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Polymorphism of *pfk13-propeller* in Niger: Detection of Novel Mutations

Ibrahim Maman Laminou[1*], Mahaman Moustapha Lamine[1,2], Boubacar Mahamadou[1], Oumou Maïga Ascofaré[3] and Alioune Dieye[2]

[1]Centre de Recherche Médicale et Sanitaire, 634 Bd de la Nation. 034 Yantala BP: 10887, Niamey, Niger.
[2]Université Cheick Anta Diop-Dakar, Sénégal.
[3]Benhard Nocht Institute for Tropical Medicine, Hamburg, Germany.

Authors' contributions

This work was carried out in collaboration between all authors. Authors IML and AD designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors MML and BM managed the analyses of the study. Author OMA managed the literature searches. All authors read and approved the final manuscript.

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Short Research Article

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ABSTRACT

Aim: Artemisinin resistance was confirmed in Southeast Asia in 2009, and a molecular marker of resistance to artemisinin as point mutations in the region of the *kelch13* gene (*k13*) coding for the C-terminal propeller domain of *Kelch13* (*K13-propeller*) gene, was discovered in Cambodia in 2013.
Methods: In this context, we examined the polymorphism of *k13-propeller* by sequencing 602 *Plasmodium falciparum* isolates collected from patients with uncomplicated malaria in Niamey, Niger, during the rainy season of 2013.
Results: We observed thirteen single-nucleotide polymorphisms (SNPs) including eight specific to Niger at a low frequency from 0.02% to 2.7%. The key mutations associated with delayed parasite clearance time in Southeast Asia (SEA) and with decrease of the ring-stage sensitivity to artemisinin *in vitro* (C580Y, R539T, Y493H, I543T and N458Y) were not observed. However, five

**Corresponding author: E-mail: lamine@cermes.org;*

particular non-synonymous mutations were found in our study area: M472I, Y558C, K563R, P570L and P615S and were not reported elsewhere.
Conclusion: These results suggest a genetic variability of *P. falciparum k13-propeller* domain in Niamey, Niger.

Keywords: Malaria; artemisinin resistance; P. falciparum k13-propeller; SNPs; Niger.

1. INTRODUCTION

Artemisinin-based Combination Therapies (ACT) are currently the most effective drugs against malaria. Since 2005, World Health Organization (WHO) recommends them as first-line treatment for uncomplicated malaria and artesunate i.v. followed by oral ACT for severe malaria. Artemisinin resistant *P. falciparum* strains have emerged recently at the Thai-Cambodian border in Southeast Asia (SEA), where also resistance to chloroquine and sulfadoxine-pyrimethamine emerged before spreading to Africa [1,2,3]. A team from the Pasteur Institute in Cambodia has identified a molecular marker associated with *P. falciparum* resistant to artemisinin and slow parasite clearance half-life after ACT: point mutations in the propeller domain of the gene *kelch 13* (*PF3D7_1343700*) or *pfk13* [4]. Mutations shown to be strongly associated with artemisinin resistance are: C580Y, R539T, Y493H, I543T and N458Y as they are correlated with increased clearance time [4]. Niger is a crossroad of migration in Africa, and molecular surveys to quickly identify any signals of selection of mutant *pfk13* in local or imported malaria is of prime importance.

In this context characterized by the emergence of artemisinin resistance in Southeast Asia (SEA) and the recent discovery of its molecular marker in *pfK13*, the baseline of *pfK13* sequence needs to be assessed in malaria endemic areas, especially in sub-Saharan Africa.

Malaria is endemic in Niger, which is also at the cross road of migration in Africa. Artemisinin resistance survey using screening of *pfk13* mutations would quickly identify any signals of selection pressure on *pfk13*. Therefore, the aim of this study is to analyze the polymorphism of *pfK13* in urban area of Niamey (Niger) where ACT has been used for more than ten years as first-line therapies for malaria treatment.

2. METHODS

2.1 Type of Study and Sample

Samples were collected from patients with uncomplicated malaria in twelve health centers (Bangabana, Banigoungou, Boukoki, Gamkalé, Karadjé, Koiratagui, Lamordé, Madina, Republic, Tondibia, Wadata and Yantala) in the urban area of Niamey during the rainy season (June to October) of 2013. The laboratory confirmation was made by rapid diagnostic test (SD Bioline Malaria Ag/Pf). Blood samples of the patients were collected before treatment on filter paper (Whatman 3MM/ZOOPOLE).

2.2 Genotyping

DNA extraction and PF3D7_1343700 gene amplification were performed according to the protocol developed in KARMA project option 1 (K-13 Artemisinin Resistance Multicenter rapid Assessment) [5]. The primary PCR was done with primers K13-PCR_F (5'-CGGAGTGACCAAATCTGGGA-3') [100 µM] and K13-PCR_R (5'-GGGAATCTGGTGGTAACAGC-3') [100 µM]. The secondary PCR was done with K13_N1_F primer (5'-GCCAAGCTGCCATTCATTTG-3') [100 µM] and K13_N1_R primer (5'GCCTTGTTGAAAGAAGCAGA-3') [100 µM]. The secondary PCR products (band size = 849 bp) were sequenced by Macrogen (Seoul, South Korea) [5].

2.3 Ethical and Data Analysis

This study received approval from the Ministry of Health of the Republic of Niger. Informed consent was obtained from all patients or from the children's parent or legal guardian included in the study. Data were managed with Microsoft Excel and Epi Info Version 7.1.1.

3. RESULTS

3.1 Sample Distribution and *pfk13*-propeller Polymorphisms

A total of 602 malaria-infected patients were included in this study. The gender ratio was 0,8 (M/F) and the mean age was 11.4 years, ($\Omega = 12,15$; [0,08; 75]). Out of 602 samples sequenced, 366 yielded an interpretable result. A total of 13 single nucleotide polymorphisms

(SNPs) were observed: Six were non-synonymous (NS) mutations and seven were synonymous (S). The prevalence of these mutations was low (Table 1). Among the thirteen SNPs observed, eight were specific to Niger, including five NS-mutations (M472I; Y558C; K563R; P570L and P615S). Notably, two samples carried the A578S mutation, which was also observed in other surveys conducted in Africa. These mutations were observed in *different P. falciparum* isolates.

4. DISCUSSION

This study analyzed for the first time the variability of *pfk13* gene in Niger. We showed a polymorphism of *pfK13* mutations in Niger, where *in vivo* and *in vitro* artemisinin resistance has not been reported yet. Eight of the SNPs detected have not been shown in other studies, five were NS mutations (M472I, Y558C, K563R, P570L and P615S). These mutations may modify the structure or the function of the KELCH protein. Furthermore, we must mention the observation of the A578S mutant allele adjacent to the C580Y allele [6], which is associated with slow parasite clearance in Cambodia [4,7]. However, the main SNPs (C580Y, R539T and Y493H) that have been discovered in SEA and involved in resistance to artemisinin *in vivo* and *in vitro* [4,8] have not been found in Niger.

Our study showed the existence of SNPs other than the one described in Cambodia by Ariey et al. [4,9]. This diversity of mutations observed in the *pfk13* could suggest de novo emergence of artemisinin resistance [10,11]. The mutations observed in Niger do not have a high prevalence, like those observed in previous studies of African isolates [12,13,14]. This low prevalence could be explained by the fact that phenotypic resistance of *P.falciparum* to artemisinin has not been yet established in Africa. Nevertheless, the A578S mutant allele was observed in several multicenter studies including isolates of *P. falciparum* from sub-Saharan Africa [6,12,13,14,15]. This mutation is of interest as it is located two amino acids prior to one of the key mutation associated with artemisinin resistance in SEA [6].

The presence of polymorphism in the *pfk13* gene of Nigerien isolates could suggest a possible selection process of the parasite to artemisinin resistance. Indeed, Niger introduced the ACTs in its policy in 2005. Several therapeutic efficacy studies were conducted at sentinel sites (Gaya and Tessaoua) [16]. Moreover, Niger has benefited from the initiative "Affordable Medicine Facility Malaria-(AMFm)" whose aims in 2008 were to make ACTs more available and accessible. This selection pressure could explain the observed polymorphisms in *Pfk13* [17]. The amplification was negative for 236 samples included in the study. Nevertheless, we were able to analyze 366 samples, which gave a solid baseline of *pfk13* polymorphism in Niamey (Niger). A limitation of the study was being restricted to Niamey, once it would be interesting to extend the survey of *pfk13* polymorphisms in sentinel sites across the country where therapeutic efficacy studies of

Table 1. *Pfk13* polymorphisms in *P. falciparum* isolates collected in Niamey, Niger in 2013

Codon	Type	Reference amino acid	Nucleotide	Mutant amino acid	n (%)	Africa	SEA
472	NS	M	cct to ctt	I	1 (0.02%)	Niger	-
558	NS	Y	tat to tgt	C	1 (0.02%)	Niger	-
563	NS	K	aaa to aga	R	1 (0.02%)	Niger	-
570	NS	P	atg to att	L	1 (0.02%)	Niger	-
615	NS	P	cca to tca	S	1 (0.02%)	Niger	-
488	S	L	ttg to tta	L	10 (2.7%)	Niger	-
510	S	V	gtg to gta	V	10 (2.7%)	Niger	-
630	S	Y	cca to tca	Y	10 (2.7%)	Niger	-
578	NS	A	gct to tct	S	2 (0.5%)	+	+
465	S	I	att to atc	I	10 (0.5%)	+	+
496	S	G	ggt to ggc	G	10 (0.5%)	+	+
471	S	R	cgt to cgc	R	1 (0.02%)	+	-
496	S	G	ggt to ggc	G	10 (2.7%)	+	+

All pfK13 sequence data were analyzed from allele 427 to 709

n = number of samples containing mutant allele. N = number of samples with an uninterpretable result; NS=non-synonymous mutation; S=synonymous mutation. (+) = presence and (-) = absence

ACTs were performed earlier. However, this study showed the need of assessment of ACT susceptibility associating therapeutic efficacy essays to the evaluation of *pfk13* polymorphism.

5. CONCLUSION

These results suggest a genetic variability of *P. falciparum k13-propeller* domain in Niamey, Niger. Polymorphism in *pfk13* in Niger does not mean a decline in the effectiveness of ACTs. Nevertheless, our results reinforce the idea of the unresolved implication of *pfk13* in artemisinin resistance in Africa. Additional studies are needed to monitor the effectiveness of ACTs.

CONSENT

As per international standard or university standard, patient's written consent has been collected and preserved by the authors.

ETHICAL APPROVAL

As per international standard or university standard, written approval of Ethics committee has been collected and preserved by the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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