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Original research article

Molecular Analysis of Dihydrofolate Reductase and Dihydropteroate Synthase Genes of *Plasmodium falciparum* Field Isolates from Afgoi and Balad, Southern Somalia

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ABSTRACT

This study aimed to investigate the prevalence of the *pfdhps* and *pfdhfr* polymorphisms in southern Somalia. The genetic polymorphisms of both genes were analyzed by nested PCR-RFLP. A total of 150 samples were collected; of these, 101 were shown to be positive for *Plasmodium* (96 *P. falciparum* and 5 *P. vivax*) by nested PCR, the remaining 49 were PCR negative. Of the 96 *Plasmodium falciparum* isolates, 88 were successfully amplified for *pfdhps* and *pfdhfr* polymorphisms. The mutations occurring in the pyrimethamine resistance gene (*pfdhfr*) at codons 51, 59 and 108 were 59 (67.0%), 51 (58.0%) and 83 (94.3%) isolates, respectively. Sulfadoxine resistance-associated mutations in the *pfdhps* gene at codons 437, 540 and 581 were found in 41 (46.6%), 43 (48.9%) and 13 (14.8%) samples, respectively. The analysis of *pfdhfr* and *pfdhps* combination revealed that 27 (30.7%) isolates harbor the quintuple mutations ($I_{51}R_{59}N_{108}$-$G_{437}E_{540}A_{581}$ and $I_{51}R_{59}N_{108}$-$G_{437}K_{540}G_{581}$). The prevalence of single mutation, triple mutations, quadruple mutations and double mutations haplotypes were 19.3%, 18.2%, 15.9% and 12.5%, respectively. Additionally, sextuple mutations were observed at 2 isolates (2.3%). This study shows that the *pfdhfr/pfdhps* mutant alleles have moderately declined compared to a previous study, but still remain high.

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Keywords: Molecular markers; *Pfdhfr*; *Pfdhps; Plasmodium falciparum*; Southern Somalia

1. Introduction

Malaria is caused by an intracellular protozoan parasite belong to the genus *Plasmodium*. *P. falciparum* malaria is notable for its high mortality rate; particularly in young children, as well as the alarming development of resistance to most available antimalarial drugs [1, 2]. In Somalia, malaria remains a significant public health problem, with 54% of the population at high risk of malaria infection. Over 95% of malaria cases in Somalia are due to *P. falciparum* [3] and approximately 28,900 confirmed malaria cases and 40–50 malaria-related deaths were reported from Somalia during 2007-2009 [3]. New cases of malaria are mainly reported from southern Somalia, especially districts located along the Shabelle and Juba rivers [4].

In 2005, sulfadoxine-pyrimethamine (SP) was replaced by chloroquine (CQ) as the first-line treatment for uncomplicated *P. falciparum* infection and two years later the combination of artesunate-SP (AS-SP) was introduced [5]. In 2016, artemether-lumefantrine (AL) was adopted as the Somali's first-line treatment due to the high degree of AS-SP resistance [5, 6]. However, SP is still used in Somalia as a prophylactic drug for intermittent preventive treatment in pregnant women and infants ($IPT_{P/I}$) according to the recommendation of the WHO for most sub-Saharan Africa (SSA) countries [7]. Providing an effective SP to pregnant women at every antenatal care visit helps to protect against the sequelae of falciparum malaria, like low birth weight (LBW) of offspring and maternal anemia [8].

SP resistance is associated with mutations in both the *pfdhfr* and *pfdhps* genes [9]. The $I_{51}R_{59}N_{108}$-$G_{437}E_{540}$, known as quintuple mutations are strongly correlated with SP therapeutic failure in sub-Saharan Africa [10]. The failure of SP has threatened falciparum malaria treatment in Somalia [5], where the majority of healthcare services are provided by private sectors with no central coordinated malaria treatment system due to lack of effective central government since the downfall of the military regime in 1992. There is also a lack of timely revised and applicable national malaria treatment policy in Somalia.

Due to the current use of SP in $IPT_{P/I}$, the monitoring of SP resistance markers is needed. This molecular surveillance will provide important information for the malaria control program in Somalia which could predict treatment outcome and help to make a decision to either continue or abolish this regimen. Therefore, the purpose of this study was to investigate the prevalence of *pfdhfr* and *pfdhps* polymorphisms in Afgoi and Balad, southern Somalia.

2. Materials and Methods

2.1 Study sites and sample collection

The current study was conducted in the high malaria transmission areas of Somalia; Afgoi and Balad (Figure 1). Ethical clearance was approved from Somali's ministry of Health and Human Services, Mogadishu, Somalia (MOH&HS/DGO/0776/May/2018).

Finger-prick blood samples were collected from consenting patients from May to July 2018.

2.2 DNA extraction

DNA from dried blood spot was extracted using a QIAamp® DNA mini kit (Qiagen, USA) according to the manufacturer's instructions. The DNA was kept at −20°C for molecular analysis.

2.3 Identification of *Plasmodium* species

The confirmation of *Plasmodium* species was performed by using a nested PCR. The primer sequences and PCR conditions were described elsewhere [11]. The first round of PCR amplified a small subunit ribosomal RNA (SSU rRNA) gene from all *Plasmodium* species, while the second round of PCR amplified the first round PCR product using species-specific primers, rFAL1 and rFAL2 for *P. falciparum*, and rVIV1 and VIV2 for *P. vivax*.

2.4 Genotyping of *pfdhfr* and *pfdhps* genes

The polymorphisms of *pfdhfr* and *pfdhps* genes were investigated by using nested PCR-RFLP following the previously described method [12, 13]. All PCRs were carried out at 25 µl final reaction volume containing 1× *Taq* buffer, 2 mM $MgCl_2$, 200 µM dNTP, 200 nM each primer, 0.02 U/µl of *Taq* polymerase and 2 µl of *P. falciparum* genomic DNA for the first round PCR. One µL of the primary PCR product was used for the nested PCR reaction. The primer sequences and PCR cycling conditions have been described previously [12, 13]. The second PCR products were digested by site-specific restriction enzymes. For *pfdhfr*, *Mlu*Cl, *Xmn*I, *Alu*I (New England Biolabs, Beverly, USA) were used for codon 51, 59 and 108, respectively. While *Ava*II, *Fok*I, *Bst*UI (New England Biolabs, Beverly, MA, USA) were used for mutation of *pfdhps* at codons 437, 540 and 581, respectively. *P. falciparum* strains (3D7 & K1) were used as control.

2.5 Statistical analysis

Each point mutation was presented as the number of isolates followed by their percentage. Chi-square was used to analyze the frequency distribution of the point mutations between the study sites (Afgoi *vs.* Balad). All statistical significance was set at $\alpha < 0.05$.

3. Results

3.1 *Plasmodium falciparum* isolates

Of the 150 samples, a total of 101 were shown to be positive for *Plasmodium* (96 *P. falciparum* and 5 *P. vivax*) by nested PCR, the remaining 49 were PCR negative. Of the 96 *P. falciparum* isolates, 88 isolates were successfully amplified for both *pfdhfr* and *pfdhps* genes. Regarding the study sites, 71 isolates were collected from Afogi and 17 isolates were from Balad.

3.2 Prevalence of mutation of the *pfdhfr* gene

Frequency distribution of the *pfdhfr* mutation is summarized in Table 1. Three codons (51, 59 and 108) of *pfdhfr* were investigated in this study. The mutations of this gene at codons 51, 59 and 108 were found in 59 (67.0%), 51 (58.0%) and 83 (94.3%) samples, respectively. Only the polymorphism of codon 51 was statistically significant difference between Afogi and Balad ($p < 0.001$). However, the sample size in Balad is much smaller than Afgoi, hence this may have affected this finding. The distribution of *pfdhfr* haplotype is shown in Table 2. High prevalence of *pfdhfr* haplotype was $I_{51}R_{59}N_{108}$ (53.4%), followed by $N_{51}C_{59}N_{108}$ (26.1%). The

$N_{51}C_{59}S_{108}$, $I_{51}C_{59}S_{108}$, $N_{51}C_{59}N_{108}$, $N_{51}R_{59}N_{108}$ haplotypes were only observed in Afgoi.

3.3 Prevalence of mutation of the *pfdhps* gene

The *pfdhps* genotypes were observed at codon 437, 540 and 581. The *pfdhps* mutations at codons; 437, 540 and 581 were 41 (46.6%), 43 (48.9%) and 13 (14.8%) isolates, respectively (Table 1). There was no statistically significant difference between these mutations among study areas. The analysis of *pfdhps* haplotype is summarized in Table 3. The wild-type haplotype corresponding to $A_{437}K_{540}A_{581}$ was the most prevalent (40.9%). The double mutations haplotype ($G_{437}E_{540}A_{581}$) was the second most prevalent (35.3%).

3.4 *Pfdhfr* and *pfdhps* haplotypes analysis

The distribution of the *pfdhfr* and *pfdhps* haplotypes in *P. falciparum* isolates in Somalia are summarized in Table 4. The observed haplotypes were grouped according to the number of mutations. Of the 88 samples, the most prevalent haplotype group, the quintuple mutations ($I_{51}R_{59}N_{108}$-$G_{437}E_{540}A_{581}$ and $I_{51}R_{59}N_{108}$-$G_{437}K_{540}G_{581}$), was found in 27 (30.7%) samples. The following haplotypes were single mutation ($N_{51}C_{59}N_{108}$- $A_{437}K_{540}A_{581}$ and $I_{51}C_{59}S_{108}$- $A_{437}K_{540}A_{581}$), triple mutations ($I_{51}R_{59}N_{108}$- $A_{437}K_{540}A_{581}$, $N_{51}C_{59}N_{108}$-$G_{437}E_{540}A_{581}$, $N_{51}R_{59}N_{108}$-$G_{437}K_{540}A_{581}$, $N_{51}C_{59}N_{108}$-$G_{437}G_{540}K_{581}$, and $N_{51}C_{59}N_{108}$-$G_{437}A_{540}G_{581}$), quadruple mutations ($I_{51}R_{59}N_{108}$-$A_{437}E_{540}A_{581}$, $I_{51}R_{59}N_{108}$-$A_{437}K_{540}G_{581}$, $I_{51}C_{59}N_{108}$-$G_{437}E_{540}A_{581}$, $N_{51}R_{59}N_{108}$-$G_{437}E_{540}A_{581}$, and $N_{51}C_{59}N_{108}$-$G_{437}E_{540}G_{581}$) and double mutations ($I_{51}C_{59}N_{108}$- $A_{437}K_{540}A_{581}$, $N_{51}C_{59}S_{108}$- $G_{437}E_{540}A_{581}$, and $N_{51}C_{59}N_{108}$-$A_{437}E_{540}A_{581}$) which were observed in 17 (19.3%), 16 (18.2%), 14 (15.9%) and 11 (12.5%) samples, respectively. The prevalence of sextuple mutations was observed at 2.3% (2 isolates).

4. Discussion

The mutations of the *pfdhfr* and *pfdhps* genes, which are molecular markers for SP treatment failure, were investigated in this study. Mutations of the *pfdhfr* gene, I_{51}, R_{59} and N_{108}, are commonly described as triple mutant alleles and confer resistance to pyrimethamine, while the double mutants of *pfdhps* (G_{437}, E_{540}) are known to cause sulfadoxine resistance. The double *dhps* mutations plus the triple *dhfr* mutations (called quintuple mutations) are strongly associated with a higher level of SP-resistance [14, 15].

In the present study, the mutation at codon 108 of *pfdhfr* (N_{108}), which is known as the initial mutant for pyrimethamine resistance [16], was observed in 94% and the I_{51} mutation was found in 67%. These results were in contrast to a previous study conducted in southern Somalia, which reported 100% prevalence for the N_{108} mutation and 87% prevalence for the I_{51} in 2011 [6]. The data from this study indicate a higher prevalence in N_{108} than detected in North Ethiopia (52.3%) [17], and East Africa (72.9%) [12]. The results for the R_{59} mutation in *pfdhfr* (58%) resemble the observed prevalence by Warsame *et al* in southern Somalia (52%) [6] but the R_{59} mutation was much more common than another study conducted in Bosaso, northeastern Somalia (22%) in 2015 [5].

For the *pfdhps* gene, 46% prevalence of G_{437} mutation was reported by this study. This mutation was depicted to be the initial mutant point associated with sulfadoxine resistance in many endemic areas [18]. The G_{437} mutation appears more frequently in this study as compared to another study conducted in southern Somalia (37.1%) in 2011 [6]. According to the criteria of the WHO, IPTp-SP can be used if the prevalence of E_{540} and G_{581} is less than 95% and 10%, respectively [19]. From these criteria, the use of IPTp-SP should be carefully monitored because 49% frequency of E_{540} was within the criteria

whereas the G_{581} was found to be higher than the recommended value (15%). The G_{581} mutation had not been reported from the previous study conducted in southern Somalia in 2011 [6]. The G_{581} mutation was assumed to be associated with IPTp-SP failure; in addition, the G_{540} mutation appeared to increase the degree of resistance [20]. The *pfdhfr* triple mutation ($G_{437}E_{540}G_{581}$) has shown a sharp decline from 64.4% in 2011 [6] to 4.6% in our data. Previous studies from several African countries have demonstrated an association between moderate SP resistance level and triple mutations in the *pfdhfr* ($G_{437}E_{540}G_{581}$) [6, 21, 22].

As per the study sites, the difference in distribution of SP resistance markers could not be clearly shown due to the disparity of sample sizes between the study sites (Afgoi=71 *vs.* Balad=17). Among all codons, only *pfdhfr* N51I showed a statistically significant difference between Afogi and Balad. However, as stated, the sample size in Balad is much smaller and this may have affected this finding.

The prevalence of $I_{51}R_{59}N_{108}$-$A_{437}K_{540}A_{581}$ haplotype, which conferred a pronounced resistance to pyrimethamine, was found in 11.5% of samples. This finding is much lower than that reported by Warsame *et al* [6] in 2011 (31.8%). The quintuple mutations ($I_{51}R_{59}N_{108}$-$G_{437}E_{540}A_{581}$), which was reported to confer a full resistance against SP in southeastern Africa [23], showed an upward trend from 15.7% in 2011 [6] to 28.4% in the current study. The finding indicates an increase in the prevalence of the quintuple mutation over a 7-year period. The level of quintuple mutation is much higher than that was previously reported in Ghana (1.4%) after long term abandonment of SP used [24].

The sextuple mutation ($I_{51}R_{59}N_{108}$-$G_{437}E_{540}G_{581}$), which is considered to confer a higher level of resistance than the quintuple mutation alone, was present in only 2 isolates (2.3%). No sextuple mutations had been identified in Somalia before [5, 6]. The high prevalence (>75%) of sextuple mutations was seen in Rwanda [25]. Only one isolate retained a wild haplotype ($N_{51}C_{59}S_{108}$- $A_{437}K_{540}A_{581}$) at all codons.

The different distributions for SP resistance molecular markers are likely due to differences in the period, geographical location of the studies and the decrease of the drug selective pressure after its discontinuation. However, the present study shows that most *pfdhfr* and *pfdhps* mutant alleles are still high after 13 years of withdrawal from SP-monotherapy for the treatment of uncomplicated falciparum malaria, although comparative studies were not conducted in the same study areas. The extensive use of different types of antifolate drugs with a similar mechanism of action as SP, such as cotrimoxazole (trimethoprim and sulphamethoxazole), in managing bacterial infections or preventing opportunistic infections among HIV-infected patients, might be another reason for the increased SP resistance [26]. Cotrimoxazole has been proven to have a cross-resistance with SP *in vitro P. falciparum* culture [26, 27].

Although there was no clinical data of SP treatment efficacy, the current results of high prevalence of SP resistance alleles are probably indicative of the low efficacy of SP mono-therapy against falciparum malaria treatment in Somalia.

Overall, most *pfdhfr*/*pfdhps* mutant alleles have moderately declined from levels described in a previous work by Warsame *et al* in 2011 [6]. The slow decline of SP-resistance alleles after 13 years discontinued SP-monotherapy was due to the remaining SP drug pressure from use as intermittent prophylaxis treatment during pregnancy and/or self-medication since the drug is still available at the local drug store.

Table 1. Frequency distribution of *pfdhfr* and *pfdhps* polymorphisms in 88 *P. falciparum* isolates from southern Somalia.

Gene	Position	Amino Acid	Number of isolates (%)		
			Afgoi	Balad	Total
pfdhfr	51*	N (Wild type)	29 (33.0)	0 (0.0)	29 (33.0)
		I (Mutation)	42 (47.7)	17 (19.3)	59 (67.0)
	59	C (Wild type)	31 (35.2)	6 (6.8)	37 (42.0)
		R (Mutation)	40 (45.5)	11 (12.5)	51 (58.0)
	108	S (Wild type)	5 (5.7)	0 (0.0)	5 (5.7)
		N (Mutation)	66 (75.0)	17 (19.3)	83 (94.3)
Pfdhps	437	A (Wild type)	40 (45.4)	7 (8.0)	47 (53.4)
		G (Mutation)	31 (35.2)	10 (11.4)	41(46.6)
	540	K (Wild type)	39 (44.3)	6 (6.8)	45 (51.1)
		E (Mutation)	32 (36.4)	11 (12.5)	43(48.9)
	581	A (Wild type)	58 (65.9)	17 (19.3)	75 (85.2)
		G (Mutation)	13 (14.8)	0 (0.0)	13 (14.8)

*The statistical significance among Afgoi and Balad; p <0.001.

Table 2. Distribution of *pfdhfr* haplotype in 88 *P. falciparum* isolates from southern Somalia.

Pfdhfr* haplotype	Number of isolates (%)		
	Afgoi	Balad	Total
$N_{51}C_{59}S_{108}$	2 (2.3)	0 (0.0)	2 (2.3)
$I_{51}C_{59}S_{108}$	3 (3.4)	0 (0.0)	3 (3.4)
$N_{51}C_{59}N_{108}$	23 (26.1)	0 (0.0)	23 (26.1)
$I_{51}C_{59}N_{108}$	3 (3.4)	6 (6.8)	9 (10.2)
$N_{51}R_{59}N_{108}$	4 (4.6)	0 (0.0)	4 (4.6)
$I_{51}R_{59}N_{108}$	36 (40.9)	11 (12.5)	47 (53.4)

*Bold letter indicate the mutation allele.

Table 3. Distribution of *pfdhps* haplotype in 88 *P. falciparum* isolates from southern Somalia.

Pfdhps* haplotype	Number of isolates (%)		
	Afgoi	Balad	Total
$A_{437}K_{540}A_{581}$	30 (34.1)	6 (6.8)	36 (40.9)
$G_{437}K_{540}A_{581}$	1 (1.1)	0 (0.0)	1 (1.1)
$A_{437}E_{540}A_{581}$	6 (6.8)	1 (1.1)	7 (7.9)
$A_{437}K_{540}G_{581}$	3 (3.4)	0 (0.0)	3 (3.4)
$G_{437}E_{540}A_{581}$	21 (23.9)	10 (11.4)	31 (35.3)
$G_{437}K_{540}G_{581}$	5 (5.7)	0 (0.0)	5 (5.7)

$A_{437}E_{540}G_{581}$	1 (1.1)	0 (0.0)	1 (1.1)
$G_{437}E_{540}G_{581}$	4 (4.6)	0 (0.0)	4 (4.6)

*Bold letter indicate the mutation allele

Table 4. Distribution of *pfdhfr* and *pfdhps* haplotypes in *P. falciparum* isolates from southern Somalia.

Pfdhfr-pfdhps* haplotype	**Number of isolates (%)**		
	Afgoi	**Balad**	**Total**
All wild type • $N_{51}C_{59}S_{108}$- $A_{437}K_{540}A_{581}$	1 (1.1)	0 (0.0)	1 (1.1)
Sextuple mutations • $I_{51}R_{59}N_{108}$-$G_{437}E_{540}G_{581}$	2 (2.3)	0 (0.0)	2 (2.3)
Quintuple mutations	17 (19.3)	10 (11.4)	27 (30.7)
• $I_{51}R_{59}N_{108}$-$G_{437}E_{540}A_{581}$	15 (17.0)	10 (11.4)	25 (28.4)
• $I_{51}R_{59}N_{108}$-$G_{437}K_{540}G_{581}$	2 (2.3)	0 (0.0)	2 (2.3)
Quadruple mutations	13 (14.8)	1 (1.1)	14 (15.9)
• $I_{51}R_{59}N_{108}$-$A_{437}E_{540}A_{581}$	4 (4.6)	1 (1.1)	5 (5.7)
• $I_{51}R_{59}N_{108}$-$A_{437}K_{540}G_{581}$	3 (3.4)	0 (0.0)	3 (3.4)
• $I_{51}R_{59}N_{108}$-$G_{437}E_{540}A_{581}$	1 (1.1)	0 (0.0)	1 (1.1)
• $N_{51}R_{59}N_{108}$-$G_{437}E_{540}A_{581}$	3 (3.4)	0 (0.0)	3 (3.4)
• $N_{51}C_{59}N_{108}$-$G_{437}E_{540}G_{581}$	2 (2.3)	0 (0.0)	2 (2.3)
Triple mutations	16 (18.2)	0 (0)	16 (18.2)
• $I_{51}R_{59}N_{108}$- $A_{437}K_{540}A_{581}$	10 (11.5)	0 (0.0)	10 (11.5)
• $N_{51}C_{59}N_{108}$-$G_{437}E_{540}A_{581}$	1 (1.1)	0 (0.0)	1 (1.1)
• $N_{51}R_{59}N_{108}$- $G_{437}K_{540}A_{581}$	1 (1.1)	0 (0.0)	1 (1.1)
• $N_{51}C_{59}N_{108}$-$G_{437}G_{540}K_{581}$	3 (3.4)	0 (0.0)	3 (3.4)
• $N_{51}C_{59}N_{108}$-$G_{437}A_{540}G_{581}$	1 (1.1)	0 (0.0)	1 (1.1)
Double mutations	5 (5.7)	6 (6.8)	11 (12.5)
• $I_{51}C_{59}N_{108}$- $A_{437}K_{540}A_{581}$	2 (2.3)	6 (6.8)	8 (9.1)
• $N_{51}C_{59}S_{108}$- $G_{437}E_{540}A_{581}$	1 (1.1)	0 (0.0)	1 (1.1)
• $N_{51}C_{59}N_{108}$- $A_{437}E_{540}A_{581}$	2 (2.3)	0 (0.0)	2 (2.3)
Single mutation	17 (19.3)	0 (0.0)	17 (19.3)
• $N_{51}C_{59}N_{108}$- $A_{437}K_{540}A_{581}$	14 (15.9)	0 (0.0)	14 (15.9)
• $I_{51}C_{59}S_{108}$- $A_{437}K_{540}A_{581}$	3 (3.4)	0 (0.0)	3 (3.4)

*Bold letter indicate the mutation allele

Fig. 1. Map of Somalia showing study area.

4. Conclusion

This is the first molecular study highlighting the mutations of the *pfdhfr* and *pfdhps* genes conducted in Afgoi and Balad, Somalia. The study shows that the *pfdhfr/pfdhps* mutant alleles have moderately declined compared to a previous study, but still remain high. Monitoring of *pfdhfr* and *pfdhps* mutations could provide important data for the drug resistance mapping and the pressure for other antifolate drugs.

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