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Detection of *Plasmodium falciparum* K13 Propeller A569G Mutation after Artesunate-amodiaquine Treatment Failure in Niger

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Authors' contributions

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

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ABSTRACT

Background: Artemisinin (ART) resistance is a problem that may compromise the elimination of malaria. It is associated with point mutations in the kelch gene *PF3D7_1343700* or *K13* propeller (*pfk13*). A recent worldwide map of *pfk13* polymorphisms revealed more than 100 non-synonymous (NS) mutations. However, it remains unclear whether these mutations are the result of drug pressure or the expression of a natural polymorphism, because of the scarcity of *in-vivo* selection of *pfK13* mutations data in Africa.
Methodology: This survey evaluates the association between mutations in *PfK13* and the response to treatment with artemether-lumefantrine (AL) and artesunate-amodiaquine (ASAQ) at Gaya, Niger. Mutations in *Pf*K13 before and after treatment were analyzed and used as evidence for the selection of drug resistance following drug pressure.
Results: A total of 161 DNA from patients included in a therapeutic efficacy survey comparing AL vs

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ASAQ at Gaya sentinel site in 2011 were amplified and sequenced. Five SNPs were identified including 3 non-synonymous (NS) mutations (R528K, A569G and V637I) and 2 synonymous (SY) mutations (C469C and Q613Q). Four SNPs were observed prior to artemisinin-based Combination Therapy (ACT) including 2 NS (R528K and V637I) and 2 SY (C469C and Q613Q) mutations. One NS mutation was selected by ASAQ (*PfK13A569G*) whereas AL treatment did not select any mutation.

Conclusion: This study suggests that the mutation *pfk13A569G* is selected by ASAQ. Further mutagenesis studies (CRISPR/Cas9 or Z-Finger Nuclease) will be needed to confirm if *pfk13A569G* confers resistance to artemisinin.

Keywords: Malaria; Artesunate-amodiaquine; Artemether-lumefantrine; pfK13-propeller; PfK13A369G; Niger.

1. INTRODUCTION

A molecular marker associated with *P.falciparum* resistance to Artemisinin-based Combination Therapy (ACT) and delayed parasite clearance was previously identified in the Kelch domain of PF3D7_1343700 or K13 Propeller. The *PfK13* gene was described in 2013. It is located on chromosome 13 and codes for a protein with a six-bladed structure. The latter would act in cyto-protective regulation and adaptation to oxidative stress [1].

A recent survey on the polymorphism of *Pfk13* gene revealed more than 100 NS point mutations [2]. A similar survey in Sub-Saharan Africa revealed 22 mutations including 7 NS mutations: A557S, V566I, A569T, S576L, A578S, L589I, Y630F (3,4). In Niger, 13 mutations were described (M472I; Y558C; K563R; P570L; A578S; P615S; I465I; C469C; R471R; L488L; G496G; V510V and Y630Y), of which 8 were specific of Niger. 5 of them were NS (M472I; Y558C; K563R; P570L; P615S) and 3 were SY (L488L; V510V and Y630Y) [3].

However, the question remains as to whether these mutations might be the result of the drug pressure or the expression of a natural polymorphism [4]. Few studies have documented *pfK13* mutations selected in therapeutic efficacy studies in Africa. The role of *pfK13* mutations in reducing the effectiveness of treatments remains controversial [5].

It is known that 4 mutations are strongly associated with both a delayed parasite clearance and a decrease *in vitro* or *ex vivo* susceptibility after culture adaptation by the Ring Stage Survival Assay (Survival rate> 1%), which are: Y493H, R539T, I543T and C580Y [6]. Moreover, more recent work has demonstrated that R539T and I543T mutations are associated with a very high level of resistance to artemisinin and C580Y mutation is associated with very variable resistance levels [7]. Nine other mutations are poorly associated with resistance to artemisinin, including P441L, F446I, G449A, N458Y, P553L, R561H, V568G, P574L and A675V [2].

Here, we analyzed the polymorphism of *PfK13* in *Plasmodium falciparum* strains from patients before and after therapeutic failure with AL or ASAQ in Gaya, Niger. The mutations before and after treatment were analyzed as evidence of natural or drug pressure selection of resistance gene.

2. METHODS

2.1 Study Type

This survey aims to establish a relationship between the response to treatment and nucleotide sequence of *pfK13* in 161 strains of *P.falciparum* from a therapeutic efficacy investigation carried out in 2011 in Gaya, Niger [8], which compared artemether-lumefantrine to artesunate-amodiaquine in a 28-day long follow-up.

2.2 Study Population

These samples were collected from 161 patients including 146 at day 0 and 15 samples at failure day at Gaya-Niger. Gaya is the sentinel site of the National Malaria Control Program (NMCP) located 144 km from Niamey. Malaria transmission is endemic and lasts about 6 months yearly. The main vector of malaria is *Anopheles gambiae* and the predominant *Plasmodium* species is *P. falciparum.* Exceptionally, *Plasmodium malariae* and *Plasmodium ovale* can be found [8].

2.3 Genotyping and Sequencing

The genotyping method is based on PCR and sequencing of a 849 bp region on chromosome 13 of kelch domain PF3D7_134370 [2] and is described in KARMA project-option 1 (K13 Artemisinin Resistance Multicenter Assessment) [2].

Briefly, DNA extraction was done by Qiagen® kit (Reference: QIAamp® DNA Blood Mini Kit (250) Cat. No. 51106. Lot No. 148020944). Genotyping was performed by nested PCR. The primary PCR was made in 25 µl comprising 13.75 µl of water, 0.625 µl of primer K13-PCR_F (5'-CGGAGTGACCAAATC TGGGA-3'), 0.625 µl of primer K13-PCR_R (5'-GGGAATCTGGTGGTAACAGC-3 '), 5 µl of Master Mix and 5 µl of DNA.

The secondary PCR (849 bp) was made in 50 µl comprising 32.5 µl of pure water, 1.25 µl of primer K13_N1_F (5'-GCCAAGCTGCCATTCATTTG-3'), 1.25µL of primer K13_N1_R (5'-GCCTTGTTGAAAGAAGCAGA-), 10 µl of master mix and 5 µl of DNA.

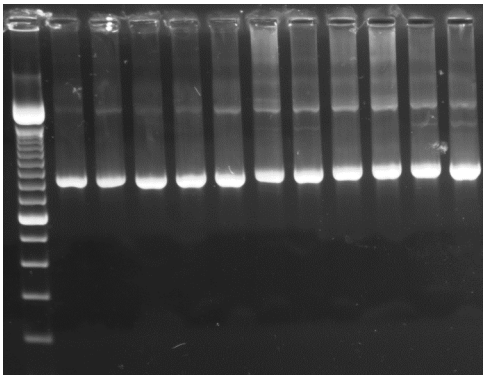

Fig. 1. Agarose gel of the nested PCR of *pfk13*

Fig. 1 present nested PCR products migrated on 2% agarose gel

All PCR products were mixed with 2 µl of blue and migrated on 2% agarose gel containing 1 µl of ethidium bromide. The generator was set at 150 volts for 1 h 30 min. A size marker (100 Pair Base Ladder, Invitrogen®) was used as a scale. The gel was visualized under an UV-trans-luminator of BIORAD® mark and a picture was taken for the archives Fig. 2. All samples were sealed in a 96-well plate and sent to sequencing. The sequencing was performed by Macrogen in Korea (http://www.macrogen.com/eng/).

Importantly, to distinguish between reinfestation and recrudescence, *msp1* and *msp2* genes were also analyzed according to Konaté, et al. [9].

2.4 Ethics Committee Approval

The samples from this genotyping study come from CERMES biobank (Genomic library). It does not require an approval of the ethics committee. However, the therapeutic efficacy study was approved by the National Consultative Ethics Committee of the Ministry of Public Health of Niger.

3. RESULTS

3.1 Population Characteristics

A total of 146 patients were included in that survey and each provided DNA extracted from a blood spot. 72 and 74 patients were treated with AL and ASAQ respectively. All patients came from the districts of Gaya. The average age of the patients was 58.20 months (Ϭ = 35.8, [3, 158]), with 52.5 months (Ϭ = 37, [3, 168]) for AL arm and 63.7 months (Ϭ = 168]) for ASAQ arm. The two arms of the survey were comparable. The sex ration was 1.17. The average weight of the patients was 16.27 kg (Ϭ = 6.74, [6.1, 44.3]). The mean of parasite density was 58.212 P/µl (Ϭ = 67052, [1520, 200000]) (Table 1).

3.2 Classification of the Samples according to the Treatment Response

A total of 146 patients was treated on day 0, when their DNA was collected. Of the 146 samples, 111 were from patients with Adequate Clinical and Parasitological Response (ACPR), which included 49 patients treated with AL and 62 treated with ASAQ. A total of 15 samples were from patients with therapeutic failures, one of whom one had an Early Therapeutic Failure (ETF), two had a Late Clinical Failure (LCF) and 12 had a Late Parasitological Failure (LPF) including nine to AL and three to ASAQ. Twenty samples were from patients treated but excluded from the study for various reasons (re-infestation, involuntary withdrawal, protocol violation etc.).

3.3 Genotyping of *pfK13*, *msp1* and *msp2*

161 samples were genotyped, 146 from patients before treatment and 15 from patients with

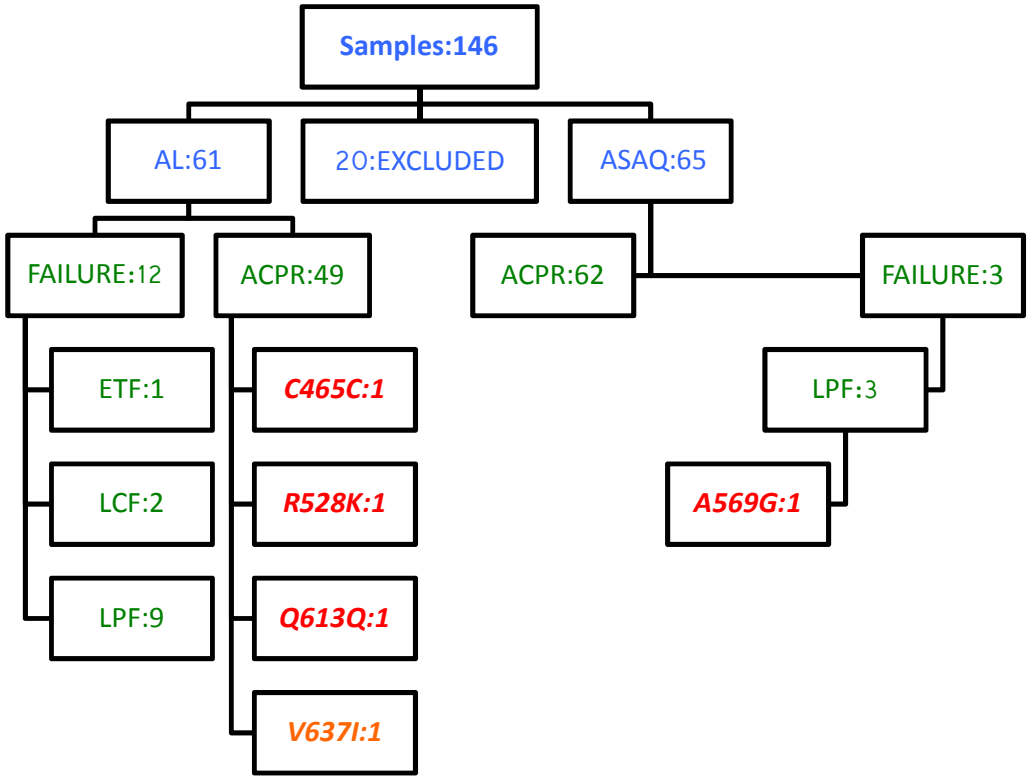

Fig. 2. flow diagram of the samples analyzed and the mutations observed

Blue: Samples, Red: Mutations, Green: Responses to treatment
ETF: Early Therapeutic Failure, LCF: Late Clinical Failure, LPF: Late Parasitological Failure, ACPR: Adequate Clinical and Parasitological Response

Table 1. Characteristics of the population

	AL arm	ASAQ arm	Total or mean
Number of patients	72	74	146
Age mean (months)	52,5	63,7	58,2
Weight mean (Kg)	13,25	17,11	16, 27
Parasite density (P/µL)	59928	56541	58212
Sex Ratio	1.4	1	1,17

AL: Artemether-lumefantrine
ASAQ: Artesunate-amodiaquine

therapeutic failure. All samples were amplified. The efficiency of the amplifications was 100%. Only eight out of 161 samples were not successfully sequenced.

Five SNPs were observed (C469C, Q613Q, R528K, A569G, and V637I), of which three were NS (R528K, A569G and V637I) and two were SY (C469C and R528K). The prevalence of all these mutations remained low (0.006%) Fig. 2.

The analyses of *msp1* and *msp2* in the sample harboring *pfK13A569G* mutation showed respectively 210 pb at day 0 and 210 bp at day 28 for *mps1*. For *msp2* gene, 500 pb at day 0 and 500 bp at day 28. Fig. 3.

3.4 Relationship between Mutation and Treatment

Four mutations were observed before treatment, including two SY (C469C, Q613Q) and two NS (R528K and V637I). *pfk13A569G* mutation was recovered after ASAQ treatment and the patient with this mutation had a late parasitological failure. AL treatment did not select any mutation (Table 2).

Table 2. Main results of the study

Codon	Nucleocid	Reference AA	Mutant AA	n/N	Type	Drug	Response	Treatment
469	TGC->TGT	C	C	1/161	SY	AL	RCPA	Before
528	AGA->AAA	R	K	1/161	NS	AL	RCPA	Before
569	GCA->GGA	A	G	1/161	NS	ASAQ	EPT	After
613	CAA->CAG	Q	Q	1/161	SY	AL	RCPA	Before
637	CAA->CAG	V	I	1/161	NS	AL	RCPA	Before

AA : amino acide, n : number of samples containing mutant allele; N : Total number of samples; NS : non synonymous mutation; SY : synonymous mutation; AL : Artemether-Lumefantrine; ASAQ : Artesunate – Amodiaquine

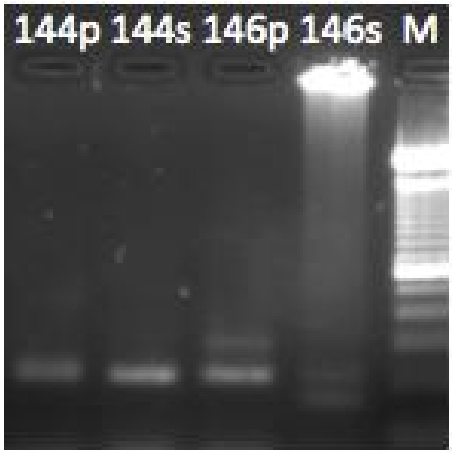

Fig. 3. Agarose gel of *msp1* PCR in the sample harboring *pfK13A560G* mutation (N°144)

*Fig. 3 present the sample harboring pfK13A560G mutation with **210** pb at day 0 (144p) and **210 bp** at day 28 (144sà for mps1)*

4. DISCUSSION

The survey aimed to investigate a possible link between the mutations in *pfK13* and the response to treatment with AL and ASAQ and to know whether the polymorphism of this gene was natural or the consequence of drug pressure.

A single non-synonymous mutation was obtained, *pfK13A569G*, which was selected by treatment with artesunate amodiaquine. Patient harboring the parasite with this mutation experienced a late parasitological failure (LPF), i.e., 1200 P/µL at day 28, and was treated with artesunate amodiaquine. Mutation selected by the treatment referred to the same, original isolate, as shown by the genotyping of *msp1* and *msp2*. These data strongly suggested that the mutation was selected in the host under drug pressure. Treatment with artemether lumefantrine did not select any mutation.

Four mutations were observed prior to treatment with ACTs including two non-synonymous (R528K and V637I) and two synonymous (C469C and Q613Q). These four mutations were observed before treatment in patients who had adequate clinical and parasitological response (ACPR). This clearly indicates a natural polymorphism of *PfK13* independent of drug pressure.

Various surveys have studied the variability of this marker. KARMA survey showed an important polymorphism of this gene [2]. Indeed, 108 non-synonymous mutations have been observed. Nine had prevalence greater than 1%. In Southeast Asia, the most strongly mutations associated with artemisinin resistance are Y493H, R539T, I543T and C580Y, C580Y being the most predominant [2,6]. In Sub-Saharan Africa, 22 major mutations have been described, including seven non-synonymous (A557S, V566I, A569T, S576L, A578S, L589I, Y630F) [10,11]. A578S mutation is the most common in African countries [1,12]. In Niger, a previous survey found 13 SNPs, including six non synonymous mutations and seven synonymous [13]. Of these 13 SNPs observed, eight were specific to Niger, including five non-synonymous: M472I; Y558C; K563R; P570L and P615S. A mutation in codon 569, as reported in our study, has already been described in Kenya [14]; however, Kenyan mutation changed the alanine residue into a threonine (T) rather than a glycine (G). In Senegal, the main non-synonymous mutations observed in 2012 were T149S and K189T, while in 2014 the mutations N554H, Q613H and V637I were observed [15]. In Mali, 26 mutations were found, the most frequent being F446I [16]. On the other hand, In Burkina Faso, no mutated genotypes of *pfk13* was reported. Indeed, 2.26% of the alleles were mutated but all mutations were synonymous: C469C, Y493Y, G496G, and V589V [17].

Now that the primary structure of the beta (β) chain of *pfk13* gene product is better described, we are interested in the effect of mutations on resistance to artemisinin *in vivo* and *in vitro*. Two main mutations, C580Y and E252Q, have been observed in patients at the border of Thailand-Myanmar, the epicenter of resistance to artemisinin, who were treated with ACT and suffered a recrudescence. The proportion of infections caused by isolates with *pfk13* polymorphism increased from 6.7% in 2003 to 83.9% in 2013 [5]. In a similar survey in Myanmar, F446I mutation was the most frequent (66%) with patients on day 0. P574L mutation was observed in patients with early therapeutic failure [18]. In South-East Asia, mutations that are strongly related to artemisinin resistance *in vitro* and *in vivo* are: Y493H, R539T, I543T and C580Y [2] and the latter was shown by mutagenesis to confer a variable level of resistance [7]. In West Africa, specifically in Togo, non-synonymous mutations of *pfk13* gene have been reported in patients treated with ACTs, which are S522M, A578S and C532S. However, patients harboring these mutations cleared their parasites before three days [19]. In Central Africa, two mutations were selected by treatment, T522G and G509A [20]. In Senegal no mutations were observed in parasites obtained three days after treatment with artesunate [21]. In Uganda too, there is no *in vivo* mutation selection of *pfk13* [22]. Mutations from Asian parasites were not found in our samples, although both types of mutations are located in the same area of the gene, which lies between codon 493 and codon 580. The question arises as to whether it is more beneficial to analyze HTGY haplotype relating to codons 493, 539,569 and 580 than to seek a single nucleotid polymorphism?

The number of mutations observed in *Plasmodium falciparum* strains before treatment is greater than those observed after treatment, which fits the hypothesis of a natural polymorphism independent of selective pressure. Indeed, studies have shown that mutations were present in strains several years before implementation of ACT [719,23]. This polymorphism is also observed in strains derived from asymptomatic carriers [24] and symptomatic carriers [7,19] The lack of polymorphism of *pfk13*, ten years after implementation of ACTs in Benin [25], Kenya [14], Yemen [26], China [27] and the complete absence of mutation in Angola also supports this hypothesis [28]. It should be noted that *pfK13A569K* mutation is observed in the samples of a 2011 study, two years before the discovery of *pfk13* and five years after the establishment of ACTs in Niger.

The results show that *pfK13* has a natural polymorphism and is subjected to selection pressure. In fact, five point mutations were detected in all the samples; three being non-synonymous and two synonymous.

The parasite that was resistant harbor a mutated genome was parasites from late parasitological failures because they resisted and survived to treatment. These parasites are difficult to collect because they come from a selection and are in very small quantity. Indeed, there is $1/10^9$ mutant in a natural population, hence the importance of our observation.

The sequenced samples were obtained from a therapeutic efficacy study using WHO/2003 protocol with a 28-day follow-up [8]. The best methodological approach for evaluating artemisinin efficacy is the method of parasitic clearance. This method consists of administering ACT to patients and a biological follow up every six hours until thick and thin smears are negative [29]. This method can be coupled with the Ring Stage Survival Assay (RSA) [29]. The main limitation of our study is that we did not use the parasite clearance approach. The adjacent microsatellites of *pfK13* or the barcode technique could also be used to distinguish reinfection to recrudescence.

Nonetheless, our results strongly favour the hypothesis of an intra-host selection of resistant parasites, which is described for the first time in Niger and commands identification of the key mutation selected by the treatment with artemisinin. Clearly, the use of parasite clearance method coupled with mutagenesis like CRISPR/Cas 9 or Z-Finger Nuclease would allow to further document the mutation(s) involved in this resistance [30].

5. CONCLUSION

This study suggests that the mutation *pfk13A569G* is selected by ASAQ. Mutagenesis studies using genomic editing with CRISPR /Cas9 or Z-Finger Nuclease will be required to confirm the role and the function of this mutation conclusively. If this hypothesis is confirmed, this mutation could be used as a marker to monitor artemisinin resistance.

DISCLAIMER

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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