Parasit Host Dis 2023;61(1):78-83
https//doi.org/10.3347/PHD.22140
pISSN 2982-5164

Brief Communication

Monitoring antimalarial drug-resistance markers in Somalia

Abdifatah Abdullahi Jalei[1], Kesara Na-Bangchang[1,2,3], Phunuch Muhamad[2], Wanna Chaijaroenkul[1,3,*]

[1]Chulabhorn International College of Medicine, Thammasat University, Klong Luang, Pathumthani 12120, Thailand; [2]Drug Discovery and Development Center, Office of Advanced Science and Technology, Thammasat University, Pathumthani 12120, Thailand; [3]Center of Excellence in Pharmacology and Molecular Biology of Malaria and Cholangiocarcinoma, Chulabhorn International College of Medicine, Thammasat University, Klong Luang, Pathumthani 12120, Thailand

Received: 12 October 2022
Accepted: 16 January 2023

***Correspondence**
(wn_ap39@yahoo.com)

Citation
Jalei AA, Na-Bangchang K, Muhamad P, Chaijaroenkul W. Monitoring antimalarial drug-resistance markers in Somalia. Parasit Host Dis 2023;61(1):78-83.

Abstract

The use of an effective antimalarial drug is the cornerstone of malaria control. However, the development and spread of resistant *Plasmodium falciparum* strains have placed the global eradication of malaria in serious jeopardy. Molecular marker analysis constitutes the hallmark of the monitoring of *Plasmodium* drug-resistance. This study included 96 *P. falciparum* PCR-positive samples from southern Somalia. The *P. falciparum chloroquine resistance transporter* gene had high frequencies of K76T, A220S, Q271E, N326S, and R371I point mutations. The N86Y and Y184F mutant alleles of the *P. falciparum multi-drug resistance 1* gene were present in 84.7 and 62.4% of the isolates, respectively. No mutation was found in the *P. falciparum Kelch-13* gene. This study revealed that chloroquine resistance markers are present at high frequencies, while the parasite remains sensitive to artemisinin (ART). The continuous monitoring of ART-resistant markers and in vitro susceptibility testing are strongly recommended to track resistant strains in real time.

Keywords: *Plasmodium falciparum*, drug-resistance, molecular marker, Southern Somalia

Malaria is a significant health problem in most tropical countries, including Somalia. *Plasmodium falciparum* is the most prevalent malaria species in sub-Saharan Africa [1]. The emergence of multidrug-resistant *P. falciparum* strains poses a challenge to malaria control plans and has considerably increased drug costs. This situation endangers affordable, safe, and cheap drugs in the least-developed countries wherein malaria control is ineffective [2]. The World Health Organization recommends using artemisinin (ART)-based combination therapies as the first-line treatment for uncomplicated falciparum malaria to confront the spread of treatment-resistant malaria strains [3]. In 2006, Somalia discontinued chloroquine (CQ) as the first-line therapy for uncomplicated falciparum malaria because CQ has high treatment failure rates that range from 76.5 to 88.0% in Janale, Jamame, and Jowhar. The combination of artesunate and sulphadoxine–pyrimethamine was introduced into the national malaria control program [4]. The country's first-line treatment was later switched to artemether–lumefantrine (AL) due to numerous mutations in antifolate resistance markers [5]. ART-resistance has been reported to be strongly associated with non-synonymous single nucleotide polymorphisms within the propeller region of the *P. falciparum Kelch-13* (*pfk13*) gene [6]. Of the 10 validated ART-resistance mutations, 6 (M476I, P553L, R561H, P574L, C580Y, and A675V) have been found at low frequencies in 4 African countries. However, Rwanda is the only country to report delayed parasite clearance

Author contributions
Conceptualization: Jalei AA, Chaijaroenkul W
Data curation: Chaijaroenkul W
Formal analysis: Jalei AA, Muhamad P, Chaijaroenkul W
Funding acquisition: Na-Bangchang K
Investigation: Na-Bangchang K
Methodology: Jalei AA, Muhamad P
Resources: Na-Bangchang K, Muhamad P
Supervision: Na-Bangchang K
Validation: Na-Bangchang K, Chaijaroenkul W
Writing – original draft: Jalei AA
Writing – review & editing: Na-Bangchang K, Chaijaroenkul W

Conflict of interest
We have no conflict of interest related to this work.

ORCID
Abdifatah Abdullahi Jalei (https://orcid.org/0000-0002-4701-4683)
Wanna Chaijaroenkul (https://orcid.org/0000-0002-3720-3878)

(3+ days) [7]. The emergence of *pfk13* mutations is an early warning sign for Africa given that an increase in the frequency and spread of these mutations across the continent could have catastrophic consequences for malaria control. The purpose of the present study is to determine the prevalence rates of drug-resistance molecular markers (polymorphisms in the *P. falciparum chloroquine resistance transporter* [*pfcrt*] gene, *P. falciparum multidrug resistance 1* [*pfmdr1*], *P. falciparum ATPase 6* [*pfatp6*], and *pfk13*) and the copy number variation (CNV) of *pfmdr1* in isolates from Afgoi and Balad in southern Somalia.

Approval for the study protocol was given by the Ethics Review Committee for Research in Human Subjects, Ministry of Health of Somalia (No. 0776/May/2018). Informed consent for study participation was obtained from all participants. A total of 150 dried blood spot samples were collected in 2018 from patients with *P. falciparum* infection attending malaria clinics in southern Somalia (Afgoi and Balad; Fig. 1). DNA was extracted from blood samples spotted on filter paper by using a QIAamp DNA Mini kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's protocol then used as a template for PCR amplification. The polymorphisms of *pfcrt, pfmdr1,* and *pfatp6* were examined by using PCR with restriction fragment length polymorphism [8,9]. CNVs were assessed by utilizing a Bio-Rad iCycler (Bio-Rad, Hercules, CA, USA) [10]. The *pfk13* gene was amplified by nested PCR as described by Ariey et al. [6]. Amplified products were purified by applying a QIAquick PCR purification kit (Qiagen) then sequenced by a commercial vendor (Bioneer, Daejeon, Republic of Korea). DNA sequencing was performed in 2 directions for complete coverage. The sequences of each isolate were aligned with the 3D7 reference strain (GenBank accession number, XM_001350122) by employing BioEdit software ver7.0.5.2 (Ibis Therapeutics, Carlsbad, CA, USA). Chi-square or Fisher's exact test was used to compare the proportions of the mutated points in the 2 study sites (Afgoi vs Balad). The level of sta-

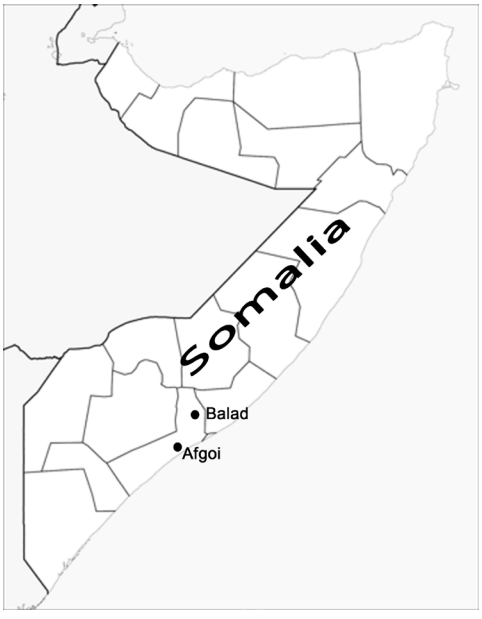

Fig. 1. Map of Somalia showing the study site.

tistical significance was set at $\alpha = 0.05$.

A total of 150 microscopically confirmed *P. falciparum* samples were collected for the study. PCR confirmed the presence of malaria species in 67.3% of the samples (96 *P. falciparum* and 5 *Plasmodium vivax*), whereas the remaining samples were PCR-negative. Thus, 96 *P. falciparum* samples were included in molecular analysis. Most of the samples (81.0%) included in the molecular analysis were from the Afgoi district in the Lower Shabelle region. Eighty-six samples were successfully genotyped at all codons of *pfcrt*. All isolates from Balad showed mutant genotypes at codons K76T, A220S, Q271E, and R371I and wild genotypes at codon I356T. The frequencies of each mutant point showed no significant difference between Afgoi and Balad isolates ($P > 0.05$) (Table 1). The frequency of the *pfmdr1* mutation among the 85 samples that were successfully amplified is shown in Table 1. Mutations in *pfmdr1* were only seen at N86Y and Y184F in the isolates from the 2 study areas. Meanwhile, the 3 other positions (S1034C, N1042D, and D1246Y) were 100.0% wild-type. Four haplotype patterns of *pfmdr1* were found: NYSND (2.4%), YYSND (35.3%), NFSND (12.9%),

Table 1. Distribution of *pfcrt, pfmdr1* and *pfatp6* in *Plasmodium falciparum* isolates from southern Somalia. Data is presented as the number of isolates (%)

Gene	Codon	Amino Acid	No. of isolates (%)		
			Afgoi	Balad	Total
pfcrt	76	K (Wild-type)	15 (17.4)	0 (0.0)	15 (17.4)
		T[b] (Mutation)	54 (62.8)	17 (19.8)	71 (82.6)
	220	A (Wild-type)	2 (2.3)	0 (0.0)	2 (2.3)
		S[b] (Mutation)	67 (77.9)	17 (19.8)	84 (97.7)
	271	Q (Wild-type)	1 (1.2)	0 (0.0)	1 (1.2)
		E[b] (Mutation)	68 (79.1)	17 (19.8)	85 (98.8)
	326	N (Wild-type)	7 (8.1)	11 (12.8)	18 (20.9)
		S[b] (Mutation)	62 (72.1)	6 (7.0)	68 (79.1)
	356	I (Wild-type)	67 (77.9)	17 (19.8)	84 (97.7)
		T[b] (Mutation)	2 (2.3)	0 (0.0)	2 (2.3)
	371	R (Wild-type)	5 (5.8)	0 (0.0)	5 (5.8)
		I[b] (Mutation)	64 (74.4)	17 (19.8)	81 (94.2)
pfmdr1	86[a]	N (Wild-type)	7 (8.2)	6 (7.1)	13 (15.3)
		Y[b] (Mutation)	62 (72.9)	10 (11.8)	72 (84.7)
	184	Y (Wild-type)	25 (29.4)	7 (8.2)	32 (37.6)
		F[b] (Mutation)	44 (51.8)	9 (10.6)	53 (62.4)
	1034	S (Wild-type)	69 (81.2)	16 (18.8)	85 (100.0)
	1042	N (Wild-type)	69 (81.2)	16 (18.8)	85 (100.0)
	1246	D (Wild-type)	69 (81.2)	16 (18.8)	85 (100.0)
	Copy no.	1 copy	65 (77.4)	16 (19.0)	81 (96.4)
		2 copies	3 (3.6)	0 (0.0)	3 (3.6)
pfatp6	37	R (Wild-type)	67 (77.9)	16 (18.6)	83 (96.5)
		K[b] (Mutation)	3 (3.5)	0 (0.0)	3 (3.5)
	639	G (Wild-type)	66 (76.7)	16 (18.6)	82 (95.3)
		D[b] (Mutation)	4 (4.7)	0 (0.0)	4 (4.7)
	769	S (Wild-type)	70 (81.4)	16 (18.6)	86 (100.0)
	898	I (Wild-type)	46 (53.5)	11 (12.8)	57 (66.3)
		I[b] (Mutation)	24 (27.9)	5 (5.8)	29 (33.7)

[a]Statistically significant difference between Afgoi and Balad isolates ($P = 0.006$).
[b]Mutation codons.
pfcr, P. falciparum chloroquine resistance transporter; pfmdr1, P. falciparum multidrug resistance 1; pfatp6, P. falciparum ATPase 6.

and YFSND (49.4%). The most dominant haplotype was the YFSND double-mutated haplotype, which represents the substitution of the amino acid tyrosine for codon 86 and phenylalanine for codon 184 [8]. On the other hand, the wild NYSND haplotype had the lowest prevalence. A total of 84 isolates were subjected to *pfmdr1* gene amplification analysis. Only 3 isolates from Afgoi were found to carry 2 copies of *pfmdr1* (Table 1). The *pfatp6* gene had mutant genotypes at codons R37K, G639D, and I898I and a wild-type S769N codon. The I898I mutation was detected in isolates from the 2 study sites, whereas mutations at codons R37K and G639D were detected only in Afgoi isolates (Table 1). All 84 samples that were successfully sequenced for *P. falciparum* isolates carried the wild-type alleles of *pfk13*.

The molecular analysis of antimalarial drug-resistance markers provides information on parasite gene mutations and/or gene copy number changes associated with drug-resistance [11]. The *pfcrt* and *pfmdr1* genes are generally considered markers of CQ resistance (CQR) [8]. The *pfmdr1* gene and the pleiotropic drug-resistance transporter *pfcrt* reside on the membrane of the parasite's digestive vacuole and are believed to prevent osmotic stress in the vacuole by exporting host-derived peptides and delivering them to the cytosol, where they are degraded into amino acids to fuel parasite growth [12]. The *pfcrt* gene is a stronger predictor of CQR than *pfmdr1*. The K76T mutation is the crucial marker of CQR, whereas the other alleles of the *pfcrt* gene confer a lesser degree of CQR [8]. In this study, the K76T mutation was found in 82.6% of isolates. All isolates from Balad carried the K76T mutation. The study also showed high mutation rates at codons A220S, Q271E, N326S, and R371I. The accumulation of point mutations in the *pfcrt* gene has been associated with a high level of CQR [13]. This finding contradicts previously reported results showing the re-emergence of CQ-sensitive *P. falciparum* populations after the withdrawal of CQ from the national treatment policy [14]. This situation might be due to the availability of the drug in local drug stores that leads to continued drug pressure on circulating malaria parasites [15]. Of the 5 polymorphisms of the *pfmdr1* gene, no mutations were detected at positions S1034C, N1042D, or D1246Y in any isolate. The wild haplotype of these 3 points is common in CQR parasites in Africa and Asia, whereas their mutant haplotypes are common in CQR parasites from South America [16]. The present study found predominant mutations of *pfmdr1* N86Y and Y184F (84.7 and 62.4%, respectively). However, in accordance with study sites, only the N86Y mutation showed a significant difference in frequencies between the isolates from Afgoi and Balad ($P < 0.05$). This discrepancy might be due to differences in sample size (Afgoi vs. Balad = 69 vs. 16). The mutation profiles of N86Y and Y184F showed similar patterns, as reported by a previous study from Tanzania [17]. The N86Y mutant allele contributes to resistance to CQ and amodiaquine but increases parasite susceptibility to lumefantrine, mefloquine, and dihydroartemisinin [16]. No previously published data on *pfcrt* and *pfmdr1* allelic variants from Somalia are available for comparison with the present study's findings. Among the *pfmdr1* haplotypes, YFSND was the most common (49.4%), followed by YYSND (35.3%). The relatively high frequency of the double mutational haplotype detected in the present study might be due to continuous selective pressure from CQ [15]. This study also showed a low prevalence rate of 2 *pfmdr1* copies (3.6%) in southern Somalia. This finding is consistent with the result of a study from Kenya, which reported a prevalence of 2 *pfmdr1* copies of less than 10.0% [18]. Notably, in-

creased *pfmdr1* copy number was associated with lower parasite susceptibility to mefloquine and partial susceptibility to lumefantrine [10]. Most likely, the low rate of parasites with 2 copies of *pfmdr1* is due to the fact that AL has been a part of the national policy for malaria treatment since 2016 [5]. The *pfk13* and *pfatp6* genes are potential markers among the candidate genes for *P. falciparum* ART-resistance [6,9]. Jambou et al. [9] observed that the mutation at S769N in the *pfatp6* gene is associated with an increased in vitro susceptibility to artemether. Although this work discovered that *pfatp6* allelic variants had a relatively high mutation rate at I898I (33.7%), none of the isolates carried a mutation at *pfatp6* S769N. No mutation in the *pfk13* gene was observed in any of the studied isolates. A cross-sectional study conducted in Kenya to determine the prevalence of *pfk13* substitutions reported that no mutations were associated with ART-resistance in Africa [19]. The present work discovered that *pfatp6* S769N, which is known to confer resistance to ART, was wild-type [9]. The absence of mutations in ART-resistance genes indicates that AL has only been in policy for malaria control for 2 years [5].

In conclusion, this study found a high prevalence of *pfcrt* and *pfmdr1* mutations in *P. falciparum* isolates from 2 endemic areas in southern Somalia, indicating that CQ remains inappropriate for *falciparum* malaria treatment in Somalia. On the other hand, no mutation in the *pfk13* gene was detected. Therefore, AL, which is currently in use in the country, is likely to be effective. However, regular surveillance is essential if the development of widespread ART-resistance is to be prevented.

Acknowledgments

We thank all the patients who participated in the study. Special thanks go to the staff of Balad Hospital and Afgoi General Hospital, especially Mr. Mohamed Omar, Mr. Ahmed Hersi, and Ms. Fartun Mohamed, for their assistance in sample collection. We also thank Chulabhorn International College of Medicine of Thammasat University, Center of Excellence in Pharmacology and Molecular Biology of Malaria and Cholangiocarcinoma of Thammasat University, for the support of this research. Kesara Na-Bangchang is supported by the National Research Council of Thailand under the Research Team Promotion grant (grant number NRCT 820/2563).

References

1. Bousema T, Drakeley C. Epidemiology and infectivity of *Plasmodium falciparum* and *Plasmodium vivax* gametocytes in relation to malaria control and elimination. *Clin Microbiol Rev* 2011;24(2):377-410. https://doi.10.1128/CMR.00051-10
2. Jalei AA, Chaijaroenkul W, Na-Bangchang K. *Plasmodium falciparum* drug resistance gene status in the Horn of Africa: a systematic review. *Afr J Pharm Pharmacol* 2018;12(25):361-373. https://doi.org/10.5897/AJPP2018.4942
3. World Health Organization. Guidelines for the Treatment of Malaria. World Health Organization. Geneva, Switzerland. 2015. https://apps.who.int/iris/handle/10665/162441
4. Warsame M, Atta H, Klena JD, Waqar BA, Elmi HH, et al. Efficacy of monotherapies and artesunate-based combination therapies in children with uncomplicated malaria in Somalia. *Acta Trop* 2009;109(2):146-151. https://doi.10.1016/j.actatropica.2008.10.009
5. Warsame M, Hassan AH, Hassan AM, Arale AM, Jibril AM, et al. Efficacy of artesunate+ sulphadoxine/pyrimethamine and artemether+ lumefantrine and *dhfr* and *dhps* mutations in Somalia: evidence for updating the malaria treatment policy. *Trop Med Int Health* 2017;22(4):415-422. https://doi.10.1111/tmi.12847
6. Ariey F, Witkowski B, Amaratunga C, Beghain J, Langlois AC, et al. A molecular marker of artemisinin-resistant *Plasmodi-*

um falciparum malaria. *Nature* 2014;505(7481):50-55. https://doi.10.1038/nature12876
7. Ndwiga L, Kimenyi KM, Wamae K, Osoti V, Akinyi M, et al. A review of the frequencies of *Plasmodium falciparum* Kelch 13 artemisinin resistance mutations in Africa. *Int J Parasitol Drugs Drug Resist* 2021;16:155-161. https://doi.org/10.1016/j.ijpddr.2021.06.001
8. Djimdé A, Doumbo OK, Cortese JF, Kayentao K, Doumbo S, et al. A molecular marker for chloroquine-resistant falciparum malaria. *N Engl J Med* 2001;344(4):257-263. https://doi.10.1056/NEJM200101253440403
9. Jambou R, Legrand E, Niang M, Khim N, Lim P, et al. Resistance of *Plasmodium falciparum* field isolates to in-vitro artemether and point mutations of the SERCA-type PfATPase6. *Lancet* 2005;366(9501):1960-1963. https://doi.org/10.1016/S0140-6736(05)67787-2
10. Win AA, Imwong M, Kyaw MP, Woodrow CJ, Chotivanich K, et al. K13 mutations and *pfmdr1* copy number variation in *Plasmodium falciparum* malaria in Myanmar. *Malar J* 2016;15:1-7. https://doi.10.1186/s12936-016-1147-3
11. Nsanzabana C, Ariey F, Beck HP, Ding XC, Kamau E, et al. Molecular assays for antimalarial drug resistance surveillance: a target product profile. *PLoS One* 2018;13(9):e0204347. https://doi.10.1371/journal.pone.0204347
12. Shafik SH, Cobbold SA, Barkat K, Richards SN, Lancaster NS, et al. The natural function of the malaria parasite's chloroquine resistance transporter. *Nat commun* 2020;11(1):1-16. https://doi.10.1038/s41467-020-17781-6
13. Durrand V, Berry A, Sem R, Glaziou P, Beaudou J, et al. Variations in the sequence and expression of the *Plasmodium falciparum* chloroquine resistance transporter (Pfcrt) and their relationship to chloroquine resistance in vitro. *Mol Biochem Parasitol* 2004;136(2):273-285. https://doi.10.1016/j.molbiopara.2004.03.016
14. Mwai L, Ochong E, Abdirahman A, Kiara SM, Ward S, et al. Chloroquine resistance before and after its withdrawal in Kenya. *Malar J* 2009;8:1-10. https://doi.10.1186/1475-2875-8-106
15. Ikegbunam MN, Nkonganyi CN, Thomas BN, Esimone CO, Velavan TP, et al. Analysis of *Plasmodium falciparum Pfcrt* and *Pfmdr1* genes in parasite isolates from asymptomatic individuals in Southeast Nigeria 11 years after withdrawal of chloroquine. *Malar J* 2019;18(1):1-7. https://doi.10.1186/s12936-019-2977-6
16. Veiga MI, Dhingra SK, Henrich PP, Straimer J, Gnädig N, et al. Globally prevalent PfMDR1 mutations modulate *Plasmodium falciparum* susceptibility to artemisinin-based combination therapies. *Nat Commun* 2016;7:1-12. https://doi.10.1038/ncomms11553
17. Kavishe RA, Paulo P, Kaaya RD, Kalinga A, van Zwetselaar M, et al. Surveillance of artemether-lumefantrine associated *Plasmodium falciparum* multidrug resistance protein-1 gene polymorphisms in Tanzania. *Malar J* 2014;13:1-6. https://doi.10.1186/1475-2875-13-264
18. Ngalah BS, Ingasia LA, Cheruiyot AC, Chebon LJ, Juma DW, et al. Analysis of major genome loci underlying artemisinin resistance and *pfmdr1* copy number in pre-and post-ACTs in western Kenya. *Sci Rep* 2015;5:1-6. https://doi.10.1038/srep08308
19. Muwanguzi J, Henriques G, Sawa P, Bousema T, Sutherland CJ, et al. Lack of K13 mutations in *Plasmodium falciparum* persisting after artemisinin combination therapy treatment of Kenyan children. *Malar J* 2016;15:1-6. https://doi.10.1186/s12936-016-1095-y