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RESEARCH ARTICLE

GENETIC ARCHITECTURE AND DYNAMICS OF PFKELCH13'S PROPELLER DOMAIN IN SENEGALESE *PLASMODIUM FALCIPARUM*CLINICAL ISOLATES

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Abstract

Plasmodium resistance to Artemisinin Combination-based Therapies (ACT) in Southeast Asia is a major public health concern that is sporadically appearing in Africa. Senegal has shifted from malaria control to elimination plans. Given notable progresses obtained through robust strategic plans,it is still crucial to assess genetic variability of the *Plasmodium falciparum*artemisinin resistance gene marker Kelch13 (*PfKelch13)*in circulating field isolates.We herereportan analysis of PfKelch13-propeller polymorphism in clinical isolates collected nine years after ACT introduction in five Senegalese regions with different malaria transmission settings. Sequencing of *PfKelch13*-propeller domainfrom 280 clinicalisolates reveals that 16% (45/280) of the parasite population harboredvariants. Dynamics of *PfKelch13* variants reveals emerging, persistent but also disappearing mutations over time. In addition to the malaria epidemiology, our survey also shows the dynamics of *PfKelch13* variants in different malaria transmission settings in Senegal. Despite the absence of *PfKelch13*associatedartemisinin resistance mutations, a shift from 86% to 68% of PfKelch13^{WT}was observed when comparing parasites collected prior vs. post ACT intensive usage in Dakar a low malaria transmission area. All together,our data confirms the need to closely monitor PfKelch13 polymorphism to anticipate and or preventemergence of*P. falciparum* resistancein Senegal.

Introduction:-

As in many other African countries, the state of Senegal has made tremendous efforts over the last two decades to revert the malaria burden throughout robust control strategies. Among these strategies, the intensive use of Rapid Diagnostic Tests (RDTs) as a point-of-care diagnostic method, the large-scale distribution of bednets, as well as the

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progressive implementation throughout the different malaria facies of vector control programs have fully contributed to the notable progress recorded against malaria within Senegal. As a result, the country has shifted from malaria control to elimination plans in Northern and central regions. To the counter growing threat of chloroquine resistant parasites, the WHO recommended artemisinin combination-based therapies (ACTs) have been progressively introduced as frontline treatment regimens in late 2000s in malaria endemic countries[1]. In Senegal Artesunate-Amodiaquine (ASAQ) and Artemether-Lumefantrine (AL) are the first two drug combinations introduced in 2006 as frontline treatment for uncomplicated malaria. In addition to these dual therapies, a third ACT drug regimen comprising theDihydroartemisinin (DHA): themetabolite of all artemisinin derivatives and Piperaquine (PQ), has been slowly distributed to prevent emergence and/or spread of artemisinin resistant *Plasmodium falciparum* isolates. However artemisinin resistance (AR) has emergedin Southeast Asia (SEA)[2] since 2006therefore becoming a global health concern. AR is defined as a reduced *P. falciparum* susceptibility towards Artemisinin derivatives manifesting with a clinical delay in parasite clearance upon malaria treatment[3].

There is now evidence that AR is due toemerging single point mutations in *P. falciparum kelch 13*gene[4]. Mutations, in the parasite gene resistant marker (PfKelch13, PF3D7_1343700), located in its propeller domain (C-terminal domain of the protein) were indeed shown to be clinically associated with a delay in parasite clearance in the first 3 days of treatmentfollowing artemisinin monotherapy or ACT. Next the elevated survival rate based on the *in vitr*o ring survival assay (RSA)[5] was developed and subsequently used as a powerful tool to measure AR*in vitro*. In conjunction with PfKelch13's propeller mutations, both clinical parasite clearance delay and high RSA level are commonly used to define parasite resistance phenotypes in the field and in the laboratories respectively[6].

PfKelch13^{C580Y} and PfKelch13^{F446I} are the most common variants in the propeller domain of the parasite gene marker known to be associated with residual and viable parasites upon ACT treatments in SEA[7]. These mutations were shown in addition to PfKelch13^{R539T}to confer increased*in vitro* survival rate expressed by the RSA in both clinical and laboratory engineered strains, while parasites with PfKelch13^{WT} showed a sensitivity to DHA[7]. AR spreads in China Myanmar border while a decreased in vitro artemisinin sensitivity of *P. falciparum*isolates across India was reported[8].Next, isolated cases of treatment failure have been reported in other regions of the world in conjunction or independently from PfKelch13[9]. All together, these findings make tracking of AR not only an urgent public health problem that needs a robust tracking system, but also a concern that does require scientific tools to provide insights on the biological relevance of each mutation. In parallel, both impact of different parasite background and the identification of other markers that could play a critical role inthe artemisinin resistance mechanism will be critical to delay spread of AR in malaria endemic regions.

In sub-Saharan Africa, drug clinical efficacy studies reveals that ACTs are still efficacious against *P. falciparum* parasites. Indeed, PfKelch13 genetic diversityof thousands African *P. falciparum* isolates has shown a very low frequency of SEA mutations in circulating parasites[10–12]. Nevertheless, sporadic cases of either clinical treatment failure upon treatment or increased in *vitro* RSA levels have been documented in African background parasites[13–15]. The emergence of indigenous mutations, associated with ACT treatment failure cases in Equatorial Guinea was observed in 2017[13]. The same year four Ugandan-importedartemether-lumefantrine treatment failure cases were reported to be independent to PfKelch13 mutations in the United Kingdom[14]. Next,PfKelch13^{R622I}was identified in an Ethiopian patient with a persistantparasitemia at day 3 upon ACT treatment. However, it is important to note that all patients included in the study showed a parasite clearance at day 28 post treatment indicating a lack of correlation between PfKelch13^{R622I} mutation and resulting resistance phenotype[16]. More recently a clear early warning signs of AR in Rwanda was reported with a Pfkelch13^{R561H} mutation identifiedin 19 of 257 (7.4%) included patients[17]

All aforementioned African studies calls for an urgent need to routinely map out the genetic diversity of the AR gene marker and to establish efficient surveillance system to closely monitor parasite clearance half-life within Sub-Saharan Africa accounting for more than 90% of total malaria death[18].

A total of 280 Senegalese *P. falciparum* clinical isolates were collected between 2014 and 2015 (8-9 years after ACT introduction) from five regions with variable malaria transmission intensity. PfKelch13 genetic diversity was assessed, using Sanger sequencing,to understand the dynamics of PfKelch13 mutations in Senegal.Seventy four (74) samples were also collected before ACT introduction in the urban-Senegalese capital Dakar, a region that has shifted to malaria elimination plans, and PfKelch13 mutationscompared to post ACT samples.Our study provides a map of PfKelch13 distribution in circulating Senegalese *P. falciparum* clinical isolates from low to high malaria

transmission areas. In addition our epidemiology data on the parasitic disease shows an increase of severe malaria cases that could result from the low infection rates and loss of immunity. The prior vs. post ACTPfkelch13 mutations comparison shows a useful baseline for emerging PfKelch13polymorphism in parasites under 8 and 9 years ACT drug pressure in Senegal.

Results:-

Demographic characteristic of the study population, malaria prevalence and mortality

The data of our study were generated from 280 malaria-infected patients, recruited from five regions of variable malaria transmission intensity. From low to high transmission setting hospitals from Louga, Saint-Louis (N = 14), Dakar (N = 25), Kolda (N = 117)and Tambacounda (N = 124) were included respectively. In both the pre-elimination regions Saint-Louis and Louga (referred as Louga in our study), the low number of malaria infections (14) confirms the lowest malaria prevalence. All 280 blood samples were collected between 2014 and 2015 i.e. 8 to 9 years following ACT introduction in Senegal. All included patients were tested and confirmed to be Pf-RDT positive on collection/visit day. Both demographic and clinical characteristics of the study participants are summarized in **Table 1**. The median sex ratiowas 1.52 across the five sampling sites (Chi-square, $p = 0.118$). The age of participants ranged between 0 to 85 years with a median of 17 years. There was a significant difference in the mean age across sites (ANOVA, $p = 0.03$) with the youngest participants from Tambacounda (mean age 8 years) and the eldest in Kolda(mean age, 18 years). This finding is indicating that in the highly malaria endemic sites there is in addition to the vulnerable 0 to 5 years old populations, a susceptibility to malaria infections in the 10 to 18 years old group. With respect to the disease outcome at inclusion level, the percentage of severe malaria cases (SM) was higher in Dakar (96%), followed by Kolda (75.2%) and Louga (64.2%), while this outcome was lower in Tambacounda (53.2%). Overall, seven participants died of malaria (2.5%) during the study period and, Dakar, once again accounted for the highest number of deaths recorded across the study sites (16%), followed by Kolda and Tambacounda (1.7 and 0.81%, respectively (**Table 1**). These results indicate,between 2014 and 2015,an increasing severity of malaria infection that is probably associated with the loss of immunity in malaria and decreased infection rates in low malaria endemic areas and higher transmission rate respectively. As the results our data shows that despite notable progress that Senegalese populations remain at risk for severe malaria infections.

Table 1:- Demographic characteristics of the study population between 2014 and 2015 for PfKelch13 mapping in Senegal.

		Louga	Dakar	Tambacounda	Kolda	Total
Number of subjects		**14**	**25**	**124**	**117**	**280**
Median Age (years old) (min-max)*		**10 (4-51)**	**11,5 (1-61)**	**8 (0-84)**	**18 (0-85)**	**17 (0-85)**
Sex Group	**Male (M)**	**10**	**17**	**55**	**60**	**142**
	Female (F)	**4**	**8**	**62**	**57**	**131**
	ND	**0**	**0**	**7**	**0**	**7**
	Ratio (M/F)	**2.5**	**2.1**	**0.9***	**1**	**1.1***
Clinicaloutcomes	**UM (%)**	**5 (35.7)**	**1 (4)**	**58 (46.8)**	**29 (24.8)**	**93 (33.2)**
	SM (%)	**9 (64.3)**	**24 (96)**	**66 (53.2)**	**88 (75.2)**	**187 (66.8)**
Issue	**Survival (%)**	**14 (100)**	**21 (84)**	**116 (93.5)**	**115 (98.3)**	**266 (95)**
	Died (%)	**0**	**4 (6)**	**1 (0.8)**	**2 (1,7)**	**7 (2.5)**
	ND (%)	**0**	**0**	**7 (5.7)**	**0**	**7 (2.5)**

*ND corresponds to missing information.

Geographical distribution of PfKelch13mutations 8 and 9 years following ACT introduction

PfKelch13 propeller domain was successfully genotyped for all the 280-collected*P. falciparum* fieldisolates. Fifteen variable mutations were detected, with 16% of the isolates (45/280) harboring at least one mutationwhen compared to the reference Pf3D7-PfKelch13 sequence from plasmdoDB (PF3D7_1343700). Seven of these mutationswere synonymous, while the remaining eight were non-synonymous. Of all non-synonymous mutations reported here, the N689Y mutation was the most frequent variant (48.33%), followed by C447Y (18.33%) and V666I, while D252N, A282T, L678F and N689C were present at similar proportions (1.67%) (**Fig. 1-a**). PfKelch13^{A578S} the frequent African background mutation known not to confer AR[10]was detected at a relatively low frequency (3.33%). The respective positions of these individual mutations within PfKelch13propeller domain are depicted in **Fig. 1-b**. None

of PfKelch13mutations previously associated with delay in parasite or increased level of *in vitro* survival rate (RSA)was found in these 280 post ACT (2014 and 2015) samples. The geographical map indicates a high prevalence ofPfKelch13^{WT} isolates across the study sites between 2014 and 2015 (**Fig. 1-c**). Across all sites, Louga and Dakar had the least diverse parasites populations with respectively one and three PfKelch13 variants detected, while Kolda and Tambacounda presented the most highly diverse parasite populations with respectively seven and eleven PfKelch13 variants reported. Eleven samples were found to harbor multiple PfKelch13 variants, of these, five samples hadthe combination N689Y/C447Y, two had V666I/G690G and onehad N689Y/V510V, respectively, while one and two samples harbored the following variant combinations, D452N/A582T/N689Y/C447Y and G690G/N689Y/C447Y, respectively. Further analysis revealed no association betweenPfKelch13 variants and disease severity or deaths, as only 0.14% (4/280) of the enrolled patients infected with mutant PfKelch13 isolates died of malaria**.** Among all sites the area of low transmission and low malaria immunity city and capital Dakar had a higher rate of PfKelch13 polymorphism (24%) (**Fig. 1-d**) although none of documented PfKelch13 and Artemisinin resistance associated mutations was detectable.

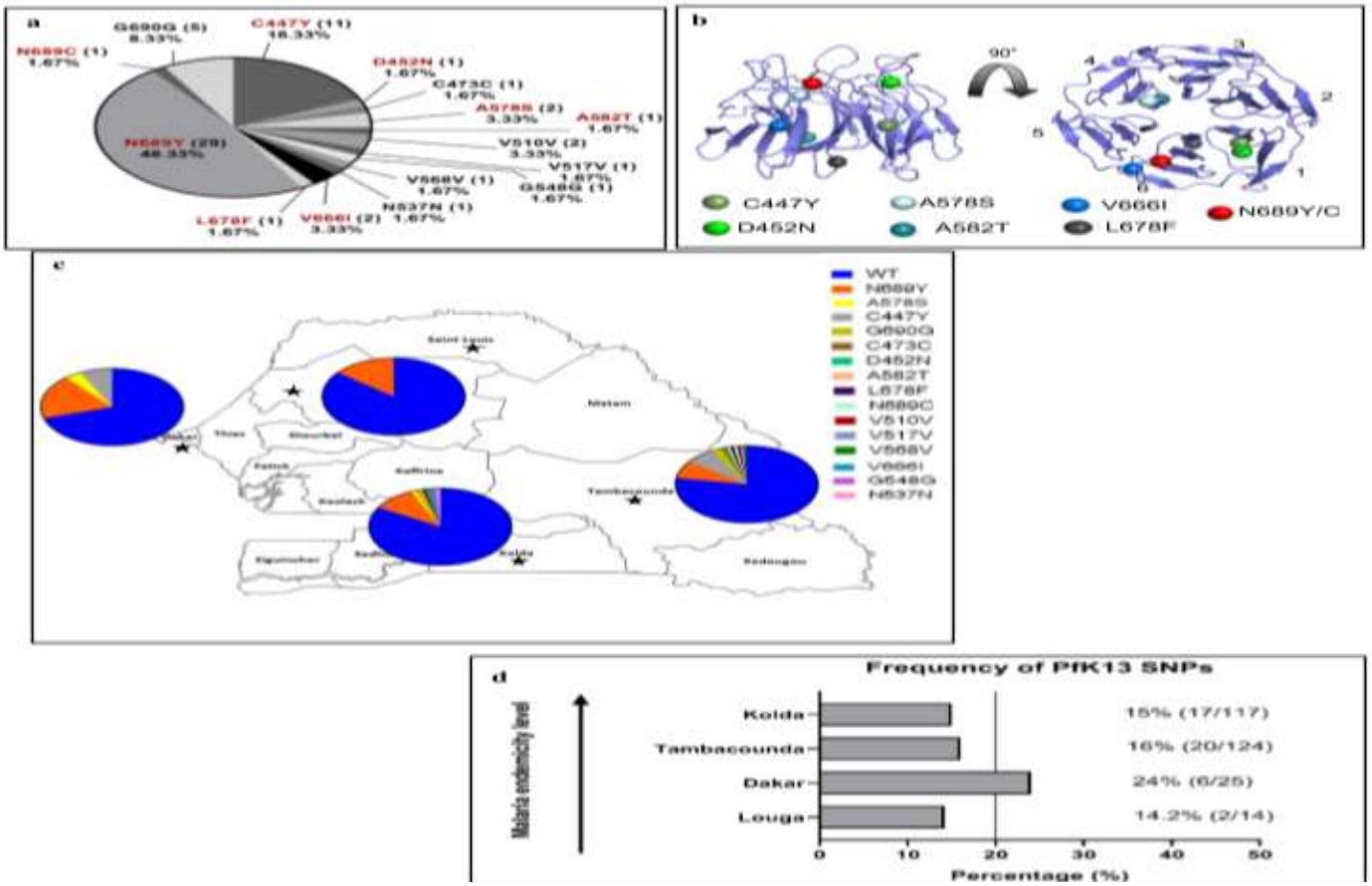

Fig 1:- Geographical distribution of PfKelch13mutations between 2014 and 2015.

a.Variants found in 280 clinical isolates are represented. Synonymous mutations are written in black and nonsynonymous mutations are in red. ***b.*** Location of the 7 Ns-SNPs on the propeller domain are shown using PfKelch13 structure pdb code: 4ZGC. ***c.*** Mapping of PfKelch13 SNPs in our study sites reveals a high frequency of mutations in highly endemic regions (Kolda and Tambacounda). PfKelch13^{WT} (blue) remains highly frequent in all sites indicating efficacy of ACTs in collected parasite lines. ***d.*** Frequency of all SNPs per region is shown. Dakar the capital and low malaria endemic city shows a higher frequency of SNPs with 24%.

Dynamics of PfKelch13 polymorphism in Dakar across time (Prior vs. Post ACT introduction)

The prevalence of *PfKelch13*mutations was also assessed across time using samples collected from Dakar. Here we sought to understand the profile of emerging mutations in the parasites by comparing clinical isolates collected between 2004 and 2005 i.e two and one year prior to the introduction of ACTs as frontline treatment regimens in

Senegal and the previously analyzed samples from Dakar (collected 8 to 9 years post ACT introductions). The pre-ACT samples was made of 74 samples obtained from patients with a median age of 27 years (range 2-74 years) (**Table 2**). Of these samples, 36 were collected from patients with UM, while the remaining 38 patients suffered from SM. Our analysis revealed a significantly lower prevalence of SM cases in the pre-ACT (51.3%) samples as compared to the post-ACT samples (96%). Further analysis of the patients' clinical issue revealed that 20% (15/74) died from the malaria onset (Table 1). This further emphasizes the sharp decrease in malaria-associated mortality in the 2014-2015 cohort where only 2.5% mortality was recorded (**Tables 1 & 2**).

Table 2:- Demographic characteristics of the study population priorly to ACT introduction in Dakar (between 2004 & 2005).

Pre ACT Samples (Dakar)		
Number of subjects		**74**
Median Age (years old) (min-max)*		**27 (2-74)**
Sex Group	**Male (M)**	**40**
	Female (F)	**27**
	ND	**7**
	Ratio (M/F)	**1,5**
Clinicaloutcomes	**UM (%)**	**36 (48.6)**
	SM (%)	**38 (51.4)**
Issue	**Survival(%)**	**59 (80)**
	Died(%)	**15 (20)**
	ND	**0**

*ND corresponds to missing information.

PfKelch13 genotyping revealed five mutations from the pre-ACT samples. These comprised two synonymous (A504A and G638G) and three non-synonymous mutations (A578S, G639C and N689Y). Of these, only two (A578S and N689Y) have persisted in the parasites population over the covered period of study. Indeed both variants were found in our post-ACT samples, while the remaining three mutations (A504A; G638G and G639C) disappeared along with ACT drug pressure (**Table 3**). Interestingly our retrospective reveals emergence of the C447Y under ACTs drug pressure (0 to 8%). This variant replacement of a cysteine to a tyrosine is similar to the most frequent C580Y mutation known to be associated with delay in parasite clearance and *in vitro* high survival rate. Moreover, it is important to mention that the prevalence of the $K13^{WT}$ variant shifted from 86 to 68% in Dakar (**Table 3**).

Table 3:- Evolution of PfKelch13mutations.

	Pre ACT (n=74)		**Post ACT (n=25)**	
SNPs	Presence	Frequency (%)	Presence	Frequency (%)
A578S	+	1.35	+	4
N689Y	+	9.45	+	20
C447Y	**-**	**0**	**+**	**8**
A504A	+	1.35	-	0
G638G	+	1.35	-	0
G639C	+	1.35	-	0
WT	+	86	+	68

Discussion:-

The growing threat associated to AR calls for an urgent need to monitor its potential spread or emergence in Africa, where over 90% of the global malaria cases occur yearly[18]. Resistance to artemisinin has first been reported from SEA[2] where resistance to most antimalarials has been always firstly described before its spread to other malaria endemic regions. Although ARassociated mutations are yet to be declared in Africa, there are increasing studies describing appearance of either SEA associated mutations in African settings or emerging mutations causing worrying delay in parasite clearance upon ACT treatments[13–15,17].In Africa, tremendous efforts have been done to reduce malaria burden. We are currently facing new challenges that if not controlled might postpone the malaria

elimination plans adopted by few countries including Senegal. The possibility that AR could emerge independently in Africa makes its surveillance crucial. Our study provides a geographical mapping of K13 variants in four regions with variable malaria transmission settings. With fifteen mutations inPfKelch13-propeller domain of 280 clinical isolates collected 8 to 9 after ACT introduction we are adding valuable information for the antimalarial tracking strategies of the Senegalese National malaria control program. None of the SEA AR or newly PfKelch13 variant found in Africa and reported to be causing delayed parasite clearance upon ACT treatment was detected in our samples. This is in phase with previous reports indicating the absence of SEA-associated mutations in Senegal[19–24].Both the malaria epidemiology and physiopathology of the tropical disease reveal an increasing proportion of severe malaria that results from a loss of malaria immunity in low malaria transmission settings. Indeed, the highest rate of PfKelch13 polymorphism was found in Dakar which is an area of low transmission and low malaria immunity city[25]. Although none of documented PfKelch13and AR associated mutations was detectable, this finding is in phase with previous observations corroborating historical observations of antimalarial resistance in low malaria transmission areas[26].The frequency of severe malaria cases in the 2014-2015 cohort is confirming a loss of malaria immunity as seen in Dakar and Louga. It is important to note that we found emerging PfKelch13 in other regions with high incidence. Overall these results illustrate the plasticity of the malaria genome of parasite circulating in Senegal. This phenomenon is confirmed in the parasites collected priory to ACT introduction in Senegal. Comparison of PfKelch13 mutations pre vs. post ACT indicates the impact of ACT drug pressure on PfKelch13 genetic diversity. Contrarily to PfKelch13^{A578S}, ^{N689Y} and ^{C447Y} the variantsPfKelch13^{A504A,} ^{G638G} and ^{G639C} did not persist over time. Among all these mutationsPfKelch13^{C447Y} is the only variant that has clearly emerged under ACT drug pressure. Overall our study bringsinsights on the dynamics of PfKelch13 mutations in parasite circulating in Senegal. So far no clinical delay in parasite clearance was reported in Senegal[23]. This is confirming that ACTs remain efficacious in Senegal. The fact that none of the SEA-artemisinin resistance-associated mutations were found among clinical isolates under 10 years of drug pressure collected samples indicates that despite the isolated cases of AR in Africa and the usage of the three ACT drug regimen the Senegalese background parasites are still sensitive to the cornerstone of antimalarial drugs. However given both the low malaria transmission settings and the loss of immunity known to give rise to a more rapid expansion of artemisinin-resistant parasites we need to strengthen the surveillance system to anticipate antimalarial resistance in Senegal against the cornerstone of all antimalarial currently available on the market.

Methods:-

Sample collection and processing

Institutional Review Board (IRB) of Cheikh Anta Diop University on behalf of the National Research Ethics Committee approved the use of human samples for this study. Informed written consent was obtained from all participants or their legal guardians. Malaria positive patients were recruited from areas varying transmission intensities. Kolda and Tambacounda present the highest transmission intensity, while the two Northern regions Saint Louis and Louga (referred to hereafter as Louga), where malaria pre-elimination campaigns are being launched[27], were included as lowest malaria transmission intensity areas. The transmission intensity in Dakar lies somewhere between these two extremes. Sampling was undertaken in regional hospitals of each region. In Dakar the study was done in collaboration with the Principal Military Hospital (HPD) and Diamniadio Children Hospital (DCH). Samples from these two centers will subsequently be referred to as Dakar as they are both located within the same region the urban Senegalese capital. Venous blood samples were collected in EDTA tubes for each enrolled patient at admission day before treatment. Malaria diagnosis was performed by a rapid diagnostic test (SD BIOLINE Malaria Ag P.f) provided to all health services by the National Malaria Control program. All *P. falciparum* positive samples were further confirmed by microscopy.Severity of infection was defined by clinicians following WHO guidelines and eligibility criteria[28]. Uncomplicated malaria (UM) and severe malaria cases (SM) were reported for each malaria infected patient.

DNA extraction and PfKelch13 genotyping

*Plasmodium*genomicDNAwas extracted from blood of all confirmed malaria infected patients, using Qiagen Kit following manufacturer's instructions. Nested PfKelch13 PCR was carried out to amplify the propeller domain of the gene. First amplification was done using the primary primer set PfKelch13_PCR_F 5'-CGGAGTGACCAAATCTGGGA-3' and PfKelch13_PCR_R 5'-GGGAATCTGGTGGTAACAGC-3'. The resulting products were used as matrices for the PfKelch13nested amplification. The nested amplification was carried out for the propeller domain with PfKelch13_N1_F 5'-GCCAAGCTGCCATTCATTTG-3' and PfKelch13_N1_R 5'-GCCTTGTTGAAAGAAGCAGA-3' primers[4]. PCR reactions were prepared in a final volume of 25µl comprising 13.75µl of nuclease-free water, 0.625µl of each primer, 5µl of 5x HOT FirePol Master Mix and

5µl of gDNAfor each reaction. Thermocycling conditions were carried out with an initial denaturation at 95 °C for 15 minutes, followed by 30 cycles with 30 seconds denaturation at 95°C, 2 minutes annealing at 58°C, 2 minutes extension at 72°C; and a final 10 minutes extension at 72°C for the first PCR. The nested PCR was performed using the following settings, 15 minutes initial denaturation at 95°C followed by 40 cycles with 30 seconds denaturation at 95°C, 1 minute of 40 cycles annealing at 60°C, 1 minute of 40 cycles extension at 72°C and a final 10 minute extension at 72°C. PCR products were sequenced (Sanger) and analysed with the Genalys software version 2.0b[29].Sequencing reactions were performed according to the Dye terminator v3.1 method using an ABI PRISMs 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Mix sequencing contained 2µl PCR product, 0.5µl BigDye v3.1, 1.87µl buffer 5X, 0.5µl primers (10µm), up to 5.17µl with water. Conditions were set as follows, 96 °C for 5mn, 25 cycles of 96°C, 10 sec; 60°C for 4mn and 15°C forever. Sequencing products were purified with Sephadex G50 superfine (GE Healthcare). The resulting sequences were next analysed and the corresponding chromatograms closely checked for mutationdetections. All nucleotide blasts were done using NCBI blast and Plasmodb 3D7 (PlasmoDB 46 version released Nov 6th 2019) (www.plasmodb.org). One-way ANOVA was performed using GraphPad Prism version 8.0.0 (GraphPad Software, San Diego, California USA, www.graphpad.com).

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Author's contribution

A.D. conceived the study design. M.N.P., G.D. and A.M. wrote the manuscript. M.N.P. andF.T. performed the experiments.M.N.P. and A.M. made the data analysis. B.M. and A.T. were in charge of data management.

Competing interests

The authors declare no competing interests.

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