

Molecular assessment of *kelch13* non-synonymous mutations in *Plasmodium falciparum* isolates from Central African Republic (2017–2019)

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Abstract

Background: Over the last decade, Artemisinin-based Combination Therapies (ACT) have contributed substantially to the decrease in malaria-related morbidity and mortality. The emergence of *Plasmodium falciparum* parasites resistant to artemisinin derivatives in Southeast Asia and the risk of their spread or of local emergence in sub-Saharan Africa are a major threat to public health. This study thus set out to estimate the proportion of *P. falciparum* isolates, with PfKelch13 gene mutations associated with artemisinin resistance previously detected in Southeast Asia.

Methods: Blood samples were collected in two sites of Bangui, the capital of the Central African Republic from 2017 to 2019. DNA was extracted and nested PCR were carried out to detect *Plasmodium* species and mutations in the propeller domain of the PfKelch13 gene.

Results: A total of 255 *P. falciparum* isolates were analyzed. Among them, *P. ovale* DNA was found in four samples (1.57%, 4/255). Of 187 samples with interpretable PfKelch13 sequences, four isolates presented a mutation in the PfKelch13 gene (2.1%, 4/187), including one non-synonymous mutation (Y653N) (0.5%, 1/187). This mutation has never been described as associated with artemisinin resistance in Southeast Asia and its in vitro phenotype is unknown.

Conclusion: This preliminary study indicates the need for a larger study on samples collected across the whole country along with the evaluation of in vitro and in vivo phenotype profiles of PfKelch13 mutant parasites to estimate the risk of artemisinin resistance in the CAR.

Background

The introduction of Artemisinin-based Combination Therapies (ACT) as the first-line treatment for *Plasmodium falciparum* malaria worldwide has greatly helped to reduce, between 2000 and 2017, the morbidity and the mortality to 16.4% and 48.2%, respectively [1, 2]. The global implementation of ACT has thus inspired hope, but soon became an issue of concern due to the emergence of artemisinin-resistant parasites in 2006–2007 in the Greater Mekong subregion and the risk of spreading of these resistant strains to sub-Saharan Africa where malaria transmission and burden are high [3, 4]. Since then, many sub-Saharan African countries have enhanced their surveillance system to assess the efficacy of ACT treatments in clinical drug efficacy studies and to detect artemisinin resistant parasites by in vitro susceptibility testing or PfKelch13 genotyping [5, 6].

In the Central African Republic (CAR), ACT have been used since 2006 as first- and second-line treatments [7]. Set up in October 2016 and based on the WHO Global Technical Strategy for Malaria 2016–2030, the national CAR policies for malaria control emphasize surveillance of clinical efficacy, in vitro susceptibility testing and screening for molecular markers associated with antimalarial-drug resistance. Nonetheless, there are very few reliable data available. Two studies on in vitro sensitivity tests conducted in Bangui in 2004 (before ACT were introduced) and in 2014 showed that 100% of the circulating *P. falciparum* strains were sensitive to the main antimalarial drugs [8; 9]. A clinical study assessing the efficacy of ACT was

conducted in Bangui in 2010 and showed a high level of clinical and parasitological response rate estimated to 100% for the artemether–lumefantrine (AL) and artesunate–amodiaquine (ASAQ) [10]. The sole study assessing the prevalence of mutant PfKelch13 parasites was conducted in 2014 in Bangui and showed a frequency of 4.5% non-synonymous mutations (Q468R, W470Stop, K480R, L505S, Y519C, S522C, N537D, G545E, I552M, W565Stop, V566I, S577P, A578S, F583L, V589I, G591D, E606G, E612G, Q633R, I640V, D641G) [11].

In addition to the lack of data on artemisinin resistance in the CAR, since 2012, the CAR has been experiencing unprecedented social unrest and political instability, leading to the arrival of thousands of expatriate civilians and foreign troops, some of whom come from countries located in known multidrug-resistant malaria areas. The presence in the CAR of troops from Cambodia and Thailand, both of which are located in the area where artemisinin-resistance have emerged, and from Bangladesh, Bhutan and Nepal, countries that neighbor this epicenter of emergence, may lead to the potential spread of multidrug-resistant *P. falciparum* in Africa in general and to the CAR in particular. This concern is further heightened by studies (Didier Ménard, personal communication) that have shown a proportion of 0.9% asymptomatic carriers in the Cambodian military personnel sent to Africa for peacekeeping missions (18 *P. falciparum* infections among the 1950 military personnel tested between June 2014 and June 2017). Moreover, there are a multiplicity of factors that may favor the emergence, the introduction and – more importantly, the selection – of drug-resistant malaria strains, particularly those resistant to artemisinin derivatives. These factors include population migration within the CAR or emigration to neighboring countries, the deplorable living conditions in displaced persons and refugee camps, the illicit trafficking of fake antimalarial drugs in local markets and the use of local pharmacopeia or traditional medicine and self-medication practices. Since 2014, several single non-synonymous mutations in the propeller domain of the PfKelch13 gene have been associated with resistance to artemisinin derivatives, expressed clinically by a delayed clearance of *P. falciparum* during the three-day course of ACT treatment or by the increase in the number of parasites (ring stages) resistant to a pulse of 700 nM of DHA as expressed in the Ring-stage Survival Assay (RSA) [12]. Since then, *in vivo* clinical drug efficacy study and *in vitro* parasite susceptibility testing along with screening for specific mutations in the PfKelch13 gene are the recommended approach for the surveillance of the efficacy of ACT [13]. However, *in vivo* and *in vitro* approaches have some practical issues regarding their elaborate protocols and the follow-up of patients for more than one month post-infection. Therefore, screening for mutations to detect potential resistance markers may be a useful, efficient alternative. This study thus set out to investigate kelch13 polymorphism in *P. falciparum* isolates collected in Bangui, the capital of the CAR.

Methods

Study site and period.

The study was carried out in Bangui, the capital of the CAR where malaria transmission is holoendemic, on samples taken from symptomatic malaria patients at two health centers during two different periods: (1) at the Institut Pasteur in Bangui (IPB) between September 2017 and February 2018 and (2) at the

Bangui Pediatric Complex (BPC) between November 2018 and March 2019. Both centers are located in the 1st district of Bangui, but patients come from all districts.

Study population and sampling. The study population was made up of patients visiting the IPB and the BPC for malarial symptoms. Patients included in the study showed positive Giemsa-stained thick blood smears, and blood samples and demographic data were available for them. All blood samples were collected in EDTA blood collection tubes. Some of the sampled blood was then spotted on filter paper for further analyses.

Plasmodium species identification. DNA was extracted using the Chelex-100 method on the dried blood spot samples [14]. Extracted DNA was used to identify Plasmodium species using the technique described in ref. [15]. The targeted 18S (SSU) rRNA gene common to all four Plasmodium species was amplified using a specific primer pair (PCR1) and then species-specific primers were used to screen for each individual Plasmodium species (PCR2) (Table 1). After migration, PCR products were observed under UV light and the bands were compared with the positive controls for species identification.

Table 1
Primers sequences and size of PCR products

	PCR	Names of Primers	Primers sequences	Size of PCR products
P. Genus	PCR1	rPLU5 rPLU6	5'-CCTGTTGTTGCCTTAAACTTC-3' 5'-TTAAAATTGTTGCAGTTAAAACG-3'	-
P. falciparum	Nested	rFAL-F rFAL-R	5'- CTTTTGAGAGGTTTTGTTACTTTGAGTAA- 3' 5'-TATTCCATGCTGTAGTATTCAAACAAAA-3'	205 bp
P. ovale		rOVA-F rOVA-R	5'- TTTTGAAGAATACATTAGGATACAATTAATG- 3' 5'-CATCGTTCCTCTAAGAAGCTTTACCCT-3'	800 bp
P. vivax		rVIV-F rVIV-R	5'-ACGCTTCTAGCTTAATCCACATAACT-3' 5'- ATTTACTCAAAGTAACAAGGACTTCCAAGC- 3'	120 pb
P. malariae		rMAL-F rMAL-R	5'- ATAACATAGTTGTACGTTAAGAATAACCGC- 3' 5'- AAAATTCCCATGCATAAAAAATTATACAAA-3'	144 bp
PfKelch13	PCR1	K13_PCR_F K13_PCR_R	5'-CGGAGTGACCAAATCTGGGA-3' 5'-GGGAATCTGGTGGTAACAGC-3'	-
	Nested	K13_N1_F K13_N1_R	5'-GCCAAGCTGCCATTCATTTG-3' 5'-GCCTTGTTGAAAGAAGCAGA-3'	849 pb

Detection of mutations in the PfKelch13 gene. A portion of the PfKelch13 gene was amplified from the DNA extract on confirmed *P. falciparum* species using the method described in ref. [12]. PCR products were run on agarose gels and observed under UV light. The amplicons were then sequenced by Sanger sequencing technique and analyzed to screen for polymorphism with respect to the reference sequence from the 3D7 *P. falciparum* strain (PF3D7_1343700).

Data processing and statistical analyses. The data were compiled in a Microsoft Excel spreadsheet (ver. MS Office 2010). The data were analyzed using descriptive statistics (mean, frequency standard deviation and the confidence intervals).

Results

Demographic characteristics of the study population. The mean age of the patients included in our study was 9.17 years (range: 2 to 71) at IPB and 3.75 years (range: 0.12 to 15) at the BPC. The sex ratio (M/F) was 1.5 at the IPB and 1.15 at the CPB. The mean parasite density was five times higher in patients recruited at the IPB than those at the CPB (Table 2).

Table 2
Baseline characteristics of Patients

Sites Parameters	IPB 2017–2018	BPC 2018–2019
Sample size	100	155
Gender (M/F) (Ratio sex)	60/40 (1.5)	83/72 (1.15)
Mean age (95% CI), Years	9.17 (7.18 – 11.16)	3.75 (3.19–4.31)
Geometric mean of parasite density [Range], μl^{-1}	8243 [62–99820]	1542 [40–18000]

CI: Confidence interval.

Prevalence of Plasmodium species. Of the positive smear samples, 255 could be PCR-amplified using the Plasmodium primers: 100% (255/255) were positive for *P. falciparum* and 1.57% (4/255) for *P. ovale*. At the IPB, *P. falciparum* prevalence was 100% (100/100) and *P. ovale* prevalence was 3% (3/100). At the BPC, *P. falciparum* prevalence was 100% (155/155) and *P. ovale* prevalence was 0.65% (1/155). The other two Plasmodium species (*P. malariae* and *P. vivax*) were not observed.

Frequency of mutations in the PfKelch13 gene. A total of 192 amplicons were sequenced to screen for mutations. Of the 187 interpretable sequences, four mutations (2.14%) were detected, including one non-synonymous mutation (0.54%) and three synonymous mutations (1.6%) (Table 3): C469C, D464D, A627A and Y653N. The Y653N mutation was observed in a sample collected in 2019 at the CPB.

Table 3

Polymorphism observed in the PfKelch13 in two sites samples collected in Bangui CAR 2017–2019.

Sites	Number of samples	SNPs	Codons positions and nitrogenous base	Type of mutations	Frequency (%)
BPC	117	3	D464D C469C Y653N	S S NS	2.56
IPB	70	1	A627A	S	1.43
Total	187	4	-	-	2.14

S: synonymous and NS: non-synonymous

Discussion

The present study provides recent data on the Plasmodium species circulating in Bangui, CAR, as well as on the presence of parasites with PfKelch13 mutations.

First, we demonstrate that *P. falciparum* and *P. ovale* are circulating in Bangui. Although *P. falciparum* was found in all malaria-positive cases, this species was associated with *P. ovale* in 1.57% of malaria cases. This figure is higher than that of previous observations. A study carried out in 2010 in Bangui estimated a prevalence of 0.3% *P. ovale* [16]. Of note, a co-infection of *P. ovale*, *P. falciparum* and *P. malariae* was observed in Rouen, France in 2017 in an imported malaria case in two children from the CAR [17]. Studies on malaria in monkeys have also demonstrated the circulation of *P. ovale*, indicating the possible transmission from monkeys to humans [18].

Since 2006–2007, resistance to artemisinin has emerged in Southeast Asia, along the Thai-Cambodian border. This resistance, first identified as an increase in parasite clearance times after treatment with artesunate monotherapy or with ACT, is now better understood. It involves early ring-stages that resist treatment by ceasing to grow when exposed to the drug. This phenomenon has been demonstrated by the development of a new in vitro test called the ring-stage survival assay (RSA). Resistant parasites show a proportion of > 1% of parasites that survive after 72 h compared with susceptible parasites (< 1%). Since 2014, these two phenotypes (clinical and in vitro) have been clearly associated with the presence of several non-synonymous mutations in the propeller domain of the PfKelch13 gene. The two main hotspots of emergence are located in the Greater Mekong subregion in Southeast Asia, where the parasites carrying the C580Y mutation are now dominant [12]. Other single point mutations (N458Y, Y493H, R539T, I543T, F446I, M476I, P553L and R561H) have also been validated as conferring resistance to artemisinin [19]. In addition, some mutations (P441L, G449A, C469F, P527H, N537I, G538V, V568G, P574L, F673I and A675V) are candidates suspected to be associated with artemisinin resistance [19]. In sub-Saharan Africa, validated or candidate mutations associated with resistance (R539T, P574L) have been observed in Angola, Equatorial Guinea and in Rwanda, whereas mutations potentially associated

with artemisinin resistance (M476I) have been detected in Senegal [20, 21, 22, 23]. A particular case was the observation of a local mutation (M579I) in Equatorial Guinea [24]. This mutation was shown to be associated with artemisinin resistance, while the A578S mutation, common in Africa, was not found to be associated with artemisinin resistance [25]. Our study on samples collected in Bangui between 2017 and 2019 demonstrate the presence of one non-synonymous mutation (Y653N) and three synonymous mutations (frequency of 2.1%). This frequency of PfKelch13 mutants is similar to those observed in neighboring countries (Brazzaville Congo, certain regions of Cameroon), which showed frequencies of 1.57% and 2.9%, respectively [26, 27], confirming the absence of artemisinin resistance in Central Africa [20]. The only previous data on PfKelch13 polymorphism performed in 2014 in the CAR (K13 artemisinin resistance multicenter resistance, KARMA study), revealed a 4.5% prevalence of non-synonymous mutations [12]. The difference or the fluctuation in the frequency of non-synonymous mutations in the CAR in 2014 and that of Bangui in 2017–2019 is likely due to the representativeness of the samples tested, but also to the fact that mutants appear at random and disappear probably because they do not have a selective advantage compared with wild strains. The previously validated or candidate resistance-associated mutations were not detected in our study. Interestingly, the A578S codon, common in Africa, was not observed either. The sole non-synonymous mutation detected here has never been reported in other African countries. It remains to be seen if this mutation is associated with artemisinin resistance. It is possible that local mutant strains resistant to artemisinin can emerge in addition to the risk of spread of the resistant Southeast Asian strain, as observed previously with other strains resistant to chloroquine or sulfadoxine–pyrimethamine. The dynamics of resistance and its emergence are likely to be complex, particularly due to the interactions specific to each world (sub) region [28]. Similar studies in Cameroon and Nigeria have shown large differences according to region and time period, which makes it difficult to determine the spatio-temporal dynamics on polymorphism [29, 30]. Some countries in this African subregion (Congo, Gabon, and DRC) have revealed the presence of parasites polymorphic for the A578S allele, others have detected novel alleles and still others, particularly in Benin, have not detected any polymorphism [31, 32].

If the Y653N mutation increases in frequency in subsequent studies, it will then be necessary to assess its susceptibility *in vitro* to confirm or disprove its association with artemisinin resistance. A wider study is now needed, particularly in terms of the number and the representativeness of samples in Bangui and provincial cities and towns.

Conclusion

Our study demonstrates the presence of a strain carrying a non-synonymous mutation in Bangui. Neither non-synonymous mutations involved in previously demonstrated *in vitro* and *in vivo* resistance nor the candidate resistance mutations were detected. The A578S mutant, although not associated with resistance but frequently found in Africa, was not observed in our samples. The novel mutation detected here is yet another mutation to add to the limited list of non-synonymous mutations detected in Africa. Another study with a larger sample size and with samples collected elsewhere in Bangui and throughout the CAR is needed to confirm this narrow polymorphism profile.

Abbreviations

ACT

Artemisinin-based Combination Therapies

CAR

Central African Republic

IPB

Institut Pasteur in Bangui

WHO

World Health Organization

PCB

Pediatric Complex in Bangui

PfKelch13

Plasmodium falciparum kelch 13

RSA

Ring-stage Survival Assay

Declarations

Ethics approval and consent to participate

The study protocol was accepted by malaria experts from the National Malaria Control Program, in the absence of the institutional and national ethics committee that did not exist at the start of the study. The consent of each adult and the parents of child were obtained before inclusion.

Consent for publication

Not applicable.

Availability of data and materials

The database of this study was available from the corresponding author upon request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

RNB and DM conceived and designed the study, CN, CN2, JCG and JPL contributed to the design and analysis plan. CBGPW and UV deployed, collected the sample and baseline characteristics data. RNB, CBGPW, EL and NP did laboratory work. AM analyzed data. All authors interpreted, critically the data and read and approved the final version of manuscript.

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References

1. Cibulskis ER, Alonso P, Aponte J, Aregawi M, Barrette A, Bergeron L. Malaria: Global progress 2000-2015 and future challenges. *Infect Dis Poverty*. 2016; 5: 61.
2. World Malaria Report. Geneva, World Health Organization; 2019.
3. Noedl H, Se Y, Schaecher K, Smith BL, Socheat D, Fukuda MM, Artemisinin Resistance in Cambodia 1 (ARC1) Study Consortium. Evidence of artemisinin-resistant malaria in western Cambodia. *N Engl J Med*. 2008; 359(24):2619-20.
4. Hassett MR, Roepe PD. Origin and Spread of Evolving Artemisinin-Resistant *Plasmodium falciparum* Malarial Parasites in Southeast Asia. *Am J Trop Med Hyg*. 2019, 101(6):1204-1211.
5. Balikagala B, Mita T, Ikeda M, Sakurai M, Yatsushiro S, Takahashi N et al. Absence of in vivo selection for K13 mutations after artemether-lumefantrine treatment in Uganda. *Malar J*. 2017; 16 (1):23.
6. Djaman JA, Olefongo D, Ako AB, Roman J, Ngane VF, Basco LK, Tahar R. Molecular Epidemiology of Malaria in Cameroon and Côte d'Ivoire. XXXI. Kelch 13 Propeller Sequences in *Plasmodium falciparum* Isolates before and after Implementation of Artemisinin-Based Combination Therapy. *Am J Trop Med Hyg*. 2017; 97 (1):222-224.
7. Manirakiza A, Njuimo SP, Le Faou A, Malvy D, Millet P. Availability of antimalarial drugs and evaluation of the attitude and practices for the treatment of uncomplicated malaria in Bangui, Central African Republic. *J Trop Med*. 2010; 2010: 510834.

8. Menard D, Djalle D, Manirakiza A, Yapou F, Siadoua V, Sana S, et al. Drug-resistant malaria in Bangui, Central African Republic: an *in vitro* Am J Trop Med Hyg. 2005; 73 (2):239-43.
9. Javelle E, Madamet M, Gaillard T, Velut G, Surcouf C, Michel R, et al. Delayed Onset of *Plasmodium falciparum* Malaria after Doxycycline Prophylaxis in a Soldier Returning from the Central African Republic. Antimicrob Agents Chemother. 2016; 60 (4):2592-3.
10. Nambei WS, Lango Yaya E, Pounguinza S, Achonduh O, Bogon A, Lengande R, et al. Efficacy and safety of antimalarial combinations for treatment of uncomplicated malaria in children in Bangui, Central African Republic]. Med Sante Trop. 2013; 23 (3):313-9.
11. Ménard D, Khim N, Beghain J, Adegnika AA, Shafiu-Alam M, Amodu O, A Worldwide Map of *Plasmodium falciparum* K13-Propeller Polymorphisms. N Engl J Med. 2016; 374(25):2453-64.
12. Ariey F, Witkowski B, Amaratunga C, Beghain J, Langlois AC, Khim N, et al. A molecular marker of artemisinin-resistant *Plasmodium falciparum* Nature. 2014; 505(7481):50-5.
13. Global Technical Strategy for Malaria 2016-2030 – World Health Organization 2016.
14. Walsh PS, Metzger DA, Higuchi R. Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. Biotechniques. 1991; 10 (4):506-13.
15. Singh B, Bobogare A, Cox-Singh J, Snounou G, Abdullah MS, Rahman HA. A genus- and species-specific nested polymerase chain reaction malaria detection assay for epidemiologic studies. Am J Trop Med Hyg. 1999;60: 687–692.
16. Djallé D, Gody JC, Moyon JM, Tekpa G, Ipero J, Madji N, et al. Performance of Paracheck™-Pf, SD Bioline malaria Ag-Pf and SD Bioline malaria Ag-Pf/pan for diagnosis of falciparum malaria in the Central African Republic. BMC Infect Dis. 2014; 14: 109.
17. Bichara C, Flahaut P, Costa D, Bienvenu AL, Picot S, Gargala G. Cryptic *Plasmodium ovale* concurrent with mixed *Plasmodium falciparum* and *Plasmodium malariae* infection in two children from Central African Republic. Malar J. 2017; 16 (1):339.
18. Mapua MI, Qablan MA, Pomajbíková K, Petrželková KJ, Hůzová Z, et al. Ecology of malaria infections in western lowland gorillas inhabiting Dzanga Sangha Protected Areas, Central African Republic. Parasitology. 2015; 142(7):890-900.
19. World Health Organization. (2018). Artemisinin resistance and artemisinin-based combination therapy efficacy: status report. World Health Organization.
20. Yang C, Zhang H, Zhou R, Qian D, Liu Y, Zhao Y, et al. Polymorphisms of *Plasmodium falciparum* k13-propeller gene among migrant workers returning to Henan Province, China from Africa. BMC Infect Dis. 2017; 17(1):560.
21. Kamau E, Campino S, Amenga-Etego L, Drury E, Ishengoma D, Johnson K et al. K13-Propeller Polymorphisms in *Plasmodium falciparum* Parasites From Sub-Saharan Africa. J Infect Dis. 2015; 211(8): 1352–1355.
22. Ouattara A, Kone A, Adams M, Fofana B, Maiga AW, Hampton S1 Polymorphisms in the K13-Propeller Gene in Artemisinin-Susceptible *Plasmodium falciparum* Parasites from Bougoula-Hameau and Bandiagara, Mali, Am J Trop Med Hyg. 2015; 92(6): 1202–1206.

23. Taylor SM, Parobek CM, DeConti DK, Kayentao K, Coulibaly SO, Greenwood BM et al. Absence of putative artemisinin resistance mutations among *Plasmodium falciparum* in Sub-Saharan Africa: a molecular epidemiologic study. *J Infect Dis.* 2015; 211(5):680-8.
24. Lu F, Culleton R, Zhang M, Ramaprasad A, von Seidlein L, Zhou H, et al. Emergence of Indigenous Artemisinin-Resistant *Plasmodium falciparum* in Africa. *N Engl J Med.* 2017; 376(10):991-3.
25. Mishra N, Prajapati SK, Kaitholia K, Bharti RS, Srivastava B, Phookan S et al. Surveillance of Artemisinin Resistance in *Plasmodium falciparum* in India Using the kelch13 Molecular Marker, Antimicrobial Agents and Chemotherapy 2015 ;59(5):2548-53.
26. Mayengue PI, Niama RF, Kouhounina Batsimba D, Malonga-Massanga A, Louzolo I, Loukabou Bongolo NC, et al. No polymorphisms in K13-propeller gene associated with artemisinin resistance in *Plasmodium falciparum* isolated from Brazzaville, Republic of Congo. *BMC Infect Dis.* 2018; 18(1):538.
27. Eboumbou Moukoko CE, Huang F, Nsango SE, Kojom Foko LP, Ebong SB, Epee Eboumbou P, et al. K-13 propeller gene polymorphisms isolated between 2014 and 2017 from Cameroonian *Plasmodium falciparum* malaria patients. *PLoS One.* 2019; 14 (9):e0221895.
28. Hastings IM. Complex dynamics and stability of resistance to antimalarial drugs. *Parasitology.* 2006; 132(Pt 5):615-24.
29. Djaman JA, Olefongo D, Ako AB, Roman J, Ngane VF, Basco LK, Tahar R. Molecular Epidemiology of Malaria in Cameroon and Côte d'Ivoire. XXXI. Kelch 13 Propeller Sequences in *Plasmodium falciparum* Isolates before and after Implementation of Artemisinin-Based Combination Therapy. *Am J Trop Med Hyg.* 2017; 97(1):222-224.
30. Oboh MA, Ndiaye D, Antony HA, Badiane AS, Singh US, Ali NA et al. Status of Artemisinin Resistance in Malaria Parasite *Plasmodium falciparum* from Molecular Analyses of the Kelch13 Gene in Southwestern Nigeria. *Biomed Res Int.* 2018; 2018: 2305062.
31. Maïga-Ascofaré O, May J. Is the A578S Single-Nucleotide Polymorphism in K13-propeller a Marker of Emerging Resistance to Artemisinin Among *Plasmodium falciparum* in Africa? *The Journal of Infectious Diseases*, Volume 213, Issue 1, 2016, 165–166.
32. Ogouyèmi-Hounto A, Damien G, Deme AB, Ndam NT, Assouhou C, Tchoulin D, et al. Lack of artemisinin resistance in *Plasmodium falciparum* in northwest Benin after 10 years of use of artemisinin-based combination therapy. 2016; 23:28.