfr* codons 51I and 108N and 85% for isolates carrying the 437G *Pfdhps* mutation [32]. The National Malaria Control Program recommended the use of artesunate–amodiaquine and artemether–lumefantrine instead of CQ as treatment of uncomplicated malaria, and SP only as IPT during pregnancy, which has been effective since 2006 [33].

In Brazzaville, the political capital of the Republic of the Congo located in the south of the country, a recent study conducted on febrile children aged 4–9 years reported a high level of CVIET *Pfcrt* haplotype following the implementation of ACT, with a non-significant decrease from 2010 to 2015 [34]. Four years after the introduction of ACT, the prevalence of the *Pfcrt* 76T mutation was 92% in isolates from asymptomatic patients [35]. The *Pfdhfr* (codons A16V, N51I, C59R, S108N and I164L) and *Pfdhps* (S436A, A437G, K540E, A581G and A613S) mutations are associated with resistance to sulphoxide and pyrimethamine, respectively. The IRNI *Pfdhfr* haplotype (with mutations at codons 51, 59 and 108) was reported in 79% of isolates from asymptomatic pregnant women and 98.5% of isolates carried the 437G *Pfdhps* mutation [36]. In febrile patients, the frequencies of the N86Y and D1246Y mutations of *Pfmdr1* were, respectively, 73% and 22% in 2010 and 27% and 0% in 2015 [34]. Delayed *P. falciparum* parasite clearance following artemisinin treatment associated with Mal10-688956 and Mal13-1718319 has been evaluated in samples from febrile patients, and no SNPs associated were observed [37]. Thirteen new mutations were observed in the propeller domain of *Pfk13* in the Republic of Congo [34] but none of the mutations associated with artemisinin resistance in Southeast Asia were identified [23].

Moreover, the efficacy of ACT is >92% in the north of the Republic of the Congo, but no data on the distribution of molecular markers associated with resistance, except for Mal10-688956 and Mal13-1718319, are available for this part of the country [37,38]. No more is known on this area [39].

Asymptomatic infection and associated *P. falciparum* resistance is not well described in the Republic of the Congo from north to south [39]. All available data on *P. falciparum* are in relation to the suburban area of Brazzaville in the south, and isolates collected from asymptomatic infections are available only for 2010 for *Pfcrt* and 2013 for *Pfdhfr* and *Pfdhps* [35,36,40]. Data on *Pfmdr1* and *PfK13* mutations were only obtained from isolates from febrile patients [21,37].

The aim of this study was to determine the diversity of haplotypes of antimalarial drug resistance molecular markers such as the *P. falciparum* chloroquine resistance transporter (*Pfcrt*), *P. falciparum* multidrug resistance protein 1 (*Pfmdr1*), *P. falciparum* dihydropteroate synthase (*Pfdhps*), *P. falciparum* dihydrofolate reductase (*Pfdhfr*) and the propeller domain of *P. falciparum* Kelch 13 (*PfK13*) in 2010 and 2015, i.e. 4 years and 9 years following the implementation of ACT in the south of the Republic of the Congo.

2. Methods

2.1. Isolate collection

A cross-sectional study was performed in health centres in the Republic of the Congo to collect peripheral blood samples from asymptomatic children under 15 years of age in 2010 and 2015 in Brazzaville (4°16′41.94″S–15°16′40.95″E) in the south of the country and from asymptomatic patients in 2013 in Lopola (6°59′28″E–3°4′48″N), Ouésso (0°28′59.98″S–15°54′0.35″E) and Thanry (2°44′42.0″S–16°57′57.9996″E) in the north. In this study, only positive samples from previous studies were analysed.

2.2. Diagnosis

Malaria diagnosis was based on the detection of *P. falciparum* in blood smears.

2.3. DNA extraction

DNA was extracted using an E.Z.N.A® Blood DNA Mini Kit (Omega Bio-tek, Norcross, GA) as previously described [12]. Briefly, 250 μL of blood, 25 μL of protease K (20 mg/mL) and 250 μL of lysis buffer were mixed and were heated to 71 °C for 45 min. Then, 260 μL of isopropanol was added and the mixture was transferred to a column and was centrifuged at 8000 × *g* for 1 min. The column was washed twice and DNA was eluted with 100 μL of sterile water pre-heated to 70 °C. DNA aliquots were kept at −20 °C until use.

2.4. Determination of single nucleotide polymorphisms

The *Pfcrt*, *Pfdhfr*, *Pfdhps*, *Pfmdr1* and *PfK13* sequences were amplified using previously described primers [41–43]. The reaction mixture and conditions for thermal cycling were as previously described [41–43].