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Assessment of Pfmdr1 and Pfcrt Mutations After Six Years of Implementation of Artemisinin-Based Combination Therapy in Dakar Senegal.

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ABSTRACT

Background: Artemisinin-based combination therapies (ACTs) have been shown to be effective for uncomplicated P. falciparum malaria in Africa including Senegal. In South East Asia, Pfmdr1 Single Nucleotide Polymorphism (SNPs) are frequent and tentatively associated with reduced susceptibility to ACT partner drugs mefloquine and lumefantrine. In Africa where amodiaquine is one the most partner drugs of ACT, studies on molecular marker of AQ resistance are urgent. The objective of this study is to monitor molecular markers of AQ the partner drug of ACT in Senegal.

Methods: Blood samples were collected from patients with uncomplicated malaria in Deggo health post in 2010 (N=124) and 2012 (N=160). Pfmdr1 and Pfcrt SNPs were determined by PCR-RFLP in Plasmodium falciparum positive samples.

Results: A total of 284 samples positives were analyzed for various Pfmdr1 and Pfcrt SNPs.. Pfcrt-76T mutant type haplotype was present at 12.90% and 15.62% in 2010 and in 2012 respectively. Prevalence of 16.94% and 15.62% were found for Pfmdr1-86Y in 2010 and 2012 respectively. Low prevalence of Pfmdr1-184F was noted in 2010 (7.26%) and in 2012 (6.88%)..

Conclusions: Overall a low prevalence of Pfcrt and Pfmdr1 SNPs associated with CQ and AQ resistance were noted in our study area. Similar results were found in west West Africa. Results suggested that partner drug of ACT still be effective in Senegal, however a regular monitor of antimalarial drug is essential in the context of while use of ACT.

KEYWORDS :

Introduction

The emergence and spread of drug-resistance parasites oblige many African countries to change their heath policy. Since 2001, WHO has recommended artemisinin-based combination therapies (ACTs) as first-line treatment for uncomplicated *P. falciparum* malaria[1]. For instance, these African countries have through their National Malaria Control Program (NMCP) implemented the use of ACTs. In Senegal this strategy is effective since 2006[2] and ACTs were widely use in all Senegalese health facilities with Artemether-lumefantrine (AL) and Artesunate-Amodiaquine (ASAQ) as first line treatment and Dihydro-artemisinin-piperaquine DHAPQ as second line treatment. The two most commonly used ACTs worldwide are artemether-lumefantrine (AL) and artesunate amodiaquine (ASAQ)[4]. Polymerase chain reaction (PCR)–adjusted efficacy for both combinations remains high in most regions.[5–7] However, there have been some reports of decreasing AL cure rates in Africa [8–11] and Asia[12] and reports of high levels of treatment failures of ASAQ [13–14]. Resistance to ACT partner drugs has historically manifested before that of artemisinins, whose short half-lives result in the exposure of residual parasites to sub-therapeutic levels of the partner drug alone. Response to the partner drug is therefore a key component of overall ACT efficacy. Mutations in the gene encoding the *P. falciparum* chloroquine resistance transporter (pfcrt) are associated with chloroquine resistance [15]. In the presence of pfcrt 76T, chloroquine resistance is modulated by point mutations in the gene that encodes the *P. falciparum* multidrug resistance transporter 1 (pfmdr1), primarily at codon 86 [16] and also by mutations at positions 184, 1034, 1042, and 1246 [17]. Decreased susceptibility to lumefantrine has been linked to polymorphisms in these two genes [18-19]. Selection of Pfmdr1 86Y and Pfcrt 76T alleles in recurrent parasites after treatment with amodiaquine alone or in combination with artesunate has been observed in a number of studies [20-22]. It has also been suggested that parasites that carry chloroquine-resistant Pfmdr1 alleles may be more susceptible to artesunate in classical in vitro assays [23], an effect that could counteract the increased risk of amodiaquine failure when these drugs are combined in ASAQ.

Therefore it's become important to monitor molecular markers of AQ resistance in the context of widely use of ASAQ and AL in Senegal. The aim of this study was to assess de prevalence of Pfmdr1 and Pfcrt SNPs associated with AQ and CQ resistance in the context of large use of ACTs for uncomplicated malaria treatment in Senegal.

Methods

Study area and population enrolment

Samples for this study were collected in Deggo health post located in the suburb of Dakar the capital, from patient with uncomplicated malaria in 2010 and 2012. Deggo health post is part of Guediawaye hospital area. It is a sentinel site of NMCP for malaria surveillance. In this area malaria is highly seasonal with pick of transmission during the rainy season from August to November. This area is also characterised by the existence of water basins favourable for the development of malaria vector. *Plasmodium falciparum* is the predominant specie and malaria transmission is mainly due to *Anopheles gambiae s.l.*

Were included in this study any patient visiting Deggo health post with symptoms of mild malaria.

Samples collection

Finger prick blood samples were collected from each study participant and blotted onto Whatman filter paper 3MM packed in ziplock bags with desiccant after they dried. Thick and thin smears blood films were also collected for malaria parasites quantification and identification. Thick and thin film were stain in Giemsa and read in Health center laboratory by qualify microscopists. Whatman filter papers were sent to the Parasitology Mycology central laboratory for *Pfmdr1* and *Pfcrt* SNPs analysis by PCR-RFLP.

P. falciparum DNA extraction

DNA was extracted from filter paper by Chelex-100 method described by Wooden et al [24] . Briefly, 1X PBS with 0.5% saponin was added to small pieces of blood-impregnated filter paper, shake for 10mn (150 rpm) and incubated at room temperature overnight. The Supernatant was removed and wash twice with PBS buffer. 150µL milli-Q H2O and 75µl 20% of chelex mix (5g chelex in 25 ml milli-Q H2O) were added in the 96 deep plate wells and gently seal. Plates were boiled for 8mn (2×4) and cool for 10mn at room temperature. Spin down for 5mn, freeze deep well plate with DNA and carefully transpose 50µl of the supernatant to new 96 PCR plates by leaving carefully the chelex in the original deep well plate.

Pfmdr1 and *Pfcrt* genotyping

Extracted *P. falciparum* DNA was amplified by using primers for *Pfcrt* and *Pfmdr1* genes and SNPs were analysed by using Restriction Enzyme spanning codons 76 for *Pfcrt* and 86-184 for *Pfmdr1* genes.

Pfcrt SNPs analysis

A nested PCR as described by Djimde et al (9) was done for *Pfcrt* DNA amplification.

The 25-µL *Pfcrt* outer PCR mixture consisted of the primers P1/P2 (1.0 µl /primer), 1.0X TEMPase Hot Start1 Master Mix (3.0 mM MgCl2, 0.4 mM 2′-deoxynucleoside5′-triphosphate [dNTP], and 0.2 units/ µl TEMPase Hot Start DNA Polymerase, Ampliqon III; VWR-Bie, Berntsen, Denmark, and 2 µl extracted DNA. The reaction mixture of the nested *Pfcrt* PCR was identical to the mixture of the outer PCR, and the primer set D1/D2 was used. A genomic DNA preparation of laboratory isolates 3D7 and Dd2 were used as controls for *Pfcrt-76* wild type and mutant type respectively.

Pfmdr1 SNPs polymorphism

For *Pfmdr1* a nested PCR was done for DNA amplification (10). For the first amplification the 19-µL PCR mixtures consisted of the primers Mdr2/1 New rev1 4.0 µl (0.2 µM /primer), 10.0 µl TEMPase/AH Master Mix (3.0 mM MgCl2, 0.4 mM 2′-deoxynucleoside5′-triphosphate [dNTP], and 0.2 units/ µlTEMPase Hot Start DNA Polymerase, Ampliqon III; VWR-Bie, Berntsen, Denmark), and 1 µl extracted DNA.

The reaction mixture of the nested *Pfmdr1* PCR was identical to the mixture of the first PCR, and the primer set FN1/1 Rev/C1was used.

DD2 (86F-184Y-1246D) and 7G8 (N86-184F-1246Y) were used as positive controls. Blood donors from Denmark who were never exposed to malaria were used as *P. falciparum* negative controls.

Restriction enzymes generating RFLPs

Restriction enzymes were used for SNPs determination. Endonucle-

ases *ApoI*, *DraI* and *AflIII* had been obtained from New England BioLabs™, Roche Molecular Biochemicals™ and Stratagene™ respectively. Incubations of *P. falciparum* DNA samples with restriction enzymes were setup following the manufacturer's instructions.

Following amplification of the fragments concerned, *ApoI* enzyme was used for *Pfcrt* SNPs determination. *Pfcrt* DNA was incubated with *ApoI* enzyme overnight at 53°C. The mixture products was visualised on 2% agarose gel with ethidium bromide and visualised under UV (ultraviolet) light. Samples are classified as wild type haplotypes when DNA fragment length was found at 96pb and 46 bp while mutant type was found at 261bp. Sample was classified as mixt if fragment length for wild and mutant types were found.

For *Pfmdr1* polymorphism analysis, enzymes *AflIII* and *DraI* were used for *Pfmdr1-86* and *Pfmdr1-184* respectively. For *Pfmdr1-86Y*, mutant type haplotypes were found at 200 bp while wild types were found at 521bp. For *Pfmdr1-184F*, mutant type haplotypes were found at 242 and 204 bp and wild type was found at 220 bp.

Ethical considerations

This study received the approval of the National Ethical Committee and the administrative approval of the Ministry of Health and Prevention of Senegal. All parents or legal representatives of children prior to any blood sampling exercise signed an informed consent.

Results

Characteristics of the population:

A total of 284 dried blood spots samples were selected for the study. Among them, 124 wer were collected in 2010 and 160 samples in 2012. All samples selected for this study were from *P. falciparum* positives patients confirmed by microscopy at the time of collection (Table 1).

***Pfcrt* polymorphisms**

All samples (284) were successfully amplified by nested PCR. Genotype results showed a low prevalence of *Pfcrt76T* mutant type haplotype in 2010 (12.90%) and in 2012 (15.62%) with no significant difference over year (p = 0.517) (Table 2).

Considering the age (children, adolescent and adult) there is no significant difference in the prevalence of mutation (p=0.191). However, the highest prevalence of mutant type *Pfcrt-76T* was found in children 0-10 years old (23.53%) (Figure1).

***Pfmdr1* polymorphisms**

Among 284 positives samples analysed, 16.20% (46/284) carried out Pfmdr1-86Y mutation associated with AQ resistance. Results shown a low prevalence of *Pfmdr1-86Y* mutant type in the study area over 2 years (p=0.766) (Table 2). However a slight decrease of Pfmdr-86Y was noted from 2010 *(*16.94%) to 2012 (15.62%). Similar results were found for *Pfmdr1-184.* Low prevalence of *Pfmdr1-184F* mutant type haplotype was noted in 2010 (7.26%) and 2012 (6.88%) with no significant difference over year (p=0.900). By comparing prevalence of *Pfmdr1* mutant's types (*Pfmdr1-86Y* and *184F*) by age and parasitémie, no significant difference was noted over year in our study area (Figure 1 and 2). A slight increase of the prevalence of *Pfmdr1* mutant types was noted in age group 11-18 years compare to others groups (Figure 1).

A low prevalence of haplotype double mutant *Pfmdr1-86Y+Pfmdr1-184F* was found in our study area in 2010 (3.23% (4/6)) and 2012 (1.25% (2/6)) with no significant difference (p=0.477)(Table 3).

Combination of *Pfcrt* and *Pfmdr1* mutations

Analysis of the combination of *Pfcrt/Pfmdr1* mutation shows that, the triple mutation *Pfmdr86Y+Pfcrt76T+Pfmdr184F* was not found in our study site. While we noted the presence of *Pfmdr86Y+Pfcrt76T* haplotype: 1.61% (2/5) and 1.88% (3/5) in 2010 and 2012 respectively with no significant difference (p=0.970) (Table 3).

Discussion

Due to widespread parasite resistance to chloroquine (CQ) and, subsequently, sulphadoxine pyrimethamine (SP), all malaria-endemic countries in sub-Saharan Africa including Senegal have adopted artemisinin-based combination therapy (ACT) as first-line policy for treatment of uncomplicated *Plasmodium falciparum* infection[11]. In Senegal, Artemether-lumefantrine (AL) and Artesunate-Amodiaquine (ASAQ) are used as first line treatment and Dihydro-artemisinin-piperaquine DHAPQ as second line treatment [3] depending on availability.

However, the emergence of reduced sensitivity to artemisinin in focal areas of Southeast Asia became a global concern. Therefore monitoring of parasite resistance to ACT partner drugs is vital for malaria control in the context of pre elimination. Indeed, apart ACT, there are little or no new drug or effective combinations for uncomplicated *P. falciparum* malaria treatment.

The *P. falciparum multi drug resistance gene-1* (*Pfmdr-1*) is implicated in resistance/tolerance to all antimalarial drugs including chloroquine (CQ), amodiaquine (AQ) and the artemisinin derivates. It has been shown that certain combinations of SNPs in the *Pfmdr-1* gene, at codons 86, 184, and 1246, are emerging in areas where the ACT drug combination artemether-lumefantrine (AL) is being widely used [25-26] and suggested that these *Pfmdr-1* haplotypes may be markers for the emergence of the decreased of ACTs efficacy [27].

As part of an annual monitoring of clinical efficacy of anti malaria in Senegalese sentinel sites, the objective of this study was to assess the effect of change in drug treatment policy on the molecular markers of AQ resistance.

Our results shown that a low prevalence of *Pfmdr-1* mutant type at the target acid residue 86 was found in 2010 (16.94%) and in 2012 (15.62%) (p=0.766). The prevalence of *Pfmdr1-184F* was 7.26% in 2010 and 6.88% in 2012 (p=0.900). In Burkina Faso *Barake et al* found between 2005 and 2006 similar results of *Pfmdr1-86Y* (18.7%) [28]. A low prevalence of *Pfmdr1* mutant types was also found by *Fall et al* in Dakar in 2010. These Authors show a prevalence of *Pfmdr1-86Y* of 14.9% while the prevalence of *Pfmdr1-184F* was noted at 49.4% [29]. This increase of *Pfmdr1-184F* could be done by AL drug pressure. However our results showed a low prevalence of *Pfmdr1-184F* over the 2 years. This low prevalence of *Pfmdr1-184F* found in our study area could be due by the use of three ACTs with different modes of action of their components there by reducing the prevalence of parasites mutated.

Regarding *Pfcrt-76* SNPs, our results show a low prevalence of mutant type. *Pfcrt-76T* was found in our study area respectively at 12.90% in 2010 and 2012 at 15.62% (p=0.517). Similar results were found in the southern part of Senegal [30]. *Jovel et al* in Guinea Bissau (border country of Senegal) showed that there is a decrease of the mutant type *Pfcrt-76T*. Authors found a prevalence of *Pfcrt-76T* at approximately 24% between 2003 and 2007 [31]. Similar results were noted in the capital city of Guinea with a decrease of *Pfcrt-76T* over year: 85% in 1992 and 13% in 2000 [32] .

In Senegal the drastic decrease of frequency of *Pfcrt-76T* haplotype in 2010 and 2012 can be due to the withdrawal of chloroquine for uncomplicated malaria treatment since 2003. Since there, NMCP, following the WHO guidelines for malaria prevention and treatment, adopted the SP/AQ for an interim malaria treatment and since 2006, the ACTs were used for malaria treatment. Following our funding, similar results found in the South part of Senegal and in many countries, it appeared that chloroquine could probably be used by combination with antimalarial for malaria prevention [33] .

In our study, we found low prevalence of haplotypes combination *Pfcrt-76T/Pfmdr-86Y* both in 2010 than in 2012. Our results suggested that the use of alternative ACT for uncomplicated malaria treatment can be effective on CQR and AQR parasites. Previous studies in Kilifi (Kenya) between 1995 and 2013 show a decrease of this association, 21.3% to 0% [34]. The low prevalence of this combination noted in our study show that ACTs still be effective in Senegal. However in Eastern and Southern Africa, authors have showed a significant increase in infections carrying the *Pfcrt-Pfmdr-1* combination CVMNK-NFD from 24.3% in 2009 to 45.3% in 2010 [35] and 70% in 2008. This increase of *Pfcrt/Pfmdr-1* mutant types in East Africa must be due to AL and CQ failure treatment.

The role of drug pressure on the emergence of parasite resistance to anti-malarials has been well described[25]. To avoid this, RDTs were in-

troduced in Senegal since 2008 to improve malaria diagnosis and we have noted an important reduction in ACTs consumption in Senegal after the deployment of this strategy [36].

The low prevalence of *Pfcrt-76T*, *Pfmdr1-86Y* and *Pfmdr1-184F* noted in the study area is associated with low ACT drug pressure. Furthermore in the context of malaria pre elimination, monitoring of ACT resistance by looking at the K13 mutation is essential.

TABLES AND FIGURES

Table I: Characteristics of the population

	Age (years)			sex			DP (parasites /µl)		
	minima	maxima	mean	female	man	ratio	minima	maxima	mean
2010	15	55	23.26	76	48	1.58	1001	96100	18239.82
2012	05	65	17.91	59	101	0.58	1115	96261	22079.79

Table II: Prevalence of *Pfcrt-76T*, *Pfmdr1*-86Y and *Pfmdr1-184F*

	2010	2012	p-value
Pfcrt-76T	12.90	15.62	0.517
Pfmdr1-86Y	16.94	15.62	0.766
Pfmdr1-184F	7.26	6.88	0.900

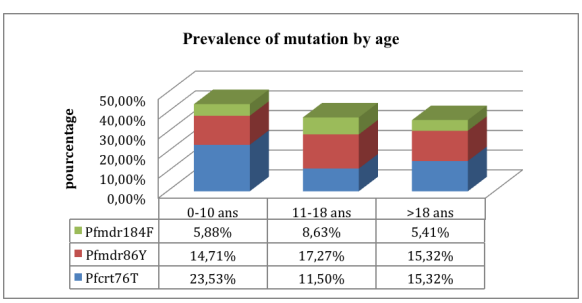

FIGURE 1: Prevalence of mutation by age

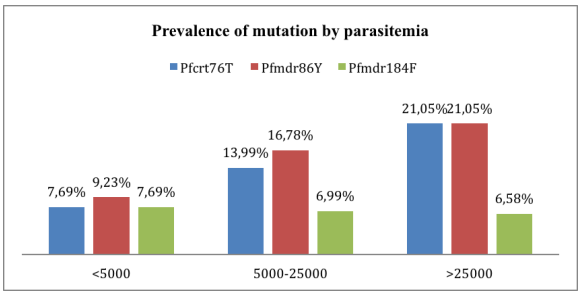

FIGURE 2: Prevalence of mutation by parasitemia

TABLE III : Prevalence of mutations combinations

Mutations combinations	2010	2012	p-value
Pfcrt-76T/Pfmdr1-86Y/Pfmdr1-184F	0%	0%	-
Pfcrt-76T/Pfmdr1-86Y	1.61%	1.88%	0.970
Pfcrt-76T/ Pfmdr1-184F	0%	0%	-
Pfmdr1-86Y/Pfmdr1-184F	3.23%	1.25%	0.477

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