RESEARCH ARTICLE

Screening for antifolate and artemisinin resistance in *Plasmodium falciparum* clinical isolates from three hospitals of Eritrea [version 1; peer review: 1 not approved]

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Abstract

Background: Antimalarial drug resistance is a major challenge hampering malaria control and elimination. *Plasmodium falciparum*, the leading causative parasite species, has developed resistance to basically all antimalarials. Continued surveillance of drug resistance using genetic markers provides important molecular data for treatment policies. This study sought to verify the genetic mechanism of resistance to sulfadoxine-pyrimethamine and assess the occurrence of point mutations associated with artemisinin resistance in *P. falciparum* clinical isolates from Eritrea.

Methods: Nineteen dried blood spot samples were collected from patients visiting Adi Quala, Keren and Gash Barka Hospitals, Eritrea. The patients were followed up after receiving treatment with first line artesunate-amodiaquine. Nested polymerase chain reaction and Sanger sequencing techniques were employed to genotype point mutations in the *P. falciparum* bifunctional dihydrofolate reductase-thymidylate synthase (*Pfdhfr*, PF3D7_0417200), dihydropteorate synthase (*Pfdhps*, PF3D7_0810800) and kelch 13 (*PfK13*, PF3D7_1343700) genes.

Results: Eight of nineteen (42%) of the dried blood spot samples were successful for PCR-amplification. Data analyses of the PCR-positive isolates revealed the following point mutations: *Pfdhfr* N51I in four isolates, C59R in one isolate, S108N in four isolates, a rare non-synonymous substitution V45A in four isolates and *Pfdhps* K540E in four isolates. No *PfK13* point mutations were reported.

Conclusions: *Pfdhfr* C59R and *Pfdhps* K540E point mutations are reliable markers for the sulfadoxine-pyrimethamine quintuple mutant haplotype combination. These findings highlight first reports in Eritrea, which verify the underlying genetic mechanism of antifolate

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Any reports and responses or comments on the article can be found at the end of the article.

resistance. Continuous monitoring of the *PfK13* marker is recommended.

Keywords

drug resistance, *Plasmodium falciparum*, antifolate, artemisinin, genetic markers, Eritrea

This article is included in the **Pathogens** gateway.

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Introduction

Malaria is a major vector-borne disease, endemic in 87 tropical and sub-tropical countries, causing over 400,000 deaths yearly (WHO World Malaria Report 2020). Eritrea, which is situated in the Horn of Africa, has experienced a significant decline in deaths and cases of malaria over the past 20 years (WHO World Malaria Report 2019). This reduction, according to the Ministry of Health (MOH) reports, is mainly due to extensive interventions employed towards the control of malaria since the establishment of the Eritrea National Malaria Control Program (NMCP) in 1995.¹ Working hand-in-hand with Roll Back Malaria (RBM) collaborators and stakeholders,² NMCP set up a combination of strategies including integrated vector management (IVM), early diagnosis and prompt treatment³ consequently leading to a remarkable decrease in incidence and mortality rates, following the gruesome 1998 malaria epidemic in the country.⁴ The disease is generally endemic in the Western lowlands of Gash Barka, Anseba, Debub and Semenawi Keih Bahri (Northern Red Sea) zobas (regions) whereas the Central highlands and Eastern lowlands of Maekel and Debubawi Keih Bahri (Southern Red Sea) zobas respectively have unstable, seasonal transmission. July–September is the common rainy season and hence malaria transmission peaks between October–November in a majority of the endemic areas while in the Coastal region the rainy season mostly occurs between December–January leading to a heightened transmission in March–April.^{5,6} About three-quarters of confirmed malaria cases in Eritrea are caused by *Plasmodium falciparum* and the remaining one-quarter is attributed to *Plasmodium vivax*, as well as small proportions of mixed infections (WHO African Region: Eritrea 2018). Currently, case management in Eritrea exclusively entails World Health Organisation (WHO) recommended first line treatment of uncomplicated malaria using artesunate-amodiaquine (AS-AQ), an artemisinin-based combination therapy (ACT) adopted in 2007, while quinine (Q) has been used for severe cases of infection since 2002 (WHO African Region: Eritrea 2018). Monitoring for drug resistance plays a major role in governing the efficacy of antimalarials, which subsequently influences their use in a population.

The emergence of drug resistance, especially among *P. falciparum* parasites, is a major hindrance to malaria control due to its increasing prevalence to essentially all antimalarials including sulfadoxine-pyrimethamine (SP) and lately artemisinins (ARTs).⁷ Genetic markers are invaluable tools in screening and detection of drug resistance, in addition to predicting the efficacy of antimalarials.⁸ Sulfadoxine-pyrimethamine *P. falciparum* resistance (SPR), which is well-studied, results from the occurrence and accumulation of mutations in the dihydrofolate reductase gene (*Pfdhfr*) and in the dihydropteroate synthase gene (*Pfdhps*) leading to a gradual reduction of sensitivity to pyrimethamine and sulfadoxine respectively.⁹ *In vitro* and *in vivo* studies have shown that SPR is mainly associated with point mutations at codons N51I, C59R, S108N and I164L of *Pfdhfr* and S436A, A437G, K540E, A581G and A613S of *Pfdhps*.^{10,11} Various combinations of these mutations have been used to classify SP resistant parasites according to different levels of resistance i.e. partially-, fully- or super resistant parasites and this has subsequently affected SP treatment policy. Partial resistance is demonstrated by a combination of triple mutant *Pfdhfr*, N51I, C59R, S108N and *Pfdhps*, A437G whereas full resistance is shown by a combination of triple mutant *Pfdhfr*, N51I, C59R, S108N and double mutant *Pfdhfr*, A437G, K540E. Finally, the sextuple mutant genotype involving a combination of triple mutant *Pfdhfr*, N51I, C59R, S108N and triple mutant *Pdhfr*, A437G, K540E and A581G defines super resistance.¹²

The development of artemisinin (ART) resistant *P. falciparum* parasites was first independently described in Western Cambodia, South East Asia.¹³ To date, resistance is commonly associated with five non-synonymous mutations including M476I, Y493H, R539T, I543T, and C580Y in the propeller domain of *P. falciparum* kelch 13 gene (*Pfk13*).^{14,15} ART resistance is primarily characterized by delayed parasite clearance rates in clinical studies as well as reduced *in vitro* drug susceptibility of the ring stage of parasite development.^{16,17} Considering the significant malaria control interventions accomplished in Eritrea, this pilot study aimed at availing supplementary molecular data by screening for SP and ART resistance-associated mutations from a cohort of patients, treated with first line AS-AQ, visiting selected hospitals located in malaria endemic regions of Eritrea. Generally, despite WHO's change in treatment policy from the chloroquine (CQ) - sulfadoxine-pyrimethamine (SP) combination, adopted in 2002 to ACT, little is documented on the genetic mechanism underlying SPR using genetic markers. Additionally, a continuous detection for ART-resistance using genetic markers is important to keep track of changes at the genetic level.

Methods

Ethical statement

The ethical approval for this study was obtained from the Eritrea Institute of Technology, Research and Postgraduate Studies (RPS) Ethics Review Committee (Reference no. RPS/169/14) and the Ethics Review Board of the National Commission for Higher Education, Eritrea (NCHE) (Reference no. BHEAIL/3/656-568/14).

Study sites and sample collection.

Sample collection was conducted from 1st July to 1st October 2014 at three hospitals located in malaria-endemic zobas of Eritrea: Adi Quala Hospital, Adi Quala (14°38'07"N, 38°50'03"E) in Zoba Debub, Keren Hospital, Keren (15°46'40"N,

38°27'03''E) in Zoba Anseba and Gash Barka Referral Hospital, Barentu (15°06'20''N, 37°35'26''E) in Zoba Gash Barka. Three time ranges were employed for the study at the three hospitals: from 1st July to 31st August 2014 for Adi Qualla Hospital, 16th July to 15th September 2014 for Keren Hospital and 15th August to 1st October 2014 for Gash Barka Referral Hospital.

Blood samples were obtained from patients with febrile illness, who visited the three hospitals within the study period. The samples were spotted on Whatman 903TM paper (GE Healthcare Bioscience Corp.), stored in individual plastic bags with silica desiccant and transported for further molecular studies at the Institute for Biotechnology Research (IBR) in Jomo Kenyatta University of Agriculture and Technology (JKUAT), Kenya.

Genomic DNA extraction and PCR amplification

Genomic DNA extraction was performed on the dried blood spot (DBS) samples using Schneeberger's protocol with slight modifications, comprising 1.5M guanidine thiocyanate and 100mM Tris with 0.1% sodium dodecyl sulfate (SDS) at pH 8.¹⁸ Concentration of DNA ranged from 0.05 ng/uL to 6.03 ng/uL whereas the ratio obtained from analysis of DNA purity (260 nm/280 nm) ranged from 1.4 to 2.17. The DNA extracts were stored at -20°C and used for PCR amplification.

Outer and nested PCR amplification was conducted using the AB 9800 Fast Thermocycler machine (Applied Biosystems, UK) on regions flanking identified point mutations of the following *P. falciparum* genes: bifunctional dihydrofolate reductase-thymidylate synthase – *DHFR-TS* (PF3D7_0417200), i.e. N51I, C59R, and S108N, hydroxymethyldihydropterin pyrophosphokinase-dihydropteroate synthase – *PPPK-DHPS* (PF3D7_0810800) i.e. K540E and kelch protein – kelch 13 (PF3D7_1343700) i.e. Y493H, R539T, I543T, and C580Y which confer drug resistance to SP and ART respectively. The respective gene sequences were retrieved from PlasmoDB release 46 (<http://PlasmoDB.org>) and primer design (Table 1) was performed using PrimerQuest and OligoAnalyzer tools from Integrated DNA technologies online platform (<https://www.idtdna.com/>). Selection of primers considered characteristics such as: Guanine-Cytosine (G+C) content of greater than 50, five degrees difference between melting temperatures and absence of hair-pin formation and self-annealing properties. A total PCR volume of 25 uL containing 12.5 uL of 2× DreamTaq PCR master mix (Thermo ScientificTM), 3.75 uL of the DNA template and 0.25 uL of the forward and reverse primers respectively were obtained for all the reactions. A volume of 3.75 uL of DNA template in the outer primary PCR reaction, as well as for the PCR amplicon in the nested secondary reaction was used. Step-down PCR cycling conditions for the outer and nested reactions were set as follows: an initial denaturation of 94°C for three minutes, a denaturation of 94°C for 15 seconds, an annealing temperature range of 55°C–60°C for 30 seconds, an elongation of 72°C for one minute and a final elongation of 72°C for 10 minutes.

Resolution of PCR amplicons was run in 1.5% agarose gel, 1× TAE buffer, at 70 V, 58 mA for one hour 30 minutes using a gel electrophoresis system (IBI-Shelton Scientific MP-1015 multipurpose) and an electrophoresis power voltage supplier (Pharmacia LKB ECPS 3000V/150mA). GelRed[®] Nucleic Acid Gel stain (Biotium) was used for pre-cast gel staining, 1 kb DNA ladder (Thermo ScientificTM) for DNA quantification of resolved PCR amplicons. *P. falciparum* 3D7 purified DNA laboratory strain obtained from Kenya Medical Research Institute (KEMRI) was used as the main control for wild-type and mutant alleles of each gene. Purification of nested PCR amplicons depicting a single band was performed using the QIAquick PCR purification kit (Qiagen) whereas for amplicons showing double bands, the targets were processed using QIAquick gel extraction kit (Qiagen) as per the manufacturer's protocol respectively. The PCR amplicons were shipped to Macrogen (Seoul, Korea) for Sanger sequencing.

Bioinformatics analysis

QIAGEN CLC Main Workbench v21.0.4 was used to perform sequence data editing, consensus sequence assembly and identification of nucleotide base conflicts against the 3D7 reference gene sequences of PF3D7_0417200, PF3D7_0810800 and PF3D7_1343700. Multiple sequence alignment (MSA), was carried out in MEGA v7.0¹⁹ using the Muscle algorithm²⁰ to identify nucleotide base changes, including translation to amino acid sequences using the standard genetic code for the identification of amino acid changes and their respective positions. Further visualisation of sequence alignments was performed in Jalview v2.11.1.4²¹ to identify non-synonymous point mutations.

Results

Sample characteristics

After consent was given for participation and follow up, 19 dried blood spot (DBS) samples were successfully collected from a total of 131 patients who visited the three hospitals during the study period,²² 10 samples from Adi Qualla Hospital (AQH = 10), three samples from Keren Hospital (KH = 3) and six samples from Gash Barka Referral Hospital (GBH = 6). Eight DBS samples were from patients treated with ACT (artesunate [AS] 100 mg + amodiaquine [AQ] 200 mg) and did not respond to treatment. These underwent re-treatment with quinine (Q) and were cured. Five DBS samples were from

Table 1. Outer and nested primer sets used for PCR amplification of target gene regions.

Gene name	Gene ID	Primer sequences	Amplicon band size (bp)	Targeted point mutations	Primer reference
Bifunctional dihydrofolate reductase thymidylate synthase – DHFR-TS (<i>Pfdhfr</i>)	PF3D7_0417200	Outer primer set: PF_0417200_OF 5' CCAACATTTCAAGATTGATAC 3'	397	N51I, C59R, N108S	This study
		PF_0417200_OR 5'CGCTAACAGAAATAATTTGATACTC3'			
		Nested primer set: PF_0417200_NF 5' GGCTAGGAAATAAAGGAG 3'			
		PF_0417200_NR 5' GATAAACACGGAACTCC 3'			
hydroxymethyldihydropterin pyrophosphokinase-dihydropteroate synthase – PPPK-DHPS (<i>Pfdhps</i>)	PF3D7_0810800	Outer primer set: PF_0810800_OF 5' GTGATTGTGGATCAGAAG 3'	457	K540E	This study
		PF_0810800_OR 5' GTTCTTCGCAATCCTAATCC 3'			
		Nested primer set: PF_0810800_NF 5' GGTGGAGAAATCCTCTGGT 3'			
		PF_0810800_OR 5' GTTCTTCGCAATCCTAATCC 3'			
Kelch protein-K-13 (<i>Pfk-13</i>)	PF3D7_1343700	Outer primer set: PF_1343700_OF 5' CGGAGTGACCAAAATCTGGGA 3'	532	C580Y, A578S, A569S, N554S, V566I	This study
		PF_1343700_OR 5' GCCTTGTGAAAGAAAGCAG 3'			
		Nested primer sets: PF_1343700_OF PF_1343700_NR1 5' GGGGATATGATGGCTCTTCT 3'			
		PF_1343700_NF2 5'AGAAGAGCCATCATATCCCCC 3' PF_1343700_NR2 5' GCCTTGTGAAAGAAAGCAG 3'			

patients who responded to ACT treatment. The remaining six were from patients presenting severe illness and were treated with quinine (Q) (Table 2).

PCR amplification and point mutation analyses

On PCR amplification of targeted gene regions, sequence data from eight samples (AQH = 2, KH = 2, GBH = 4) was eventually analyzed for point mutations (Table 3). The nucleotide base changes comprised of four *Pfdhfr* substitutions, adenine (A) to cytosine (C) at position 152, thymine (T) to cytosine (C) at position 175, guanine (G) to adenine (A) at position 323, thymine (T) to cytosine (C) at position 134; one *Pfdhps* substitutions, adenine (A) to guanine (G) at position 1618 and none identified for *Pfk-13* (Table 4). Subsequent translation to amino acid sequences constituted changes as

Table 2. Patient description and treatment regimen data for dried blood spot samples collected from the hospital sites. AS = artesunate, AQ = amodiaquine, Q = quinine.

Hospital name (code) and region	Patient serial no.	Gender	Treatment regimen		
			First treatment	Treatment outcome	Second treatment (re-treatment with quinine)
Aqi Quala Hospital (AQH): Adi Quala	AQH001	M	AS + AQ	Did not respond	Responded
	AQH002	M	AS + AQ	Did not respond	Responded
	AQH003	M	AS + AQ	Did not respond	Responded
	AQH004	F	AS + AQ	Did not respond	Responded
	AQH005	M	AS + AQ	Did not respond	Responded
	AQH006	F	AS + AQ	Did not respond	Responded
	AQH007	F	Q	Responded	
	AQH008	M	Q	Responded	
	AQH009	M	AS + AQ	Responded	
	AQH010	M	AS + AQ	Responded	
Keren Hospital (KH): Debub	KH011	M	AS + AQ	Did not respond	Responded
	KH012	M	Q	Responded	
	KH013	M	AS + AQ	Responded	
Gash Barka Hospital (GBH): Anseba	GBH014	M	AS + AQ	Did not respond	Responded
	GBH015	F	AS + AQ	Responded	
	GBH016	M	AS + AQ	Responded	
	GBH017	M	Q	Responded	
	GBH018	F	Q	Responded	
	GBH019	F	Q	Responded	

Table 3. *P. falciparum* nested-PCR results for *Pfk-13*, *Pfdhps* and *Pfdhfr* genes from the hospital sites in Eritrea.

Hospital site (code)	Total no. of samples collected	No. of PCR positive isolates N (%)	Isolate serial no.	PCR positive isolates N per molecular marker		
				<i>Pfk-13</i>	<i>Pfdhps</i>	<i>Pfdhfr</i>
Adi Quala Hospital (AQH)	10	2 (20%)	AQH009, AQH010	2	0	2
Keren Hospital (KH)	3	2 (67%)	KH012, KH013	1	2	1
Gash Barka Hospital (GBH)	6	4 (67%)	GBH014, GBH015, GBH017, GBH018, GBH019	3	4	2

follows: asparagine (N) to isoleucine (I) at codon 51, cysteine (C) to arginine (R) at codon 59, serine (S) to asparagine (N) at codon 108 and valine (V) to alanine (A) at codon 45 for *Pfdhfr*; lysine (K) to glutamate (E) at codon 540 for *Pfdhps* and wild-type amino acids retained for *Pfkelch-13* (Table 4). Multiple sequence alignment (MSA) and visualization of consensus sequence assemblies for *Pfdhfr*, *Pfdhps* and *Pfkelch-13* against their 3D7 reference sequences distinguished four non-synonymous (nsy) point mutations for *Pfdhfr* (N51I, C59R, S108N, V45A), one non-synonymous (nsy) point mutation (K540E) for *Pfdhps* while *Pfkelch-13* retained wild-type amino acids (Figure 1).

Table 4. *Pfdhfr*, *Pfdhps* and *PfK-13* results for corresponding nucleotide- and amino acid-changes across the hospital sites in Eritrea. N = asparagine, I = isoleucine, C = cysteine, R = arginine, S = serine, V = valine, A = alanine.

Molecular marker	Nucleotide base change			Amino acid change		No. of isolates per hospital			
	Position (p)	From	To	Codon (c)	Wild-type	Mutant	Adi Quala (AQH)	Keren (KH)	Gash Barka (GBH)
<i>Pfdhfr</i>	152	AaT	AtT	51	N	I	2	1	1
	175	tGT	cGT	59	C	R	0	1	0
	323	AgC	AaC	108	S	N	2	1	1
	134	GtA	GcA	45	V	A	2	1	1
<i>Pfdhps</i>	1618	AGa	AGg	540	K	E	0	1	3
<i>PfK-13</i>	G1739A	retained		580	Y	retained	0	-	0
	A1661G	retained		554	N	retained	0	-	0
	G1705A	retained		569	A	retained	0	-	0
	G1696A	retained		566	V	retained	0	-	0
	G1732A	retained		578	A	retained	0	-	0
	T1477C	retained		493	Y	retained	0	0	0
	G1615C	retained		539	R	retained	0	0	0
	T1627C	retained		543	I	retained	0	0	0

Note: The numeral '0' indicates absence of isolates with the respective nucleotide/amino acid changes The dash (-) symbol implies no sequence data generated from the respective hospital sites.

Figure 1. Jalview visualization of multiple sequence alignments depicting nsy-point mutations: *Pfdhfr* (N51I, S108N, V45A) occurred in all four isolates (KH013, GBH017, AQH010, AQH009), C59R was identified in one isolate (KH013); *Pfdhps* (K540E) occurred in all four isolates (KH013, GBH017, AQH010, AQH009), C59R was identified in one isolate (KH013) and *Pfdhps* (K540E) occurred in all four isolates (KH012, GBH014, GBH015, GBH017) *PfK-13*, established no point mutations in all six isolates, wild type amino acids retained at c.554(S), c.566(V), c.569(A), c.578(A), c.580(C), c.493(Y), c.539(R), c.543 (I).

Discussion

In this study, we present findings from a pilot survey assessing the occurrence of point mutations in *PfK-13*, *Pfdhfr* and *Pfdhps* genes from clinical isolates obtained from three zobas of Eritrea: Adi Quala (Adi Quala Hospital), Debub (Keren Hospital) and Anseba (Gash Barka Hospital). We targeted PCR-amplification of *PfK-13* point mutations associated with artemisinin (ART) resistant phenotype in western Cambodia, South-East Asia Y493H, R539T, I543T, C580Y,¹⁴ non-synonymous point mutations, V566I, A578S, identified in isolates from five Sub-Saharan countries²³ and N554S, A569S reported in a previous study from islands in Lake Victoria, Kenya.²⁴ This study found none of the corresponding point mutations in *PfK-13*, which is similar to other studies from Eritrea²⁵ and Kenya^{26,27} including other malaria endemic sub-Saharan countries.²⁸ Data from the treatment outcome with the prescribed artemisinin (artesunate [AS]) indicated susceptibility responses corresponding with our findings and suggesting a likely absence of ART resistance. Only one isolate from these analyses was obtained from a patient who did not respond to first line treatment with AS-AQ – this could be attributed to possible causes of treatment failure such as non-compliance to the treatment regimen, incorrect drug usage, drug pharmacokinetics as well as host immunity.^{29,30}

Pfdhfr point mutations, N51I, C59R and S108N observed in our study correspond with previous reports from Senegal,³¹ South Africa,³² Malawi, Mali, Kenya, Tanzania,^{33,34} including Venezuela in South America.³⁵ Additionally, the single *Pfdhfr* C59R and *Pfdhps* K540E point mutations seen in our findings, have been shown to predict the occurrence of the *Pfdhfr*-*Pfdhps* quintuple mutant haplotype (*Pfdhfr* 51I/59R/108N + *Pfdhps* 437G/540E),³⁶ which is associated with fully resistant SP parasites¹² as well as SP treatment failure.³⁷ The selection of these *Pfdhfr*-*Pfdhps* mutations from our findings, is attributable to the prior use of the CQ-SP combination as first-line treatment for clinical management of febrile disease especially at the primary health care level in Eritrea. Additionally, prior evidence shows that SP resistant parasites originated from South East Asia and consecutively spread into Sub-Saharan Africa,^{38,39} which eventually reached Eritrea too, as demonstrated in these findings. The valine (V) to alanine (A) change at codon 45 in *Pfdhfr* from this study, has not been previously reported, although, a converse occurrence of alanine (A) to valine (V) at codon 16 has been associated, both singly and doubly in combination to S108N mutation, with resistance to another antifolate, cycloguanil.^{40,41} Further investigation with a larger sample size is recommended to validate the selection of the V45A mutation in the population, as well as understand its implications to protein function in association with other established *Pfdhfr* mutations. Additionally, further detection of other SP resistance associated mutations not reported herein is recommended.

Although this study design availed treatment outcome information to compare with corresponding generated molecular data, limitation of sample size as well as DNA quality and quantity constrained further detection of *PfK13* mutations associated with artemisinin resistance. Nonetheless, the general findings reported here, are not affected by these limitations and essentially provides useful molecular inference for further investigations.

Conclusions

Here, we provide molecular data verifying the genetic mechanism underlying SP resistance from selected participants of three regions of Eritrea. *Pfdhfr* C59R and *Pfdhps* K540E are reliable markers for the quintuple mutant haplotype conferring full resistance to SP. This study provides the molecular status of SP resistance in Eritrea. Continued monitoring of artemisinin resistance is recommended. Future studies should be carried out on a larger sample size since this study was a pilot survey involving a small sample size.

Data availability

Underlying data

This project contains the following underlying data:

NCBI Gene: bifunctional dihydrofolate reductase-thymidylate synthase (DHFR-TS) [*Plasmodium falciparum* (malaria parasite)] Accession number MZ322415, <https://www.ncbi.nlm.nih.gov/nuccore/MZ322415>.

NCBI Gene: bifunctional dihydrofolate reductase-thymidylate synthase (DHFR-TS) [*Plasmodium falciparum* (malaria parasite)] Accession number MZ322416, <https://www.ncbi.nlm.nih.gov/nuccore/MZ322416>.

NCBI Gene: bifunctional dihydrofolate reductase-thymidylate synthase (DHFR-TS) [*Plasmodium falciparum* (malaria parasite)] Accession number MZ322417, <https://www.ncbi.nlm.nih.gov/nuccore/MZ322417>.

NCBI Gene: bifunctional dihydrofolate reductase-thymidylate synthase (DHFR-TS) [*Plasmodium falciparum* (malaria parasite)] Accession number MZ322418, <https://www.ncbi.nlm.nih.gov/nuccore/MZ322418>.

NCBI Gene: hydroxymethyldihydropterin pyrophosphokinase-dihydropteroate synthase (PPPK-DHPS) [*Plasmodium falciparum* (malaria parasite)] Accession number MZ322419, <https://www.ncbi.nlm.nih.gov/nuccore/MZ322419>.

NCBI Gene: hydroxymethyldihydropterin pyrophosphokinase-dihydropteroate synthase (PPPK-DHPS) [*Plasmodium falciparum* (malaria parasite)] Accession number MZ322420, <https://www.ncbi.nlm.nih.gov/nuccore/MZ322420>.

NCBI Gene: hydroxymethyldihydropterin pyrophosphokinase-dihydropteroate synthase (PPPK-DHPS) [*Plasmodium falciparum* (malaria parasite)] Accession number MZ322421, <https://www.ncbi.nlm.nih.gov/nuccore/MZ322421>.

NCBI Gene: hydroxymethyldihydropterin pyrophosphokinase-dihydropteroate synthase (PPPK-DHPS) [*Plasmodium falciparum* (malaria parasite)] Accession number MZ322422, <https://www.ncbi.nlm.nih.gov/nuccore/MZ322422>.

NCBI Gene: kelch protein (K13) (Kelch13) [*Plasmodium falciparum* (malaria parasite)] Accession number MZ322423, <https://www.ncbi.nlm.nih.gov/nuccore/MZ322423>.

NCBI Gene: kelch protein (K13) (Kelch13) [*Plasmodium falciparum* (malaria parasite)] Accession number MZ322424, <https://www.ncbi.nlm.nih.gov/nuccore/MZ322424>.

NCBI Gene: kelch protein (K13) (Kelch13) [*Plasmodium falciparum* (malaria parasite)] Accession number MZ322425, <https://www.ncbi.nlm.nih.gov/nuccore/MZ322425>.

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Extended data

Dryad: Extended data for ‘Screening for Antifolate and Artemisinin resistance in *Plasmodium falciparum* clinical isolates from three hospitals of Eritrea’, <https://doi.org/10.5061/dryad.sbcc2fr6q>.²²

This project contains the following extended data:

- the total number of patients grouped according to age, who visited the three hospitals during the study period.
- gel images of *Pfdhfr*, *Pfdhps* and *PfK13* genetic markers.

Data are available under the terms of the [Creative Commons Zero “No rights reserved” data waiver](#) (CC0 1.0 Public domain dedication).

Consent

All participants were informed concerning the aim of the study, assent and written informed consent was given by patients, voluntary participation was allowed, and confidentiality of information collected ensured.

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Mukhongo *et al.* presented a study highlighting the prevalence of *P. falciparum dhfr*, *dhps*, and K13 gene mutations in parasites collected in Eritrea. The subject of *P. falciparum* drug-resistant and gene mutations is very germane in malaria research, making this study a very important one. Unfortunately, this study is not well executed, and an extensive major review will be required.

1. The focus of this study is quite confusing. It is not clear why the authors are investigating *dhfr* and *dhps* mutations in this cohort. The country is not currently using sulfadoxine-pyrimethamine (SP) for treatment, and the cohort being investigated also did not use SP either. WHO currently recommends SP for prevention among pregnant women and children (in some cases). Neither of these groups is being studied. The author needs to justify the reason for SP mutation analysis in this study. What would have been more interesting would have been PFCRT gene mutations since amodiaquine is still being used as a partner drug for artesunate.
2. The authors stated in the abstract section that the "study sought to verify the genetic mechanism of resistance to sulfadoxine-pyrimethamine." The genetic mechanism was not performed as stated by the authors in conclusion. All that the author did was report the mutations in *dhps* and *dhfr* genes. The identified mutations were not studied for their contributions to resistance in this cohort. While these mutations are well known for their contributions to resistance, many studies have reported these mutations without much compromise in SP cure rate, meaning that other additional factors are needed for resistance to occur. So for the authors to state that "we provide molecular data verifying the genetic mechanism underlying SP resistance" is not correct.
3. The authors stated that the patients were followed up. In Malaria studies, the standard WHO method of following up patients is to be observed on days 0, 1, 2, 3, 7, 14, 21, and 28 or up to day 42. This will allow the definition of treatment failures (early, late, clinical, and parasitological) and adequate cure. The authors stated "responded" or "Did not respond."

How did they arrive at this outcome? Was this outcome based on fever or parasite detection? What type of failures are they considering? Is it re-infection or recrudescence? All these are important in the analysis of and contribution of gene mutations to resistance. In my view, since the authors did not genotype the samples collected on the days that the patient "Did not respond," this part should be expunged as it has little or no contribution to the data being presented.

4. It would be nice if the author could explain why only eight samples were successfully sequenced out of nineteen.

Is the work clearly and accurately presented and does it cite the current literature?

No

Is the study design appropriate and is the work technically sound?

No

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Not applicable

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

No

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Medical Parasitology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Author Response 27 Sep 2022

Harriet Mukhongo, Jomo Kenyatta University of Agriculture and Technology, Juja, Kenya

Response 1 (to comment 1):

- One of the objectives of this study, was to investigate for *pfdhfr* and *pfdhps* mutations associated with SPR in the present cohort, because: firstly, these SP molecular markers became validated tools for surveillance of drug resistance (Wang et al., 1997) after SPR was already widespread in many endemic regions. Therefore, their application in our study provides important molecular data retrospectively for future reference and further studies (Plowe et al., 2007) Hence, as stated in the discussion

section, this study reported the single mutant *pf dhfr* C59R and single mutant *pf dhps* K540E isolates which have been shown to accurately predict the occurrence of the quintuple SPR mutant (*pf dhfr* S108N, N51I, C59R and *pf dhps* A437G, K540E) associated with SP treatment failure (Kublin et al., 2002). Secondly, although Eritrea changed drug policy from SP to ACT in 2007, molecular investigation provides important evidence, on the impact this withdrawal of drug use and drug pressure, has exerted on parasite resistance at the genetic/molecular level, since withdrawal (Malisa et al., 2010)

- In Eritrea, SP has only been used as a first-line treatment in combination with Chloroquine in malaria treatment for the general population and not as an intervention policy neither for pregnant women nor children, as evidenced in this WHO 2020 malaria report (<https://www.who.int/publications/i/item/9789240015791>)
- This explains why our study focused on investigating for the occurrence of SPR-associated mutations in the general population visiting the hospitals during the study period and not the pregnant women and children groups.

Response 2 (to comment 2):

- “Genetic mechanism of resistance” has been used here to refer to the molecular basis underlying parasite drug resistance. From previous studies (Basco et al., 1995; Triglia et al., 1997), this has been shown to comprise identified single nucleotide polymorphisms (SNPs) or point mutations occurring in specific genes, that encode enzymes i.e. *pf dhfr* and *pf dhps* involved in the folate synthesis pathway, resulting in structural differences and thus SP resistance (Talisuna et al., 2004).
- Our study focused on applying molecular markers primarily as research tools for mapping and estimation of parasite resistance. The scope of our study did not include therapeutic drug efficacy (in vivo) studies (TES), which predicts drug cure rate based on clinical and parasitological outcomes. This is mainly because, the investigation for SP molecular markers in this study, was majorly for provision of retrospective molecular data as earlier stated in response one.
- As for the artemisinin (ART) treatment administered to study participants, investigation of validated ART molecular markers can give direct evidence of selection for resistant parasites by antimalarial drug treatment as mentioned in this article (Plowe, 2003)
- Additionally, our study relied largely on molecular analyses by PCR technique which is mainly used for research-oriented monitoring projects and is resource-friendly since samples are easily collected, stored and transported to the laboratory facility. In contrast, TES (in vivo) are mostly longitudinal and hence lengthy, resource-intensive - unless integrated in the national malaria control program - and challenging to conduct in areas of low/seasonal transmission due to limited number of patients. Finally, the evidence of molecular data is a significant contribution because it provides the basis for TES (in vivo) on the sites where molecular surveillance has been conducted, for precise prediction of the actual prevalence of resistance where necessary (Vestergaard and Ringwald, 2007).
- TES (in vivo) (Basco et al., 1998; Curtis et al., 1998; Diourte et al., 1999; Jelinek et al., 1998; Nzila et al., 2000) and in vitro (Nzila-Mounda et al., 1998) studies have demonstrated that the basis of SP treatment failure is also associated with the step-wise accumulation of identified SNPs or point mutations in *pf dhfr* and *pf dhps* genes

(as stated in the introduction section of this article). This consequently validates these SNPs/point mutations as SP molecular markers for resistance suitable for surveillance of parasite drug resistance

Response 3 (to comment 3):

- As explained previously in response two, the scope of this study did not involve therapeutic drug efficacy (in vivo) studies, which gives clinical and parasitological outcomes observed on day 0, 1, 2, 3, 7, 14, 21, 28 or up to day 42 after treatment administration.
- In view of this, neither re-infection nor recrudescence, which are incorporated in therapeutic drug efficacy studies for classifying parasitological outcomes, were considered in the present study, since no parasite testing of febrile cases was performed.
- The treatment outcome of study participants was based on manifestation of febrile illness – this is because at the period of the study, testing of all febrile cases for malaria parasites was not yet being conducted. This is also evidenced in the national malaria programme-performance review, 2013, for the state of Eritrea. The treatment response, was therefore, based on the persistence (classified as “did not respond”) or the clearance (classified as “responded”) of febrile illness in the study participants. This treatment outcome data in our study, is only applicable to artemisinin, which is the current first-line treatment – it is important in the study for comparing the outcome to administered ACT with the pfkelch-13 mutation analyses performed, in order to predict possible causes of treatment failure for individual study participants

Response 4 (to comment 4):

- Genomic DNA extraction was conducted for all the nineteen samples, however due to limitation in DNA quantity and quality, further PCR amplification and sequencing was successfully performed on eight samples.

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