Screening for antifolate and artemisinin resistance in
Plasmodium falciparum dried-blood spots from three hospitals of Eritrea [version 4; peer review: 2 approved]

Previous Title 'Screening for antifolate and artemisinin resistance in Plasmodium falciparum clinical isolates from three hospitals of Eritrea'

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Abstract

Background

Antimalarial drug resistance is a major challenge hampering malaria control and elimination. About three-quarters of Eritrea's population resides in the malaria-endemic western lowlands of the country. 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 which complements clinical studies, and strengthens control efforts. This study sought to genotype point mutations associated with P. falciparum resistance to sulfadoxine-pyrimethamine and artemisinin, in dried-blood spots from three hospitals in the western lowlands of Eritrea.

Methods

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Dried-blood spot samples were collected from patients visiting Adi Quala, Keren and Gash Barka Hospitals, between July and October, 2014. The patients were followed up after treatment with first line artesunate-amodiaquine, and dried-blood spots were collected on day three after treatment. Nested polymerase chain reaction and Sanger sequencing techniques were employed to genotype point mutations in the \( Pf\text{dhfr} \) (PF3D7_0417200), \( Pf\text{dhps} \) (PF3D7_0810800) and \( PfK13 \) (PF3D7_1343700) partial gene regions.

**Results**

Sequence data analyses of PCR-positive isolates found wild-type artemisinin haplotypes associated with resistance (Y493Y, R539R, I543I) in three isolates, whereas four mutant antifolate haplotypes associated with resistance were observed in six isolates. These included the triple-mutant \( Pf\text{dhfr} \) (S108N, C59R, N51I) haplotype, the double-mutant \( Pf\text{dhfr} \) (N51I, S108N) haplotype, the single-mutant \( Pf\text{dhfr} \) (K540E) haplotype, and the mixed-mutant \( Pf\text{dhfr}-Pf\text{dhps} \) (S108N, N51I + K540E) haplotype. Other findings observed were, a rare non-synonymous \( Pf\text{dhfr} \) V45A mutation in four isolates, and a synonymous \( Pf\text{dhps} \) R449R in one isolate.

**Conclusions**

The mutant antifolate haplotypes observed indicate a likely existence of full SP resistance. Further studies can be carried out to estimate the prevalence of SP resistance. The wild-type artemisinin haplotypes observed suggest artemisinin is still an effective treatment. Continuous monitoring of point mutations associated with delayed parasite clearance in ART clinical studies is recommended.

**Keywords**

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

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Amendments from Version 3

A statement on the in vivo findings which resulted in treatment policy change was added in the introduction.

The reference number under the ethical statement was changed.

Any further responses from the reviewers can be found at the end of the article.

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. Working hand-in-hand with Roll Back Malaria (RBM) collaborators and stakeholders, NMCP set up a combination of strategies including integrated vector management (IVM), early diagnosis and prompt treatment consequently leading to a remarkable decrease in incidence and mortality rates, following the gruesome 1998 malaria epidemic in the country.

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. Malaria transmission in the western lowlands is highly seasonal, peaking during the rainy season (June–November), and declining considerably during the dry season (December – June). The risk of malaria infection is estimated at 70 infective bites per year in the western lowlands, with high entomologic inoculation rates during the rainy season and little transmission during the dry season. Generally, malaria prevalence in the western lowlands is highly focal, with a low parasitemia proportion of 1.9% (ranging from 0.4% to 3.8%). Additionally, the prevalence of malaria infection cases in Eritrea occurs across all age groups, unlike a majority of Sub-Saharan Africa where malaria occurs mainly in children below five years and pregnant women. 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 World Malaria Report 2014). 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 World Malaria Report 2014). 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). Genetic markers are invaluable tools in screening and detection of drug resistance, in addition to predicting the efficacy of antimalarials. Sulfadoxine-pyrimethamine P. falciparum resistance (SPR), which is well-studied, results from the occurrence and accumulation of mutations in the dihydrofolate reductase gene ( Pf dhfr) and in the dihydropteroate synthase gene ( Pf dhps) leading to a gradual reduction of sensitivity to pyrimethamine and sulfadoxine respectively. In vitro and in vivo studies have shown that SPR is mainly associated with point mutations at codons N51I, C59R, S108N and double 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 Pfdhfr, A437G, K540E and A581G defines super resistance.

The development of artemisinin (ART) resistant P. falciparum parasites was first independently described in Western Cambodia, South East Asia. To date, resistance is commonly associated with five non-synonymous mutations including M476L, Y493H, R539T, I543T, and C580Y in the propeller domain of P. falciparum kelch 13 gene (Pfk13). 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. Additionally, there is the likely existence of a large reservoir of Pfk13-mutations globally, evidenced by the presence of non-synonymous mutations not associated with delayed parasite, especially in SSA. This has been demonstrated in a previous survey that involved screening of over 1000 African
**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/3/656-658/14).

**Study sites and sample collection**

This study was conducted at three hospitals located in the western malaria-endemic lowlands 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 Quala Hospital, 16th July to 15th September 2014 for Keren Hospital and 15th August to 1st October 2014 for Gash Barka Referral Hospital.

**Patient eligibility criteria**

All patients aged above twenty (20) years, with fever of temperatures > 37.5°C at the consultation visit or a history of fever within the previous 24 hours, were confirmed for malaria infection by microscopic examination of 10% Giemsa-stained thin and thick blood slides. After written assent was given, patients with *P. falciparum* mono-infection of initial density between 1000 and 100,000 asexual parasites per microliter (µL) of blood were included in the study. From these patients, those who also tested positive on rapid diagnostic testing of *P. falciparum* histidine-rich protein 2 (CareStart®, Pf/Pv, Access Bio, USA) were included. Other inclusion criteria of the study were: absence of an antimalarial treatment history in the previous two weeks, availability for follow-up after treatment prescription, absence of clinical and parasitological evidence of complicated malaria, absence of confirmed pregnancy or breastfeeding, and absence of a history of allergy or adverse reactions to the administered antimalarials or concomitant illnesses.

**Sample characteristics and collection**

A total of 131 patients (female=35; male=96) with slide-confirmed malaria infection were admitted at the three outpatient hospital sites during the study period.24 From these, 79 patients aged above 20 years (female=23; male=56), were identified as candidates for the study.24 However, 22 patients (female=8; male=14) who met the other inclusion criteria were enrolled in the study on the consultation visit (Day 0) (Figure 1). For all enrolled patients, microscopic examination and rapid diagnostic testing of *P. falciparum* mono-infection was conducted on the consultation visit and on the subsequent scheduled visits. Oral tablets of AS-AQ were prescribed once a day for three consecutive days, according to the treatment regimen. Treatment administration after Day 0 (D0) was unsupervised on D1 and D2, and sample collection was scheduled on D3. On the scheduled visit (D3), 19 blood samples (AQH=10 samples; KH=3 samples; GBH=6 samples) were successfully collected, while three patients (female=2; male=1) were lost to follow-up (Figure 1).

The blood samples collected were spotted on Whatman 903™ 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 (JBUAT), Kenya, for genomic DNA extraction and PCR amplification. On each filter paper, the date of sample collection, and the patients’ serial numbers depicted as the hospital code preceding a unique identifier (i.e. AQHxxx, KHxxx, GBHxxx), were recorded.

**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 8.25 Concentration of DNA ranged from 0.05 ng/μL to 6.03 ng/μL 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, hydroxymethyl dihydropterin 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 μL containing 12.5 μL of 2× DreamTaq PCR master mix (Thermo Scientific™), 3.75 μL of the DNA template and 0.25 μL of the forward and reverse primers respectively were obtained for all the reactions. A volume of 3.75 μL 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 Scientific™) for DNA quantification of resolved PCR amplicons. *P. falciparum* 3D7

**Figure 1. Flow diagram showing the sample characteristics.**
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 (Pfdhfr) | PF3D7_0417200    | *Outer primer set:* PF_0417200_OF 5' CCAACATTTTCAAGATTGATAC 3'  
PF_0417200_OR 5' CGCTAACAGAAATAATTGATACTC3'  
*Nested primer set:* PF_0417200_NF 5' GTCTAGAATAAAAGGAG 3'  
PF_0417200_NR 5' GATAAACAACGGAACCTCC 3' | 397            | N51I, C59R, N108S                | This study              |
| hydroxymethyldehydropterin pyrophosphokinase-dihydroloropoate synthase – PPPK-DHPS (Pfdhps) | PF3D7_0810800    | *Outer primer set:* PF_0810800_OF 5' GTGATTGTGTTGATCAGAAG 3'  
PF_0810800_OR 5' GTTCTTCGAAATCTCTATC 3'  
*Nested primer set:* PF_0810800_NF 5' GTTGGAGAATTCTCTGGT 3'  
PF_0810800_NR 5' GTTCTTCGAAATCTCTATC 3' | 457            | K540E                   | This study              |
| Kelch protein-K-13 (Pfk-13)                                               | PF3D7_1343700    | *Outer primer set:* PF_1343700_OF 5' CCGAGTGACAAATCTGGGA 3'  
PF_1343700_OR 5' GCCTTGTTGAAGAAGCAG 3'  
*Nested primer sets:* PF_1343700_OF  
PF_1343700_NR1 5' GGGGATATGATGGCTCTTCT 3'  
PF_1343700_NR2 5' GCCCTCTTGAAATCTGCT 3'  
PF_1343700_NF2 5' AGAAGAGCCTATATGCC 3'  
PF_1343700_NR2 5' GCCCTTGTTGAAGAAGCAG 3' | 532            | C580Y, A578S, A569S, N554S, V566I  
372              | Y493H, R539T, I543T, | This study              |
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.

Sequence data analyses
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 v7.026 using the Muscle algorithm27 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 v2.11.1.428 to identify non-synonymous point mutations.

Results
Microscopy analyses
Microscopic examination of samples collected from the 19 patients (AQH=10; KH=3; GBH=6) on D3 of follow-up, detected *P. falciparum* parasites in: 6 of the 10 patients (60%) from AQH, 1 of the 3 patients (33.3%) from KH, and 1 of the 6 patients (16.6%) from GBH (Table 2). These eight (8) patients who tested positive for microscopy were re-administered with Quinine for three days and on D7 of follow-up, they did not present asexual parasites (Table 2). For the other remaining 11 patients, no asexual parasites were observed microscopically for every 200 leucocytes counted (Table 2). Rapid diagnostic testing of *P. falciparum* histidine-rich protein 2 produced a similar number of patient results as microscopy.

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

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**Table 2. Microscopy analyses for D0, D3 and D7 across the hospital sites.**

| Hospital site      | Adi Qualla hospital (AQH) | Keren hospital (KH) | Gash Barka hospital (GBH) |
|--------------------|---------------------------|---------------------|---------------------------|
| Follow-up days     | D0 | D3 | D7 | D0 | D3 | D7 | D0 | D3 | D7 | D0 | D3 | D7 |
| Patients involved (n) | 11 | 10 | 6  | 4  | 3  | 1  | 7  | 6  | 1  |    |    |    |
| Isolate serial no  | AQH001-AQH011 | AQH001-AQH010 | AQH001-AQH006 | KH001-KH004 | KH001-KH003 | GBH014-GBH020 | GBH014-GBH019 | GBH014 |
| Microscopy (+)     | 11 (100) | 6 (60) | 0  | 4 (100) | 1 (33.3) | 0  | 7 (100) | 1 (16.6) | 0  |

(n), number of patients; (), percent relative frequency; (+) positive; D, Day of follow-up.

**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 |
|----------------------|-------------------------------|-----------------------------------|-------------------|---------------------------------------------|
| Adi Qualla 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 |
position 323, thymine (T) to cytosine (C) at position 134; two Pfdhps substitutions, adenine (A) to guanine (G) at position 1618 and 1347 and none identified for PfK-13 (Table 4). Subsequent translation to amino acid sequences constituted changes as 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 and arginine (R) retained at codon 449 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

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 |
|------------------|------------------------|-------------------|-----------------------------|
| Pfdhfr          |                        |                   |                             |
| Position (p)     | From                  | To                | Codon (c) | Wild-type | Mutant | Adi (AQH) | Quala (KH) | Keren (GH) | Gash (GBH) |
| 152             | AaT                   | AtT               | 51        | N         | I      | 2       | 1         | 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         |
| Pfdhps          |                        |                   |           |           |        |         |           |           |           |
| 1618            | aAA                   | gAA               | 540       | K         | E      | 0       | 1         | 3         |
| 1347            | AGa                   | AGg               | 449       | R         | R      | 0       | 0         | 1         |
| PfK-13          |                        |                   |           |           |        |         |           |           |           |
| 1738            | TgT                   | TaT               | 580       | Y         | C      | 0       | -         | 0         |
| 1660            | AaT                   | AgT               | 554       | N         | S      | 0       | -         | 0         |
| 1705            | gCA                   | aCA               | 569       | A         | S      | 0       | -         | 0         |
| 1696            | gTA                   | aTA               | 566       | V         | I      | 0       | -         | 0         |
| 1732            | gCT                   | tCT               | 578       | A         | S      | 0       | -         | 0         |
| 1477            | tAC                   | cAC               | 493       | Y         | H      | 0       | 0         | 0         |
| 1615            | AgA                   | AcA               | 539       | R         | T      | 0       | 0         | 0         |
| 1627            | ATT                   | AtT               | 543       | I         | T      | 0       | 0         | 0         |

Note: The bold depicts the respective nucleotide base changes. 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 2. Jalview visualization of multiple sequence alignments depicting nsy- and sy-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), R449R was identified in one isolate (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).
Table 5. *Pfdhfr*, *Pfdhps* and *Pfkelch-13* haplotype combination results across the hospital sites.

| Molecular marker | Haplotype combination                        | Isolate serial no. | No of isolates per hospital site |
|------------------|----------------------------------------------|--------------------|----------------------------------|
| *Pfdhfr*         | Triple mutant (S108N, C59R, N51I)            | KH013              | - - 1                            |
|                  | Double mutant (N51I, S108N)                 | AQQH009, AQQH010   | 2 - -                            |
| *Pfdhps*         | Single mutant (K540E)                       | KH012, GBH014      | - 1 1                            |
| *Pfdhfr* + *Pfdhps* | Mixed mutant (S108N, N51I + K540E)       | GBH017             | - - 3                            |
| *Pfkelch-13*     | Wild type (Y493Y, R539R, IS43)              | AQQH009, AQQH010, KH012 | 2 1 -                            |
|                  | Wild type (C580C)                           | GBH014, GBH015, GBH019 | - - 3                            |
|                  | Wild type (N554N, V566V, A569A, A578A)      | GBH014, GBH015, GBH019 | - - 3                            |

*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) and one synonymous (sy) point mutation (R449R) for *Pfdhps* while *Pfkelch-13* retained wild-type amino acids (Figure 2).

Haplotype combination analyses

Analyses of *Pfdhfr*, *Pfdhps* and *Pfkelch-13* haplotype combinations identified the following: *Pfdhfr* triple mutant (S108N, C59R, and N51I), *Pfdhfr* double mutant (N51I, S108N), *Pfdhps* single mutant (K540E), *Pfdhfr* + *Pfdhps* mixed mutant (S108N, N51I + K540E), and *Pfkelch-13* wild types. The number of isolates and distribution of haplotypes according to hospital sites is illustrated on Table 5.

Discussion

Eritrea is situated in the uppermost limit of malaria distribution in East Africa, resulting in the seasonal and focal nature of transmission in the country.10 In addition to differences in topography and altitude, disease transmission is highly influenced by rainfall and temperatures.29,30 Unlike a majority of SSA where malaria burden is higher in target populations of pregnant women and children under five years, malaria risk in Eritrea is evenly distributed across all age-groups.10 Data from our study showed a higher number of cases in the over 20 years’ group (n=79) than the under 5 years (n=4) across the hospital sites.24 Also our study found more male cases (n=96), than female (n=35), were admitted across the hospital sites.24 This finding is similar to a previous study in Eritrea which reported a higher risk of parasitemia in males than females.10 Despite this, Eritrea is one of four countries globally which has considerably reduced malaria transmission through a combination of vector and parasite management, as well as, community-led awareness campaigns.31-33 *P. falciparum*, generally has the highest transmission intensity in SSA and Eritrea, where it causes the most severe form of malaria.34,35 Molecular surveillance of point mutations associated with resistance to previous and current antimalarial drugs, is important in strengthening existing control efforts and complementing therapeutic efficacy studies.36,37

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, IS43T, C580Y,18 nSy point mutations, V566I, A578S, identified in isolates from five Sub-Saharan countries38 and N554S, A569S reported in a previous study from islands in Lake Victoria, Kenya.39 This study aimed at genotyping for ART resistance on D3 after treatment administration since, conventionally, the proportion of parasitemia on D3 is used in monitoring of therapeutic efficacy to determine a likely occurrence of *P. falciparum* artemisinin resistance.40 We also aimed to identify whether the other nSy point mutations not associated with ART resistance had spread into Eritrea. The A578S and V566I mutations targeted in our study, have been shown to have a prevalence of >1% in various sites of SSA including Kenya, Uganda, Democratic Republic of Congo, Ghana, Mali and Gabon.41 A recent review has reported A578S is most common in SSA, with a prevalence of up to 11% in 14 countries.42 Also prospective to our study, another *PfK-13* mutation R622I, first reported in Ethiopia,43 has later been seen to spread into Somalia,44 Eritrea,45 Uganda,46 and Nigeria.47 However, from our study, none of the corresponding point mutations in *PfK-13*, were detected. This is similar to other studies from Eritrea48 and Kenya49,50 including other malaria endemic sub-Saharan countries.51 Microscopy data on D3 after
treatment administration with the prescribed artemisinin (Artesunate [AS]) indicated parasite clearance for 11 patients who were sampled. This corresponded with our genotyping findings, which were negative for resistance-associated point mutations for five of the 11 samples (Table 3), suggesting a likely absence of ART resistance. One isolate (GBH014), obtained from a patient with parasitological evidence on D3 also did not have resistance-associated point mutations in the \textit{PfK-13} marker. This treatment outcome could be attributed to other possible causes of treatment failure such as non-compliance to the treatment regimen, incorrect drug usage, drug pharmacokinetics as well as host immunity.\textsuperscript{52,53}

The triple mutant \textit{Pfdhfr} haplotype (N51I, C59R and S108N) observed in our study correspond with previous reports from Senegal,\textsuperscript{54} South Africa,\textsuperscript{55} Malawi, Mali, Kenya, Tanzania,\textsuperscript{56,57} including Venezuela in South America.\textsuperscript{58} This triple mutant haplotype has been associated with \textit{in vivo} SP treatment failure in previous studies.\textsuperscript{59–61} Additionally, the single mutant \textit{Pfdhfr} C59R and single mutant \textit{Pfdhps} K540E point mutations seen in our findings, have been shown to predict the occurrence of the \textit{Pfdhfr-Pfdhps} quintuple mutant haplotype (\textit{Pfdhfr} 511I/59R/108N + \textit{Pfdhps} 437G/540E),\textsuperscript{62} which is associated with fully resistant SP parasites\textsuperscript{16} as well as \textit{in vivo} SP treatment failure.\textsuperscript{53} The \textit{Pfdhps} K540E mutation has been reported to occur together with the A437G mutation in East Africa, and they both play an important role in sulfadoxine resistance of African parasites.\textsuperscript{64} The detection of these \textit{Pfdhfr-Pfdhps} mutations from our findings, could be attributed to the development of resistance from prior use of the CQ-SP combination as first-line treatment for clinical management of malaria in Eritrea.\textsuperscript{23} Additionally, prior evidence from genotyping microsatellite loci of \textit{Pfdhps} and \textit{Pfdhfr} genes, shows that SP resistant parasites originated from South East Asia and consecutively spread into Sub-Saharan Africa,\textsuperscript{65,66} which eventually reached Eritrea too, as demonstrated in these findings. The valine (V) to alanine (A) change at codon 45 in \textit{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.\textsuperscript{57,68} Further genotyping, in vitro, and genetic transformation studies could be carried out, firstly, to validate the selection of the V45A mutation in the population, and later to understand its implications to protein function in association with other established \textit{Pfdhfr} mutations. Also, detection of other SP, resistance associated mutations, \textit{Pfdhfr} I164L, and \textit{Pfdhps} A581G, A613T/S is recommended to describe the prevalence of parasite resistance in the population.

A limitation of this study was that, PCR amplification did not occur for some samples. This could be attributed to low genomic DNA yield, as well as, storage length and conditions of the DBS samples.\textsuperscript{69,70} Nonetheless, the general findings reported here, are not affected by these limitations and essentially provides useful molecular information for further studies.

**Conclusions**

In this study neither the validated point mutations associated with ART resistance nor the other nsy mutations were detected in the \textit{PfK-13} genetic marker. However, the single mutant \textit{Pfdhfr} haplotype C59R, single mutant \textit{Pfdhps} haplotype K540E, double mutant \textit{Pfdhfr} haplotype (N51I, S108N), and mixed mutant \textit{Pfdhfr-Pfdhps} haplotype (S108N, N51I + K540E) were detected and indicate the possible occurrence of the quintuple mutant haplotype (\textit{Pfdhfr} N51I/ C59R/S108N + \textit{Pfdhps} A437G/K540E) associated with full SP resistance and \textit{in vivo} SP treatment failure. The \textit{Pfdhfr} V45A mutation identified here, has not been previously reported, and further studies could be done to validate its selection and assess its contribution to antifolate resistance. Continued monitoring of artemisinin resistance is required to track resistance-associated point mutations arising at the genetic level. Future studies can be carried out on a larger sample size to determine the mutational prevalence of SP resistance.

**Data availability**

**Underlying data**

This project contains the following underlying data:

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

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

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

NCBI Gene: bifunctional dihydrofolate reductase-thymidylate synthase (DHFR-TS) [\textit{Plasmodium falciparum} (malaria parasite)] Accession number MZ322418, https://www.ncbi.nlm.nih.gov/nuccore/MZ322418.
NCBI Gene: hydroxymethyl dihydropterin pyrophosphokinase-dihydropteroate synthase (PPPK-DHPS) [Plasmodium falciparum (malaria parasite)] Accession number MZ322419, https://www.ncbi.nlm.nih.gov/nuccore/MZ322419.

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

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

NCBI Gene: hydroxymethyl dihydropterin 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.

NCBI Gene: kelch protein (K13) (Kelch13) [Plasmodium falciparum (malaria parasite)] Accession number MZ322426, https://www.ncbi.nlm.nih.gov/nuccore/MZ322426.

NCBI Gene: kelch protein (K13) (Kelch13) [Plasmodium falciparum (malaria parasite)] Accession number MZ322427, https://www.ncbi.nlm.nih.gov/nuccore/MZ322427.

NCBI Gene: kelch protein (K13) (Kelch13) [Plasmodium falciparum (malaria parasite)] Accession number MZ322428, https://www.ncbi.nlm.nih.gov/nuccore/MZ322428.

NCBI Gene: kelch protein (K13) (Kelch13) [Plasmodium falciparum (malaria parasite)] Accession number MZ322429, https://www.ncbi.nlm.nih.gov/nuccore/MZ322429.

NCBI Gene: kelch protein (K13) (Kelch13) [Plasmodium falciparum (malaria parasite)] Accession number MZ322430, https://www.ncbi.nlm.nih.gov/nuccore/MZ322430.

NCBI Gene: kelch protein (K13) (Kelch13) [Plasmodium falciparum (malaria parasite)] Accession number MZ322431, https://www.ncbi.nlm.nih.gov/nuccore/MZ322431.

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.24

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 Pf dhfr, Pf dhps 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.
Acknowledgements
The authors are grateful to all the participants of the study from the three hospitals and to Mr. Moses Oguo (International Livestock Research Institute – Kenya) for the technical support.

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Sam L Nsobya
1 Infectious Disease Research Collaboration (IDRC),, Kampala, Uganda
2 Infectious Disease Research Collaboration (IDRC),, Kampala, Uganda

I approved the revised article.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: PhD holder in malaria drug resistance expert for the past 27 years

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 09 May 2024
https://doi.org/10.5256/f1000research.165131.r271371

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Olusola Ojurongbe
1 Department of Medical Microbiology & Parasitology, Ladoke Akintola University of Technology, Ogbomoso, Nigeria
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After reviewing the manuscript, I am pleased to see that the author has diligently incorporated my previous comments, resulting in significant improvements to the work.
I will suggest that the author should delete this statement in the introduction section- 'Hence, this study did not target the pregnant women or children groups'

'Generally, despite WHO's change in treatment policy from the chloroquine (CQ)—sulfadoxine-pyrimethamine (SP) combination, adopted in 2002, to ACT in 2007, little is documented on the point mutations underlying SPR Pfhdfr and Pfhdps using genetic markers.' The author should explain what led to this change in policy. Was it that the mutation was high or, as a result, resistant observed in vivo?

**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 confirm that it is of an acceptable scientific standard.

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**Version 2**

Reviewer Report 25 April 2023

https://doi.org/10.5256/f1000research.142771.r163672

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**Sam L Nsobya**

1 Infectious Disease Research Collaboration (IDRC), Kampala, Uganda
2 Infectious Disease Research Collaboration (IDRC), Kampala, Uganda
3 Infectious Disease Research Collaboration (IDRC), Kampala, Uganda

The study was about screening known single nucleotide polymorphisms mediating resistance in pfhdfr, pfhdps and K13 genes from the clinical samples collected from Eritrea health facilities.

**Comments:**
- The objective was not smart and study poorly designed
- The sample size was inadequate i.e only 19 samples analyzed and out of those 42% samples did not generate results and no reason was given. It is also not explained: *why after consent, 19 dried blood spot (DBS) samples were successfully collected from a total of 131 patients who visited the three hospitals during the study period*
- The inclusion criteria: blood samples were obtained from patients with febrile illness. No screening using microscopy or rapid diagnostic test.
- Methodology section was poorly written
○ Statistical and bioinformatics poorly analyzed and written

○ The conclusion does not reflect the data generated and the write up is ambiguous i.e statement like *Pfdhfr C59R and Pfdhps K540E are reliable markers for the quintuple mutant haplotype conferring full resistance to SP.*

My conclusion: This article is not worthy of indexing because the way it was designed and written.

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?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
No

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

Are the conclusions drawn adequately supported by the results?
No

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** PhD holder in malaria drug resistance expert for the past 27 years

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 01 May 2024

**Harriet Mukhongo**

Response to 1st comment:
Thank you for your feedback. The objectives have been reviewed and included in the introduction.

Response to 2nd and 3rd comment:
Thank you for your feedback. This has been reviewed in the methods.

Response to 4th comment:
Thank you for your feedback. The methodology has been reviewed.
Response to 5th comment:
Thank you for your feedback. The methodology and results have been reviewed.

Response to 6th comment:
Thank you for your feedback. The abstract and write-up have been reviewed.

Please also view the responses in the file linked here.

**Competing Interests:** No competing interests disclosed

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**Reviewer Report 20 February 2023**

https://doi.org/10.5256/f1000research.142771.r162212

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**Olusola Ojurongbe**

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Thanks for your detailed response to my review.

1. I will suggest you include some of the information in your response to my comment that justifies the need to carry out *pfhfr* and *pfhps* mutations in the introduction. For example, “Eritrea changed drug policy from SP to ACT in 2007” and the fact “SP has only been used as a first-line treatment in combination with Chloroquine in malaria treatment for the general population” this information will help the reader to appreciate the need for *pfhfr* and *pfhps* mutations surveillance in your study.

2. Although the scope of your study did not include *in vivo* study, the reported drug outcomes needed to be explained for reproducibility purposes. On what day after treatment was the “responded” or “did not respond” determined? Eight patients underwent re-treatment with quinine, how many days after the initial treatment? This information should be presented since this is a standardized research.

**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?
No

If applicable, is the statistical analysis and its interpretation appropriate?
No

Are all the source data underlying the results available to ensure full reproducibility?
No

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 15 Apr 2024

Harriet Mukhongo

Response to comment 1:
Thank you for your suggestion. This information has been included in the introduction and discussion sections of the article.

Response to comment 2:
Thank you for your suggestion. This information has been included 'sample characteristics and collection' and 'microscopy analyses' sub-sections of the article

Please also view the authors' response to Reviewer 1 (Dr. Olusola Ojurongbe) linked here.

Please kindly note that in the above PDF file, the authors' responses to Reviewer 1's report for Version 2 of the article are found under the "Responses to 2nd set of comments from reviewer 1: Olusola Ojurongbe" section.

Competing Interests: No competing interests were disclosed.
Olusola Ojurongbe

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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.

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Author Response 27 Sep 2022

**Harriet Mukhongo**

Please find the authors' response to Reviewer 1 (Dr. Olusola Ojurongbe) linked [here](#).

**Competing Interests:** No competing evidence disclosed
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