International Journal for Parasitology: Drugs and Drug Resistance 6 (2016) 54–59

Contents lists available at ScienceDirect

International Journal for Parasitology: Drugs and Drug Resistance

journal homepage: www.elsevier.com/locate/ijppaw

Limited artemisinin resistance-associated polymorphisms in *Plasmodium falciparum K13-propeller* and *PfATPase6* gene isolated from Bioko Island, Equatorial Guinea

Jian Li [a], Jiangtao Chen [b, c], Dongde Xie [c], Urbano Monsuy Eyi [d], Rocio Apicante Matesa [d], Maximo Miko Ondo Obono [d], Carlos Sala Ehapo [d], Liye Yang [e], Huitian Yang [e], Min Lin [e, f, *]

[a] *Institute of Basic Medical Sciences, College of Basic Medicine, Department of Infectious Diseases, Renmin Hospital, Hubei University of Medicine, Shiyan 442000, People's Republic of China*
[b] *Laboratory Medical Center, Huizhou Municipal Central Hospital, Huizhou 516001, People's Republic of China*
[c] *The Chinese Medical Aid Team to the Republic of Equatorial Guinea, Guangzhou 510000, People's Republic of China*
[d] *Central Blood Transfusion Service, Department of Medical Laboratory Science, Malabo Regional Hospital, Malabo 999115, Equatorial Guinea*
[e] *Laboratory Medical Center, Chaozhou Central Hospital, Southern Medical University, Chaozhou 521021, People's Republic of China*
[f] *Department of Histology and Embryology, Shantou University Medical College, Shantou 515000, Guangdong, People's Republic of China*

ARTICLE INFO

Article history:
Received 15 September 2015
Received in revised form
3 November 2015
Accepted 25 November 2015
Available online 12 January 2016

Keywords:
Plasmodium falciparum
Polymorphism
Artemisinin resistance
K13-propeller

ABSTRACT

Objective: With emergence and geographically expanding of antimalarial resistance worldwide, molecular markers are essential tool for surveillance of resistant *Plasmodium* parasites. Recently, single-nucleotide polymorphisms (SNPs) in the PF3D7_1343700 kelch propeller (*K13-propeller*) domain are shown to be associated with artemisinin (ART) resistance *in vivo* and *in vitro*. This study aims to investigate the ART resistance-associated polymorphisms of *K13-propeller* and *PfATPase6* genes in *Plasmodium falciparum* isolates from Bioko Island, Equatorial Guinea (EG).

Methods: A total of 172 samples were collected from *falciparum* malaria patients on Bioko Island between 2013 and 2014. The polymorphisms of *K13*-propeller and *PfATPase6* genes were analyzed by Nest-PCR and sequencing.

Results: Sequences of *K13-propeller* and *PfATPase6* were obtained from 90.74% (98/108) and 91.45% (139/152) samples, respectively. The 2.04% (2/98) cases had non-synonymous *K13-propeller* A578S mutation but no found the mutations associated with ART resistance in Southeast Asia. For *PfATPase6*, the mutations were found at positions N569K and A630S with the mutation prevalence of 7.91% (11/139) and 1.44% (2/139), respectively. In addition, a sample with the mixed type at position I723V was discovered (0.72%, 1/139).

Conclusions: This study initially offers an insight of *K13*-propeller and *PfATPase6* polymorphisms on Bioko Island, EG. It suggests no widespread ART resistance or tolerance in the region, and might be helpful for developing and updating guidance for the use of ART-based combination therapies (ACTs).

1. Introduction

The emergence of *Plasmodium falciparum* resistance to antimalarial drugs has been threatening the world's malaria control and elimination efforts (Young et al., 1963). Currently, *P. falciparum* have developed resistance/tolerance to antimalarial drugs chloroquine (CQ) and sulfadoxine-pyrimethamine (SP). Although World Health Organization (WHO) has recommended artemisinin (ART)-based combination therapies (ACTs) as the first-line treatment for uncomplicated *P. falciparum* malaria, *P. falciparum* are becoming insensitive to ART and its derivatives (Harinasuta et al., 1965; Wongsrichanalai et al., 2002; Amaratunga et al., 2014). At present, virtually all malaria endemic countries in sub-Saharan Africa are adopting either Artemether-Lumefantrine (AL) or Artesunate-Amodiaquine (AS-AQ) as the front-line ACTs. AS-AQ and the ART derivative dihydroartemisinin-piperaquine (DP) are used in

* Corresponding author. Department of Histology and Embryology, Shantou University Medical College, 22 Xinling Rd., Shantou, Guangdong Province 515000, People's Republic of China. Tel.: +86 0754 88900443 ; fax: +86 0754 88557562 3.
E-mail address: Konfutea@hotmail.com (M. Lin).

http://dx.doi.org/10.1016/j.ijpddr.2015.11.002

Equatorial Guinea (EG) (Barrette and Ringwald, 2010).

Emerging evidence indicate that *P. falciparum* has been developing the resistance to ART and its derivatives. In Pailin of western Cambodia and other Southeast (SE) Asia area, parasite clearance was delayed following the treatment with ART monotherapy or ACTs (Noedl et al., 2008; Dondorp et al., 2009; Amaratunga et al., 2012; Miotto et al., 2013; Ashley et al., 2014). Our recent study showed the presence of high prevalent mutations in *Pfmdr1* (91.39%) and *Pfcrt* (98.67%) which markers for antimalarial drug resistence in *P. falciparum* clinical isolates on Bioko Island, EG (Li et al., 2015). It is globally threatening for malaria prevention and treatment (Wootton et al., 2002; Roper et al., 2004). It is imperative to conduct surveillances to identify areas that are potentially developing drug resistance.

Several molecular markers for antimalarial drug resistance has been identified (Wongsrichanalai et al., 2002; Barrette and Ringwald, 2010). Polymorphisms of the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase ortholog in *P. falciparum* (*PfSERCA* or *PfATPase6*) has been associated with ART resistance, although the association of SNPs in *PfATPase6* with resistance to ART and the underlying mechanism remains to be confirmed (Jambou et al., 2005; Afonso et al., 2006; Mugittu et al., 2006; Cui et al., 2012).

Single-nucleotide polymorphisms (SNPs) in the PF3D7_1343700 kelch propeller (*K13-propeller*) have been identified to be a key causal determinant of ART resistance in SE Asia (Mok et al., 2015). From 2009 to 2015, a series of studies associated with *K13*-propeller has been published particular in Asia (Talundzic et al., 2015; Tun et al., 2015; Wang et al., 2015) and WHO Africa region (Cooper et al., 2015; Ouattara et al., 2015). In Africa, limited mutations of *K13*-propeller were found in Dakar (Torrentino-Madamet et al., 2014), Uganda (Cooper et al., 2015), Mali (Ouattara et al., 2015), and even 12 countries from sub-Saharan Africa (Kamau et al., 2015). These studies showed the mutational loci in African countries were different from those of SE Asia (Kamau et al., 2015).

Malaria is a serious health problem in EG, especially on Bioko Island. However, whether the ART resistance has been developed on the Bioko Island remains unclear. In this study, we surveyed polymorphisms in *K13-propeller* and *PfATPase6* genes in clinical isolates collected from Bioko Island, EG. Our findings may provide a clue to prevent and treat malaria using ART on Bioko Island, EG.

2. Materials and methods

2.1. Study area

Bioko Island belongs to EG and is located in the Gulf of Guinea, about 100 km off the coast of southern Nigeria and 160 km northwest of continental EG (Fig. 1). The island has a population of 266 000 inhabitants (2001 census) and a humid tropical environment. The launch of the Bioko Island Malaria Control Project (BIMCP) have had a marked impact on malaria transmission, malaria due to *P. falciparum* is still the major public health problem on the island. The entomological inoculation rates (EIRs) in Bioko Island ranged from 163 to 840, with the outdoor EIRs reaching more than 900 infective mosquito bites yearly and a malaria prevalence of 52% under the age of five years (Overgaard et al., 2012; Rehman et al., 2013).

2.2. Samples collection

Blood samples (3 ml) were collected from the confirmed malaria cases between September 2013 and March 2014. Approximately 300 μl of blood was aliquoted on 3 MM Whatman® filter paper (Whatman International Ltd., Maidstone, England), and air dried. These filters were then stored individually in Ziplock bags containing silica desiccant beads and kept at −20 °C. These samples were examined using the ICT malaria *P.f.* Cassette Test (ICT Diagnostics, South Africa) and Giemsa-stained thick and thin peripheral blood smear examination with microscope. For quality control, archived malaria positive slides were re-examined and parasitaemia was recorded. The *Plasmodium* spp. was confirmed by *Plasmodium* malaria real time PCR diagnostic kit (Shanghai Liferiver Bio-Tech Corp, China). This study was approved by the ethics committees of Malabo Regional Hospital. The informed consent was obtained from all participated subjects.

2.3. DNA extraction from blood samples

Genomic DNA (gDNA) was extracted from dried filter blood spots (DBS) by following Chelex-100 extraction procedure described in our previous report (Li et al., 2014). An 18S-rRNA-based RT-PCR was used to evaluate the quality of *P. falciparum* gDNA.

2.4. Genotyping

Nucleotide and amino-acid sequence of *K13-propeller* and *PfATPase6* used in current study has been reported in PlasmoDB (http://plasmodb.org) under Gene ID: PF3D7_1343700 and PF3D7_0106300. In order to illustrate the mutations of *K13*-propeller and *PfATPase6*, one segment of *K13*-propeller gene and one fragment from *PfATPase6* gene were amplified by a nested PCR (Zhang et al., 2008; Li et al., 2014), respectively.

The *K13-propeller* and *PfATPase6* genes were amplified by nested PCR using the primers in Table 1. For first round PCR, 0.5 μl of DNA was amplified with 10 μl 2 × NovoStar Green PCR Mix (1.25 U/μl NovoStar Taq DNA Polymerase, 0.4 mM dNTP Mixture, 2 × PCR Buffer, and 4 mM Mg^{2+}), 0.5 μl forward primer (10 μM), 0.5 μl reverse primer (10 μM), and sterile ultrapure water to a final volume of 20 μl. For the second round PCR, 0.5 μl primary PCR products were amplified with 40 μl reaction system, including 20 μl 2 × NovoStar Green PCR Mix, 1.0 μl forward primer (10 μM), 1.0 μl reverse primer (10 μM), and H_2O (up to 40 μl).

PCR reaction conditions were listed in Table 1. All PCR products were analyzed using 1.0% agar gel electrophoresis and DNA sequencing using a ABI 3730×L automated sequencer (PE Biosystems, CT, USA). The data was analyzed using the DNAstar (DNASTAR Inc., Madison, WI, USA). The 3D7 *K13*-propeller and *PfATPase6* sequences were used as the references.

2.5. Data analysis

The data was analyzed using SPSS 17.0 (SPSS Inc., Chicago, IL). The mutant and wild-type alleles of the collected clinical samples were used to generate the prevalence of the alleles. A two-tailed *P*-value is less than 0.05 was considered statistically significant. The 95% confidence intervals (95% CI) was calculated as described previously (Li et al., 2014).

3. Results

3.1. K13-propeller polymorphisms

Sequence of a total of 98 (90.74%, 98/108) *K13-propeller* nested PCR products was obtained from 108 (62.79%, 108/172) PCR-positive samples out of the 172 isolates. The *K13-propeller* SNPs were analyzed by comparing with the reference 3D7 strain

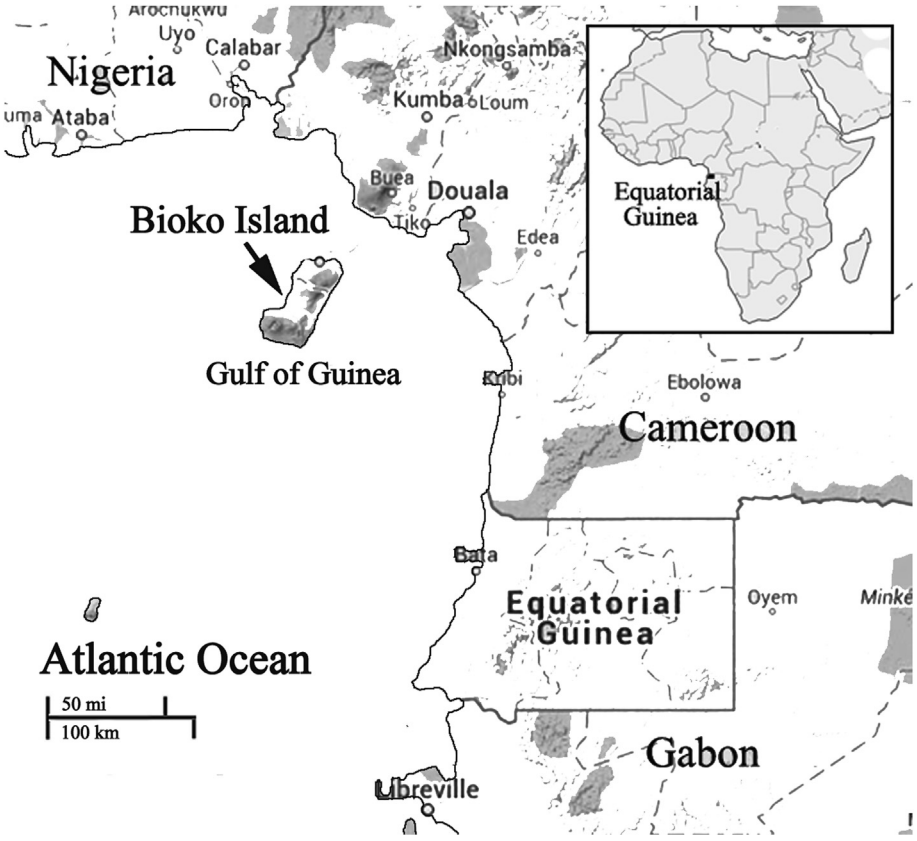

Fig. 1. Geographical map of Bioko Island, Equatorial Guinea.

Table 1
Primers and PCR conditions for genotyping.

Gene	Primer	Sequence (5′–3′)	Size (bp)	Mutation	PCR condition
K13-propeller	**1st round PCR**				
	K13-1	CGGAGTGACCAAATCTGGGA	2097		95 °C 3 min; followed by 30 cycles (95 °C 30 s, 55 °C 30 s, 72 °C 30 s); 72 °C 5 min; then store 12 °C.
	K13-4	GGGAATCTGGTGGTAACAGC			
	2nd round PCR				
	PfK13_inF2	TCAACAATGCTGGCGTATGTG	501	T474I, M476I, A481V, Y493H, T508N, P527T, G533S, N537I, R539T, I543T, P553L, R561H, V568G, P574L, A578S, and C580Y	95 °C 3 min; followed by 30 cycles (95 °C 30 s, 55 °C 30 s, 72 °C 30 s); 72 °C 5 min; then store 12 °C.
	PfK13_inR2	TGATTAAG GTAATTAAAAGCTGCTCC			
PfATPase6	**1st round PCR**				
	PfATPase6-N1F	AATATTGTTATTCAGAATATGATTATAA	896		95 °C 3 min; followed by 30 cycles (95 °C 30 s, 55 °C 30 s, 72 °C 50 s); 72 °C 5 min; then store 12 °C.
	PfATPase6-N1R	TGGATCAATAATACCTAATCCACCTA			
	2nd round PCR				
	PfATPase6-N2F	AGCAAATATTTTCTGTAACGATAATA	798	K561N, N569K, A623E, A630S, G639D, N683K, I723V, and S769N	95 °C 3 min; followed by 30 cycles (95 °C 30 s, 58 °C 30 s, 72 °C 45 s); 72 °C 5 min; then store 12 °C.
	PfATPase6-N2R	TGTTCTAATTTATAATAATCATCTGT			

(PF3D7_1343700) and listed in Table 2. There was a mutation at position 578. The frequency of A578S was 2.04% (2/98) (Table 2). No *K13-propeller* mutation was detected at positions 474, 476, 493, 508, 527, 537, 539, 543, 553, 561, 568, 574, and 580. Notably, the C580Y, R539T, and Y493H substitutions that were associated with ART resistance *in vitro* or delayed *P. falciparum* parasite clearance *in vivo* in SE Asia were not detected on the samples from Bioko Island, EG.

3.2. *PfATPase6* polymorphisms

Sequence of a total of 139 (91.45%, 139/152) *PfATPase6* nested PCR products was obtained from 152 (88.37%, 152/172) PCR-positive samples out of the 172 isolates. The *PfATPase6* SNPs were analyzed by comparing with the reference 3D7 strain (PF3D7_0106300) and listed in Table 2. The *PfATPase6* SNPs were found at positions N569K and A630S with the mutation prevalence

Table 2
K13-propeller and PfATPase6 polymorphisms in *Plasmodium falciparum* isolates on Bioko Island, Equatorial Guinea.

Gene	Reference						No. of isolates			
	Codon position	AA	Codon	AA	Codon	Base position	PCR positive	Sequencing	Mutation	Prevalence (%, 95% CI)
K13-propeller	578	A	**g**ct	S	**T**ct	1732	108	98	2	2.04, −0.76 to 4.84
PfATPase6	569	N	aa**a**	K	aa**T**	1707	152	139	11	7.91, 3.42 to 12.4
	630	A	**g**ct	S	**T**ct	1888	152	139	2	1.44, −0.54 to 3.42
	723	I	**a**ta	V	**G**ta	2167	152	139	1	0.72, −0.69 to 2.13

Bold, AA and No. represent single nucleotide polymorphism mutation, amino acid residue and number.

of 11 (7.91%, 11/139), and 2 (1.44%, 2/139), respectively (Table 2). In addition, a sample with the mixed type at position I723V was also discovered with the mutation prevalence of 1 (0.72%, 1/139) (Table 2). The remaining mutations of K561N, A623E, G639D, N683K, and S769N, all the investigated samples verified the wild-type genotype. Notably, the S769N substitution that was connected with ART resistance was not found from these samples.

4. Discussion

For EG, both the AS-AQ and DP are considered as the front-line treatment for uncomplicated *falciparum* malaria. It is essential to understand molecular mutation profiles of *P. falciparum* parasite for ART resistance and use this initial information for molecular assessment under antimalarial drug pressure. In current study, we only find limited mutation of *K13-propeller* and low frequency of *PfATPase6* mutation of the clinical isolates from Bioko Island, EG. It is the initial report focusing on the molecular markers of *K13-propeller* and *PfATPase6* for ART resistance on this Island.

The ART resistance considers as a major risk to public health, with the most rigorous potential effect in Sub-Saharan Africa, where the burden is seriously. Furthermore, the international investments and domestic investments, the prevention and elimination system for malaria are also insufficient in the region. The hazard of ART-resistant parasites scattering from western Cambodia to the Greater Mekong Subregion and then to Africa particularly Sub-Saharan Africa, as happened previously with CQ and SP-resistant parasites (Wootton et al., 2002; Roper et al., 2004), is worrying (Ariey et al., 2014). A significant action of the WHO Global Plan for Artemisinin Resistance Containment is to increase monitoring and molecular surveillance (Talisuna et al., 2012). However, it is difficult to evaluate how long or when ART resistance mutations will appear in Africa including EG, and molecular detection can offer a profile to speedily discover for the appearance or importation of resistance alleles (Taylor et al., 2014). In SE Asia, the mutations of *K13-propeller* are found in both western Cambodia (Ariey et al., 2014; Straimer et al., 2015) and Bangladesh (Mohon et al., 2014). In Africa, only limited polymorphisms of *K13-propeller* are detected (Conrad et al., 2014; Taylor et al., 2014; Torrentino-Madamet et al., 2014). However, the *K13-propeller* polymorphism in returned migrant workers from Ghana is found in Shanglin of China (Feng et al., 2015). Thus, it is very necessary to strengthen *Plasmodium* parasites genotypic resistance surveillance with *K13-propeller* polymorphism. If there is no sufficient attention to long-term survey, it will be a disaster for human health.

The survey of *P. falciparum K13-propeller* polymorphisms primarily explore a diversity of mutations across on Bioko Island, EG. Limited polymorphisms associated with ART resistance from SE Asia are observed in the clinical isolates. Recent study reports that the SNP mutations at Y493H, I543T, R539T, and C580Y are powerfully connected with prolonging *P. falciparum* parasite survival time *ex vivo*; ART resistance *in vitro* has to be M476I mutation-related, which indicates that mutations of *K13-propeller* can generate an ART-resistance phenotype *in vitro* with genetic background from African parasite (Ariey et al., 2014). Although the five mutations play crucial role during ART-resistance to *P. falciparum* parasite *in vitro* and *in vivo*, we observe none of these mutations in the survey parasite samples. Only one *K13-propeller* A578S mutation (2.04%, 2/98) that previously reported from Cambodia is discovered in the six *K13-propeller* blades. This mutation is previously found in 0.75% (1/133) of the isolates tested in Uganda and also presents in parasites from DRC, Gabon, Ghana, Kenya, and Mali (Ariey et al., 2014; Conrad et al., 2014; Kamau et al., 2015). Although the prevalence of A578S mutant allele from Bioko Island is lower than Kenya at 2.7%, it is still higher in parasites compared to 1% in the other four countries of Sub-Saharan Africa. This unusual polymorphism also merits further characterization (Taylor et al., 2014). Mutations of A481V, G533C and A578S are confirmed and adjacent to the Y493H, R539T, C580Y mutation, and propose the mutations may have a significant effect on three-dimensional structure of the *K13-propeller*. Furthermore, the mutations of S522S, Y558H report in Ugandan children and A557S in Congo have not detected on Bioko Island (Conrad et al., 2014; Taylor et al., 2014). The V520A mutation identified from West, Central and East Africa is also not found on the Island, Dakar and Uganda (Sylla et al., 2013; Taylor et al., 2014). These results encourage and suggest ART resistance is not yet established in Africa particular on Bioko Island, EG. Although none of the mutations associated with ART resistance in SE Asia are detected in Africa (Conrad et al., 2014; Taylor et al., 2014), numerous novel *K13-propeller* coding substitutions bothers in the whole continent of Africa. The phenotypes of these coding polymorphisms remain unclear and will require further characterization to better characterize the clinical impact on ART resistance in Africa. Further analysis of phylogenetic tree or haplotype network is needed to trace the origin of the *K13-propeller* mutations and to determine whether the ART resistance is widely spreaded from SE Asia or emerged independently in Bioko Island, EG (Nyunt et al., 2014).

Polymorphisms evaluation of *PfATPase6* in Africa have occurred rarely (Mugittu et al., 2006; Legrand et al., 2008; Happi et al., 2009; Menegon et al., 2010; Kamugisha et al., 2011; Zatra et al., 2012). The current study initial describes for the *PfATPase6* polymorphism on Bioko Island, EG. Among the three different observed mutations, only one, the N569K mutation, is relatively frequent 7.91% of isolates. The previous studies have also found a high prevalence of this mutation in Zanzibar (36%) and Tanzania (29%) (Dahlstrom et al., 2008), Niger (17.2%) (Ibrahim et al., 2009). The *PfATPase6* S769N mutation is absent in all Bioko samples and consistent with previous results in Africa countries (Mugittu et al., 2006; Legrand et al., 2008; Happi et al., 2009; Menegon et al., 2010; Kamugisha et al., 2011; Zatra et al., 2012) and South America (Adhin et al., 2012). It demonstrates that the Africa and South America share the similar molecular pattern of *PfATPase6* for ART resistance.

5. Conclusions

The present study shows that the low prevalence polymorphism mutations of *PfATPase6* and limited mutations of *K13-propeller*,

potentially associated with ART resistance, are obviously observed on Bioko Island, EG. Continuous molecular surveillance with *K13*-propeller gene as ART resistance marker is exceedingly recommended on Bioko Island, EG. Furthermore, it might be helpful for developing and updating guidance for the use of ACTs.

Conflicts of interest

We declare that we have no conflict of interest.

Financial support

This study was supported by the China Postdoctoral Science Foundation (M.L. Grant Number 2013M542195); Medical Science Fund of Guangdong Province (M.L. Grant Number A2013780); Scientific Research Foundation for the Returned Overseas Chinese Scholars (J.L. Grant Number JYB201448HBMU01), State Education Ministry; the Natural Science Foundation of Hubei Province of China (J.L. Grant Number 2014CFB648), and the Foundation for Innovative Research Team of Hubei University of Medicine (J.L. Grant Number 2014 CXZ02).

Acknowledgments

The authors thank the Department of Health of Guangdong Province and Department of Aid to Foreign Countries of Ministry of Commerce of People's Republic of China for their help. The authors also thank Dr. Xinsheng Gu for revising the manuscript.

References

Adhin, M.R., Labadie-Bracho, M., Vreden, S.G., 2012. Status of potential PfATP6 molecular markers for artemisinin resistance in Suriname. Malar. J. 11, 322.

Afonso, A., Hunt, P., Cheesman, S., Alves, A.C., Cunha, C.V., do Rosario, V., Cravo, P., 2006. Malaria parasites can develop stable resistance to artemisinin but lack mutations in candidate genes atp6 (encoding the sarcoplasmic and endoplasmic reticulum Ca2+ ATPase), tctp, mdr1, and cg10. Antimicrob. Agents Chemother. 50, 480–489.

Amaratunga, C., Sreng, S., Suon, S., Phelps, E.S., Stepniewska, K., Lim, P., Zhou, C., Mao, S., Anderson, J.M., Lindegardh, N., Jiang, H., Song, J., Su, X.Z., White, N.J., Dondorp, A.M., Anderson, T.J., Fay, M.P., Mu, J., Duong, S., Fairhurst, R.M., 2012. Artemisinin-resistant Plasmodium falciparum in Pursat province, western Cambodia: a parasite clearance rate study. Lancet. Infect. Dis. 12, 851–858.

Amaratunga, C., Witkowski, B., Khim, N., Menard, D., Fairhurst, R.M., 2014. Artemisinin resistance in Plasmodium falciparum. Lancet. Infect. Dis. 14, 449–450.

Ariey, F., Witkowski, B., Amaratunga, C., Beghain, J., Langlois, A.C., Khim, N., Kim, S., Duru, V., Bouchier, C., Ma, L., Lim, P., Leang, R., Duong, S., Sreng, S., Suon, S., Chuor, C.M., Bout, D.M., Menard, S., Rogers, W.O., Genton, B., Fandeur, T., Miotto, O., Ringwald, P., Le Bras, J., Berry, A., Barale, J.C., Fairhurst, R.M., Benoit-Vical, F., Mercereau-Puijalon, O., Menard, D., 2014. A molecular marker of artemisinin-resistant Plasmodium falciparum malaria. Nature 505, 50–55.

Ashley, E.A., Dhorda, M., Fairhurst, R.M., Amaratunga, C., Lim, P., Suon, S., Sreng, S., Anderson, J.M., Mao, S., Sam, B., Sopha, C., Chuor, C.M., Nguon, C., Sovannaroth, S., Pukrittayakamee, S., Jittamala, P., Chotivanich, K., Chutasmit, K., Suchatsoonthorn, C., Runcharoen, R., Hien, T.T., Thuy-Nhien, N.T., Thanh, N.V., Phu, N.H., Htut, Y., Han, K.T., Aye, K.H., Mokuolu, O.A., Olaosebikan, R.R., Folaranmi, O.O., Mayxay, M., Khanthavong, M., Hongvanthong, B., Newton, P.N., Onyamboko, M.A., Fanello, C.I., Tshefu, A.K., Mishra, N., Valecha, N., Phyo, A.P., Nosten, F., Yi, P., Tripura, R., Borrmann, S., Bashraheil, M., Peshu, J., Faiz, M.A., Ghose, A., Hossain, M.A., Samad, R., Rahman, M.R., Hasan, M.M., Islam, A., Miotto, O., Amato, R., MacInnis, B., Stalker, J., Kwiatkowski, D.P., Bozdech, Z., Jeeyapant, A., Cheah, P.Y., Sakulthaew, T., Chalk, J., Intharabut, B., Silamut, K., Lee, S.J., Vihokhern, B., Kunasol, C., Imwong, M., Tarning, J., Taylor, W.J., Yeung, S., Woodrow, C.J., Flegg, J.A., Das, D., Smith, J., Venkatesan, M., Plowe, C.V., Stepniewska, K., Guerin, P.J., Dondorp, A.M., Day, N.P., White, N.J., Tracking Resistance to Artemisinin, C, 2014. Spread of artemisinin resistance in Plasmodium falciparum malaria. N. Engl. J. Med. 371, 411–423.

Barrette, A., Ringwald, P., 2010. Global Report on Antimalarial Drug Efficacy and Drug Resistance 2000-2010. WHO Press, Switzerland.

Conrad, M.D., Bigira, V., Kapisi, J., Muhindo, M., Kamya, M.R., Havlir, D.V., Dorsey, G., Rosenthal, P.J., 2014. Polymorphisms in K13 and falcipain-2 associated with artemisinin resistance are not prevalent in Plasmodium falciparum isolated from Ugandan children. PLoS One 9, e105690.

Cooper, R.A., Conrad, M.D., Watson, Q.D., Huezo, S.J., Ninsiima, H., Tumwebaze, P., Nsobya, S.L., Rosenthal, P.J., 2015. Lack of artemisinin resistance in plasmodium falciparum in Uganda based on parasitological and molecular assays. Antimicrob. Agents Chemother. 59, 5061–5064.

Cui, L., Wang, Z., Jiang, H., Parker, D., Wang, H., Su, X.Z., Cui, L., 2012. Lack of association of the S769N mutation in Plasmodium falciparum SERCA (PfATP6) with resistance to artemisinins. Antimicrob. agents Chemother. 56, 2546–2552.

Dahlstrom, S., Veiga, M.I., Ferreira, P., Martensson, A., Kaneko, A., Andersson, B., Bjorkman, A., Gil, J.P., 2008. Diversity of the sarco/endoplasmic reticulum Ca(2+)-ATPase orthologue of Plasmodium falciparum (PfATP6). Infect. Genet. Evol. J. Mol. Epidemiol. Evol. Genet. Infect. Dis. 8, 340–345.

Dondorp, A.M., Nosten, F., Yi, P., Das, D., Phyo, A.P., Tarning, J., Lwin, K.M., Ariey, F., Hanpithakpong, W., Lee, S.J., Ringwald, P., Silamut, K., Imwong, M., Chotivanich, K., Lim, P., Herdman, T., An, S.S., Yeung, S., Singhasivanon, P., Day, N.P., Lindegardh, N., Socheat, D., White, N.J., 2009. Artemisinin resistance in Plasmodium falciparum malaria. N. Engl. J. Med. 361, 455–467.

Feng, J., Li, J., Yan, H., Feng, X., Xia, Z., 2015. Evaluation of antimalarial resistance marker polymorphism in returned migrant workers in china. Antimicrob. Agents Chemother. 59, 326–330.

Happi, C.T., Gbotosho, G.O., Folarin, O.A., Sowunmi, A., Hudson, T., O'Neil, M., Milhous, W., Wirth, D.F., Oduola, A.M., 2009. Selection of Plasmodium falciparum multidrug resistance gene 1 alleles in asexual stages and gametocytes by artemether-lumefantrine in Nigerian children with uncomplicated falciparum malaria. Antimicrob. agents Chemother. 53, 888–895.

Harinasuta, T., Suntharasamai, P., Viravan, C., 1965. Chloroquine-resistant falciparum malaria in Thailand. Lancet 2, 657–660.

Ibrahim, M.L., Khim, N., Adam, H.H., Ariey, F., Duchemin, J.B., 2009. Polymorphism of PfATPase in Niger: detection of three new point mutations. Malar. J. 8, 28.

Jambou, R., Legrand, E., Niang, M., Khim, N., Lim, P., Volney, B., Ekala, M.T., Bouchier, C., Esterre, P., Fandeur, T., Mercereau-Puijalon, O., 2005. Resistance of Plasmodium falciparum field isolates to in-vitro artemether and point mutations of the SERCA-type PfATPase6. Lancet 366, 1960–1963.

Kamau, E., Campino, S., Amenga-Etego, L., Drury, E., Ishengoma, D., Johnson, K., Mumba, D., Kekre, M., Yavo, W., Mead, D., Bouyou-Akotet, M., Apinjoh, T., Golassa, L., Randrianarivelojosia, M., Andagalu, B., Maiga-Ascofare, O., Amambua-Ngwa, A., Tindana, P., Ghansah, A., MacInnis, B., Kwiatkowski, D., Djimde, A.A., 2015. K13-propeller polymorphisms in Plasmodium falciparum parasites from sub-Saharan Africa. J. Infect. Dis. 211, 1352–1355.

Kamugisha, E., Sendagire, H., Kaddumukasa, M., Enweji, N., Gheysari, F., Swedberg, G., Kironde, F., 2011. Detecting adenosine triphosphatase 6 (pfATP6) point mutations that may be associated with Plasmodium falciparum resistance to artemisinin: prevalence at baseline, before policy change in Uganda. Tanzan. J. Health Res. 13, 40–47.

Legrand, E., Volney, B., Meynard, J.B., Mercereau-Puijalon, O., Esterre, P., 2008. In vitro monitoring of Plasmodium falciparum drug resistance in French Guiana: a synopsis of continuous assessment from 1994 to 2005. Antimicrob. Agents Chemother. 52, 288–298.

Li, J., Chen, J., Xie, D., Eyi, U.M., Matesa, R.A., Obono, M.M., Ehapo, C.S., Yang, L., Yang, H., Lin, M., Wu, W., Wu, K., Li, S., Chen, Z., 2015. Molecular mutation profile of Pfcrt and Pfmdr1 in Plasmodium falciparum isolates from Bioko Island, Equatorial Guinea. Infect. Genet. Evol. J. Mol. Epidemiol. Evol. Genet. Infect. Dis.

Li, J., Chen, J., Xie, D., Monte-Nguba, S.M., Eyi, J.U., Matesa, R.A., Obono, M.M., Ehapo, C.S., Yang, L., Lu, D., Yang, H., Yang, H.T., Lin, M., 2014. High prevalence of pfmdr1 N86Y and Y184F mutations in Plasmodium falciparum isolates from Bioko Island, Equatorial Guinea. Pathogens Glob. Health 108, 339–343.

Menegon, M., Talha, A.A., Severini, C., Elbushra, S.M., Mohamedani, A.A., Malik, E.M., Mohamed, T.A., Wernsdorfer, W.H., Majori, G., Nour, B.Y., 2010. Frequency distribution of antimalarial drug resistance alleles among Plasmodium falciparum isolates from Gezira State, central Sudan, and Gedarif State, eastern Sudan. Am. J. Trop. Med. Hyg. 83, 250–257.

Miotto, O., Almagro-Garcia, J., Manske, M., Macinnis, B., Campino, S., Rockett, K.A., Amaratunga, C., Lim, P., Suon, S., Sreng, S., Anderson, J.M., Duong, S., Nguon, C., Chuor, C.M., Saunders, D., Se, Y., Lon, C., Fukuda, M.M., Amenga-Etego, L., Hodgson, A.V., Asoala, V., Imwong, M., Takala-Harrison, S., Nosten, F., Su, X.Z., Ringwald, P., Ariey, F., Dolecek, C., Hien, T.T., Boni, M.F., Thai, C.Q., Amambua-Ngwa, A., Conway, D.J., Djimde, A.A., Doumbo, O.K., Zongo, I., Ouedraogo, J.B., Alcock, D., Drury, E., Auburn, S., Koch, O., Sanders, M., Hubbart, C., Maslen, G., Ruano-Rubio, V., Jyothi, D., Miles, A., O'Brien, J., Gamble, C., Oyola, S.O., Rayner, J.C., Newbold, C.I., Berriman, M., Spencer, C.C., McVean, G., Day, N.P., White, N.J., Bethell, D., Dondorp, A.M., Plowe, C.V., Fairhurst, R.M., Kwiatkowski, D.P., 2013. Multiple populations of artemisinin-resistant Plasmodium falciparum in Cambodia. Nat. Genet. 45, 648–655.

Mohon, A.N., Alam, M.S., Bayih, A.G., Folefoc, A., Shahinas, D., Haque, R., Pillai, D.R., 2014. Mutations in Plasmodium falciparum K13 propeller gene from Bangladesh (2009–2013). Malar. J. 13, 431.

Mok, S., Ashley, E.A., Ferreira, P.E., Zhu, L., Lin, Z., Yeo, T., Chotivanich, K., Imwong, M., Pukrittayakamee, S., Dhorda, M., Nguon, C., Lim, P., Amaratunga, C., Suon, S., Hien, T.T., Htut, Y., Faiz, M.A., Onyamboko, M.A., Mayxay, M., Newton, P.N., Tripura, R., Woodrow, C.J., Miotto, O., Kwiatkowski, D.P., Nosten, F., Day, N.P., Preiser, P.R., White, N.J., Dondorp, A.M., Fairhurst, R.M., Bozdech, Z., 2015. Drug resistance. Population transcriptomics of human malaria parasites reveals the mechanism of artemisinin resistance. Science 347, 431–435.

Mugittu, K., Genton, B., Mshinda, H., Beck, H.P., 2006. Molecular monitoring of Plasmodium falciparum resistance to artemisinin in Tanzania. Malar. J. 5, 126.

Noedl, H., Se, Y., Schaecher, K., Smith, B.L., Socheat, D., Fukuda, M.M., Artemisinin Resistance in Cambodia 1 Study, C, 2008. Evidence of artemisinin-resistant

malaria in western Cambodia. N. Engl. J. Med. 359, 2619–2620.
Nyunt, M.H., Hlaing, T., Oo, H.W., Tin-Oo, L.L., Phway, H.P., Wang, B., Zaw, N.N., Han, S.S., Tun, T., San, K.K., Kyaw, M.P., Han, E.T., 2014. Molecular assessment of artemisinin resistance markers, polymorphisms in the K13 propeller, and a multidrug-resistance gene in the eastern and western border areas of Myanmar. Clin. Infect. Dis. off. Publ. Infect. Dis. Soc. Am.
Ouattara, A., Kone, A., Adams, M., Fofana, B., Maiga, A.W., Hampton, S., Coulibaly, D., Thera, M.A., Diallo, N., Dara, A., Sagara, I., Gil, J.P., Bjorkman, A., Takala-Harrison, S., Doumbo, O.K., Plowe, C.V., Djimde, A.A., 2015. Polymorphisms in the K13-propeller gene in artemisinin-susceptible Plasmodium falciparum parasites from Bougoula-Hameau and Bandiagara, Mali. Am. J. Trop. Med. Hyg. 92, 1202–1206.
Overgaard, H.J., Reddy, V.P., Abaga, S., Matias, A., Reddy, M.R., Kulkarni, V., Schwabe, C., Segura, L., Kleinschmidt, I., Slotman, M.A., 2012. Malaria transmission after five years of vector control on Bioko Island, Equatorial Guinea. Parasites Vectors 5, 253.
Rehman, A.M., Mann, A.G., Schwabe, C., Reddy, M.R., Roncon Gomes, I., Slotman, M.A., Yellott, L., Matias, A., Caccone, A., Nseng Nchama, G., Kleinschmidt, I., 2013. Five years of malaria control in the continental region, Equatorial Guinea. Malar. J. 12, 154.
Roper, C., Pearce, R., Nair, S., Sharp, B., Nosten, F., Anderson, T., 2004. Intercontinental spread of pyrimethamine-resistant malaria. Science 305, 1124.
Straimer, J., Gnadig, N.F., Witkowski, B., Amaratunga, C., Duru, V., Ramadani, A.P., Dacheux, M., Khim, N., Zhang, L., Lam, S., Gregory, P.D., Urnov, F.D., Mercereau-Puijalon, O., Benoit-Vical, F., Fairhurst, R.M., Menard, D., Fidock, D.A., 2015. Drug resistance. K13-propeller mutations confer artemisinin resistance in Plasmodium falciparum clinical isolates. Science 347, 428–431.
Sylla, K., Abiola, A., Tine, R.C., Faye, B., Sow, D., Ndiaye, J.L., Ndiaye, M., Lo, A.C., Folly, K., Ndiaye, L.A., Gaye, O., 2013. Monitoring the efficacy and safety of three artemisinin based-combinations therapies in Senegal: results from two years surveillance. BMC Infect. Dis. 13, 598.
Talisuna, A.O., Karema, C., Ogutu, B., Juma, E., Logedi, J., Nyandigisi, A., Mulenga, M., Mbacham, W.F., Roper, C., Guerin, P.J., D'Alessandro, U., Snow, R.W., 2012. Mitigating the threat of artemisinin resistance in Africa: improvement of drug-resistance surveillance and response systems. Lancet. Infect. Dis. 12, 888–896.
Talundzic, E., Okoth, S.A., Congpuong, K., Plucinski, M.M., Morton, L., Goldman, I.F., Kachur, P.S., Wongsrichanalai, C., Satimai, W., Barnwell, J.W., Udhayakumar, V., 2015. Selection and spread of artemisinin-resistant alleles in Thailand prior to the global artemisinin resistance containment campaign. PLoS Pathog. 11, e1004789.
Taylor, S.M., Parobek, C.M., DeConti, D.K., Kayentao, K., Coulibaly, S.O., Greenwood, B.M., Tagbor, H., Williams, J., Bojang, K., Njie, F., Desai, M., Kariuki, S., Gutman, J., Mathanga, D.P., Martensson, A., Ngasala, B., Conrad, M.D., Rosenthal, P.J., Tshefu, A.K., Moormann, A.M., Vulule, J.M., Doumbo, O.K., Ter Kuile, F.O., Meshnick, S.R., Bailey, J.A., Juliano, J.J., 2014. Absence of Putative artemisinin resistance mutations among plasmodium falciparum in sub-Saharan africa: a molecular epidemiologic study. J. Infect. Dis.
Torrentino-Madamet, M., Fall, B., Benoit, N., Camara, C., Amalvict, R., Fall, M., Dionne, P., Ba Fall, K., Nakoulima, A., Diatta, B., Dieme, Y., Menard, D., Wade, B., Pradines, B., 2014. Limited polymorphisms in k13 gene in Plasmodium falciparum isolates from Dakar, Senegal in 2012-2013. Malar. J. 13, 472.
Tun, K.M., Imwong, M., Lwin, K.M., Win, A.A., Hlaing, T.M., Hlaing, T., Lin, K., Kyaw, M.P., Plewes, K., Faiz, M.A., Dhorda, M., Cheah, P.Y., Pukrittayakamee, S., Ashley, E.A., Anderson, T.J., Nair, S., McDew-White, M., Flegg, J.A., Grist, E.P., Guerin, P., Maude, R.J., Smithuis, F., Dondorp, A.M., Day, N.P., Nosten, F., White, N.J., Woodrow, C.J., 2015. Spread of artemisinin-resistant Plasmodium falciparum in Myanmar: a cross-sectional survey of the K13 molecular marker. Lancet. Infect. Dis. 15, 415–421.
Wang, Z., Wang, Y., Cabrera, M., Zhang, Y., Gupta, B., Wu, Y., Kemirembe, K., Hu, Y., Liang, X., Brashear, A., Shrestha, S., Li, X., Miao, J., Sun, X., Yang, Z., Cui, L., 2015. Artemisinin resistance at the China-Myanmar border and association with mutations in the K13 propeller gene. Antimicrob. Agents Chemother. 59, 6952–6959.
Wongsrichanalai, C., Pickard, A.L., Wernsdorfer, W.H., Meshnick, S.R., 2002. Epidemiology of drug-resistant malaria. Lancet. Infect. Dis. 2, 209–218.
Wootton, J.C., Feng, X., Ferdig, M.T., Cooper, R.A., Mu, J., Baruch, D.I., Magill, A.J., Su, X.Z., 2002. Genetic diversity and chloroquine selective sweeps in Plasmodium falciparum. Nature 418, 320–323.
Young, M.D., Contacos, P.G., Stitcher, J.E., Millar, J.W., 1963. Drug resistance in plasmodium falciparum from Thailand. Am. J. Trop. Med. Hyg. 12, 305–314.
Zatra, R., Lekana-douki, J.B., Lekoulou, F., Bisvigou, U., Ngoungou, E.B., Ndouo, F.S., 2012. In vitro antimalarial susceptibility and molecular markers of drug resistance in Franceville, Gabon. BMC Infect. Dis. 12, 307.
Zhang, G.Q., Guan, Y.Y., Sheng, H.H., Zheng, B., Wu, S., Xiao, H.S., Tang, L.H., 2008. Multiplex PCR and oligonucleotide microarray for detection of single-nucleotide polymorphisms associated with Plasmodium falciparum drug resistance. J. Clin. Microbiol. 46, 2167–2174.