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# Return of chloroquine-sensitive *Plasmodium falciparum* parasites and emergence of chloroquine-resistant *Plasmodium vivax* in Ethiopia

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## Abstract

**Background:** Increased resistance by *Plasmodium falciparum* parasites led to the withdrawal of the antimalarial drugs chloroquine and sulphadoxine-pyrimethamine in Ethiopia. Since 2004 artemether-lumefantrine has served to treat uncomplicated *P. falciparum* malaria. However, increasing reports on delayed parasite clearance to artemisinin opens up a new challenge in anti-malarial therapy. With the complete withdrawal of CQ for the treatment of *Plasmodium falciparum malaria*, this study assessed the evolution of CQ resistance by investigating the prevalence of mutant alleles in the *pfmdr1* and *pfcrt* genes in *P. falciparum* and *pvmdr1* gene in *Plasmodium vivax* in Southern and Eastern Ethiopia.

**Methods:** Of the 1,416 febrile patients attending primary health facilities in Southern Ethiopia, 329 febrile patients positive for *P. falciparum* or *P. vivax* were recruited. Similarly of the 1,304 febrile patients from Eastern Ethiopia, 81 febrile patients positive for *P. falciparum* or *P. vivax* were included in the study. Of the 410 finger prick blood samples collected from malaria patients, we used direct sequencing to investigate the prevalence of mutations in *pfcrt* and *pfmdr1*. This included determining the gene copy number in *pfmdr1* in 195 *P. falciparum* clinical isolates, and mutations in the *pvmdr1* locus in 215 *P. vivax* clinical isolates.

**Results:** The *pfcrt* K76 CQ-sensitive allele was observed in 84.1% of the investigated *P.falciparum* clinical isolates. The *pfcrt* double mutations (K76T and C72S) were observed less than 3%. The *pfcrt* SVMNT haplotype was also found to be present in clinical isolates from Ethiopia. The *pfcrt* CVMNK-sensitive haplotypes were frequently observed (95.9%). The *pfmdr1* mutation N86Y was observed only in 14.9% compared to 85.1% of the clinical isolates that carried sensitive alleles. Also, the sensitive *pfmdr1* Y184 allele was more common, in 94.9% of clinical isolates. None of the investigated *P. falciparum* clinical isolates carried S1034C, N1042D and D1246Y *pfmdr1* polymorphisms. All investigated *P. falciparum* clinical isolates from Southern and Eastern Ethiopia carried only a single copy of the mutant *pfmdr1* gene.

**Conclusion:** The study reports for the first time the return of chloroquine sensitive *P. falciparum* in Ethiopia. These findings support the rationale for the use of CQ-based combination drugs as a possible future alternative.

**Keywords:** Malaria, *Plasmodium falciparum*, *Plasmodium vivax*, Ethiopia, *pfcrt*, *pfmdr1*, *pvmdr1*, *pfmdr1* gene copy number

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## Background

Malaria remains one of the major health problems in sub-Saharan Africa [1,2]. Though there are encouraging reports that malaria morbidity and mortality are declining [3], it is still an overwhelming public health problem, with an estimated 207 million cases and 627,000 deaths every year worldwide. Of all malaria deaths, 90% occur in sub-Saharan Africa, and 77% in children under five years of age [4]. One of the main obstacles to malaria control is the ability of the parasites to develop resistance to the administered anti-malarial drugs. Chloroquine (CQ) was the first antimalarial to be widely used in endemic areas, but CQ resistance was documented in Thailand in the late 1950's, spread to African in 1974 [5], and subsequently came to East Africa in the early 1980's [6]. Since 2009, there are increasing concerns and reports of delayed parasite clearance to administered artemisinin, especially in parts of Southeast Asia [7].

In Ethiopia, CQ treatment failure for *Plasmodium falciparum* and *Plasmodium vivax* was documented in 1996 [8]. Therefore, in 1998 sulphadoxine-pyrimethamine (SP) replaced the CQ triple-dose (25 mg base/kg) regimen as the first-line drug for the treatment of uncomplicated *P. falciparum* and mixed infection of *P. falciparum and P. vivax* malaria. However, in a short time span *P. falciparum* also developed resistance to SP, leading to therapeutic failure of SP [9-12]. Artemether-lumefantrine (AL) (Coartem®) replaced SP in 2004 as a first-line drug to treat uncomplicated *P. falciparum* and mixed infection of *P. falciparum and P.vivax* malaria in Ethiopia [13]. However CQ still remains the first-line drug to treat *P. vivax* malaria in Ethiopia and sub-Saharan Africa. Although significant progress has been made to understand the resistance mechanisms to administered anti-malarial drugs, the genetic basis of drug resistance is vital to implement strategies for efficient malaria control.

Point mutations in the genes "*P. falciparum* multidrug resistance 1" (*pfmdr1*), located in chromosome 5 [14-17], "*P. falciparum* chloroquine resistance transporter" (*pfcrt*), on chromosome 7 [18-20], and "*P. vivax* multi-drug resistance 1" (*pvmdr1*) on chromosome 10 [21-23] are the most investigated genes for anti-malarial drug resistance. *Pfmdr1* encodes a trans-membrane transporter, and point mutations in this gene modulate the level of CQ resistance [24,25]. Other mutations are also associated with anti-malarial drug resistance and delayed parasite clearance [15,17,26,27]: in the codons 86 (Asn86Tyr), 184 (Tyr184Phe), 1034 (Ser1034Cys), 1042 (Asn1042Asp), 1246 (Asp1246Tyr) in *pfmdr1*; codons 72 (Cys72Ser), 74 (Met74Ile), 75 (Asn75Glu) and 76 (Lys76Thr) in *pfcrt*; and codons 976 (Tyr976Phe) and 1076 (Phe1076Leu) in *pvmdr1*. Additionally, several other studies have also reported that *pfcrt* haplotypes are associated with anti-malarial drug resistance [19,25,28]. Gene copy number variations (CNV) in the *P. falciparum* genome have been shown to influence parasite phenotypes, and increased gene copy numbers are associated with reduced susceptibility to anti-malarial drugs.

Mutations in the *pfmdr1* locus are associated with anti-malarial resistance to chloroquine, mefloquine, quinine or artemisinin derivatives, whereas *pfcrt* mutations are associated with chloroquine, amodiaquine, mefloquine and artemisinin combination therapy [18]. Increased pfmdr1 gene copy numbers are associated with reduced susceptibility of P. falciparum to anti-malarial drugs, especially with artemether-lumefantrine and artesunate-mefloquine [29]. In many countries that are prone to malaria endemics, *P. vivax* malaria is treated with CQ, as the drug is well tolerated, affordable, has a longer half-life, and protects from early relapses [30-32]. A decline in CQ efficacy for *P. vivax* malaria was reported in Papua New Guinea in 1989 [33] and in northwestern Sumatra, Indonesia in 1995 [34]. CQ resistance in *P. vivax* in Ethiopia was reported in 2008, when four patients out of 83(4.8%) had recurrent parasitaemia on Day 28 [35].

Several studies at regular intervals are required to evaluate drug resistance based on the genetic diversity of circulating *P. falciparum* strains and their molecular markers for *P. falciparum* resistance to CQ and AL. Such studies will lay a basis to re-evaluate existing strategies in malaria control [36]. In the present study, the prevalence of mutations in *pfcrt* and *pfmdr1*, the gene copy number of *pfmdr1* in *P. falciparum*, and point mutations in *pvmdr1* in *P. vivax* were investigated in clinical isolates from Southern and Eastern Ethiopia.

## Methods

### Study sites

A health institution-based study targeting febrile patients attending primary health facilities was conducted in Southern and Eastern Ethiopia. Samples from Southern Ethiopia were collected between the months of August and December, 2011, at three health centres: Omo Nada, Bala Wajo, and Arba Minch. In Eastern Ethiopia samples were collected in the Harar health centre between the months of October and December 2009. Malaria transmission in these study sites is seasonal, with higher transmission following the rainy seasons (between September and December, and between April and May).

### Study samples

Study participants were recruited from the routine health delivery system. Self-presenting febrile patients attending health centers in Omo Nada (n = 713), Bala Wajo (n = 312), and Arba Minch (n = 391), and whose age was at least six months between August and December 2011, were eligible for the current study. Additionally self-presenting febrile patients attending Harar health centre

(n = 1,304) in eastern Ethiopia were also eligible for the study. An axillary temperature was measured to check for a fever. Patients with an axillary temperature of ≥37°C were considered febrile. Any patient reported as being infected either with *P. falciparum* or *P. vivax* or with a mixed infection using the existing health care delivery system within the target health centres were approached for consent immediately after receiving their laboratory result. For those who consented to be part of the study, approximately 5–10 μL of a finger-prick blood sample was collected. No additional clinical assessment was carried out at the time of recruitment. A total of 410 Southern and Eastern Ethiopian finger-prick blood samples collected on Whatman 3 mm filter paper tested positive by microscopy for either *P. falciparum* or *vivax*. Of these, 195 tested positive for *P. falciparum* (170 from Southern Ethiopia and 25 from Eastern Ethiopia), and 215 for *P. vivax* (159 *P. vivax* from Southern Ethiopia and 56 from Eastern Ethiopia). The blood spots were air-dried and stored in a separate, clean, sealed plastic bag at room temperature for further molecular analysis.

### Blood film management

After fixing the thin film in methanol, both smears were stained with 3% Giemsa for 30 min and examined under oil immersion for malaria parasites. Thick films were used for parasite detection, and thin film for species identification. At least 100 fields examined under an oil-immersion lens were examined before ruling out infection.

### Detection of *pfmdr1*, *pfcrt* and *pvmdr1* polymorphisms

DNA was isolated using the Qiagen DNA Mini Kit for blood and tissue (QIAGEN, Germany), following the manufacturer's instructions, and it was then stored at −20°C until use. Nested PCR was carried out as previously described elsewhere [37]. DNA samples were amplified by species-specific primer pairs (Table 1). For *pfcrt, pfmdr1* and *pvmdr1* both the primary and nested amplifications were carried out in a 20 μL reaction volume containing 1X buffer, 2.5 mM $MgCl_2$, 200 μM dNTPs, 200nM primers, 1U Taq DNA-Polymerase, and 10 ng of DNA template on a PTC-200 Thermal cycler (MJ Research, USA). Each genus-specific amplification for *Plasmodium* was followed by a species-specific PCR. For amplifications, the study followed standard nested PCR procedure (Table 1). The PCR product of the first reaction was used as the template for the second in all nested PCRs. Amplicons that resulted from the nested PCRs were separated by electrophoresis on a 1.2% agarose gel, run with a 100 bp DNA ladder (Invitrogen, Karlsruhe, Germany). The presence of different *Plasmodium* species was re-confirmed by checking amplicon size. To identify relevant Single Nucleotide Polymorphisms (SNPs) in *pfmdr1, pfcrt* and *pvmdr1*, PCR products were cleaned using Exo-SAP-IT (USB, Affymetrix, USA), and 1 μL of the purified product was used as a template for direct sequencing using the Big Dye terminator v. 2.0 cycle sequencing kit (Applied Biosystems, USA) on an ABI 3130XL DNA sequencer, according to the manufacturer's instructions [38]. All investigated

**Table 1 Primer pairs used to amplify and sequence *pfcrt, pfmdr1* and *pvmdr1***

| Gene fragment | Primer Name | Primer sequence | PCR cycling conditions |
|---|---|---|---|
| Pfcrt SNPs at | OF P1 | 5′CCGTTAATAATAAATACACGCG | 35 cycles of 94°C for 30 s; 56°C |
| Codon 72&76 | OR P2 | 5′CGGATGTTACAAAACTATAGTCC | for 30 s; and 62°C for 1 min; |
| | NF P3 | 5′-AGGTTCTTGTCTTGGTAAATTTGC | 30 cycles of 94°C for 30 s; 56°C |
| | NR P4 | 5′-CAAAACTATAGTTACCAATTTTG | for 30 s; and 65°C for 1 min; |
| Pfmdr1 SNPs at | OF P5 | 5′-AGGTTGAAAAAGAGTTGAAC | 30 cycles of 94°C for 30 s; 55°C |
| Codon 86&184 | OR P6 | 5′-ATGACACCACAAACATAAAT | for 30 s; and 65°C for 1 min; |
| | NF P7 | 5′-ACAAAAAGAGTACCGCTGAAT | 30 cycles of 94°C 30 s; 60°C |
| | NR P8 | 5′-AAACGCAAGTAATACATAAAGTC | for 30 s; and 65°C for 1 min; |
| Pfmdr1 SNPs at | OF P9 | 5′-GTGTATTTGCTGTAAGAGCT | 34 cycles of 94°C for 30 s; 55°C |
| Codon 1034 | OR P10 | 5′-GACATATTAAATAACATGGGTTC | for 1 min and 72°C for 1.5 |
| 1042 and 1246 | NF P11 | 5′ CAGATGATGAAATGTTTAAAGATC | 29 cycles of 94°C for 30 s; 60°C |
| | NR 12 | 5′-TAAATAACATGGGTTCTTGACT | for 30 s; and 65°C for 1 min; |
| pvmdr1at codon | OF P13 | 5′-GCGAACTCGAATAAGTACTCCCTCTA | 45 cycles of 94°C for 5 min, |
| 976 and 1076 | OF P14 | 5′GGCGTAGCTTCCCGTAAATAAA | 530C for 1 min and 720C for 1 min |
| | NF P15 | 5′-GGATTGCTGTCAGCACATATTAACA | 45 cycles of 94°C for 5 min, |
| | NF P16 | 5′AGAGGGATTTCATAAAGTCATT | 650C for 1 min and 720C for 1 min |

OF: Outer Forward; OR: Outer Nested; P1-P12: Primers; NF: Nested Forward; NR: Nested Reverse; bp: base pair; pfmdr1: *Plasmodium falciparum* multi-drug resistance 1; pfcrt: *Plasmodium falciparum* chloroquine resistance transporter; pvmdr1: *Plasmodium. vivax multi-drug resistance 1.*

point mutations in *pfmdr1, pfcrt and pvmdr1* are illustrated in Table 2.

### Detection of *pfmdr1* gene copy number variations

The *pfmdr1* gene copy number was estimated by TaqMan real-time PCR, as previously described [29]. Briefly, 10 ng of genomic DNA was amplified in a 25 μL volume of reaction mixture containing 1x TaqMan buffer (8% glycerol, 0.625 U DNA polymerase, 5.5 mmol/L MgCl2, 300 μmol/L dNTPs, 600 nmol/L passive reference dye ROX (5-carboxy-X-rhodamine), pH 8.3), 300 nmol/L of each forward and reverse primer, 100 nmol/L of each probe, and 5 μL of template DNA on a Corbett Research RG-3000 (Qiagen, Hilden, Germany). Thermal cycling parameters were as follows: pre-incubation at 95°C for 5 minutes, followed by 50 cycles of 15 seconds at 95°C and 1 minute at 58°C. Genomic DNA from *P. falciparum* reference 3D7, reported to have only one copy, was used as the calibrator [39], and *P. falciparum* ß-tubulin as the house-keeping gene. For multiple *pfmdr1* copy number control, DNA from the Dd2 clone was used. The $2^{-\Delta\Delta Ct}$ method of relative quantification [40] was used to estimate copy number. Using this cycle threshold (Ct) method to estimate copy numbers of unknown samples, a calibrator DNA template with known copies of the interest and a house-keeping gene which has the same copy number was utilized. The ΔΔCt calculation was as follows: ΔΔCt = (Ct target gene - Ct Pf ß-tubulin) X - (Ct target gene - Ct Pf ß-tubulin) Y, where X = unknown sample and Y = *P. falciparum* 3D7. The result was expressed in N-fold changes in X target gene copies normalized to *P. falciparum* ß-tubulin according to the equation (amount of target) = $2^{-\Delta\Delta Ct}$. Each sample was run as triplicate, and the mean and standard deviation of the three Ct values were calculated and computed for specificity as suggested earlier [29].

### Ethics

Ethical approvals were obtained from the review boards of Aklilu Lemma Institute of Pathobiology (ALIPB), College of Health Sciences of Jimma University, College of Health and Medical Sciences of Haramaya University and Armauer Hansen Research Institute (AHRI). After obtaining informed consent and assent from either the patient or legal guardian, blood samples were collected from individuals positive for *P. falciparum* or *P. vivax*. The study participation was voluntary and had no influence on the treatment provision at the respective health facilities. All patient information was kept confidential. All individuals positive for *P. falciparum* were treated with AL, while *P. vivax* positive patients were treated with CQ.

## Results

The observed frequencies of the investigated SNPs in the *pfmdr1, pfcrt* and the *pvmdr1* loci are summarized in Table 3. The *pfcrt* K76T (Lys76Thr) mutation was observed in only 15.9% (31/195) of the clinical isolates investigated compared to 84.1% (164/195) wild type allele that were sensitive to CQ. The *pfcrt* C72S (Cys72Ser) was observed only in 3.6% (7/195) compared to 96.4% (188/195) wild type allele that were sensitive to CQ. The *pfcrt* double mutations (K76T and C72S) were observed in less than 2.6% of individuals (5/195) Table 3. For the investigated *pfmdr1* locus N86Y(Asn86Tyr), the mutation was observed in 14.9% (29/195) of the clinical isolates compared to 85.1% (166/195) wild type that carried sensitive alleles. The investigated *pfmdr1* locus Y184 (Tyr184Phe), only 5.1% (10/195) of the clinical isolates carried the mutant. None of the investigated 195 *P. falciparum* clinical isolates carried S1034C, N1042D and D1246Y of *pfmdr1* polymorphisms. For the investigated *pvmdr1* locus Y976 (Tyr976Phe), the mutation was observed in 32.6% (70/215) of the clinical isolates compared to 67.7% (145/215) wild type that carried sensitive alleles, whereas the investigated F1076 (Phe1076Leu) carried no drug resistant mutants Table 3. Of the all investigated *P. falciparum* clinical isolates from Southern and Eastern Ethiopia, only one copy of *pfmdr1* gene copy number was observed.

The frequency of SNPs in the *pfmdr1, pfcrt* and the *pvmdr1* loci in Southern and Eastern Ethiopia are summarized in Table 4. The *pfcrt* K76T mutation in Southern Ethiopia was 13.5% (23/170) of the clinical isolates investigated compared to 86.5% (147/170) wild type allele that were sensitive to CQ. The *pfmdr1* locus N86Y was observed in 12.9% (22/170) compared to 87.1% (148/170) wild type allele that were sensitive to CQ. In Eastern Ethiopia, SNPs in the *pfmdr1* locus N86Y and *pfcrt* locus K76T were 28.0% (7/25) and 32.0% (8/25) respectively. On the other hand, for the investigated *pvmdr1* locus Y976 (Tyr976Phe), the mutation was observed in 37.1% (59/

**Table 2 Investigated point mutations in *pfmdr1, pfcrt* and *pvmdr1*genes**

| | ***Pfmdr1* locus** | | | | | **Pfcrt locus** | | | | ***Pvmdr1* locus** | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Codon position | 86 | 184 | 1034 | 1042 | 1246 | 72 | 74 | 75 | 76 | 976 | 1076 |
| Wild type/Mutant | TGA/TG***T*** | ATA/AT***T*** | CAG/C***T***G | TTA/TT***G*** | GAG/GA***T*** | TGT/***A***GT | ATG/AT***T*** | AAT/***G***A***A*** | AAA/A***C***A | TAC/T***T***C | TTT/***C***TT |
| ns substitution | Asn/Tyr | Tyr/Phe | Ser/Cys | Asn/Asp | Asp/Tyr | Cys/Ser | Met/Ile | Asn/Glu | Lys/Thr | Tyr/Phe | Phe/Leu |

ns: non synonymous ; *pfmdr1*: *Plasmodium falciparum* multi-drug resistance 1; *pfcrt*: *Plasmodium falciparum* chloroquine resistance transporter; *pvmdr1*: *Plasmodium vivax* multi-drug resistance 1.

**Table 3 Distribution of *pfcrt*, *pfmdr1* and *pvmdr1* point mutations in the investigated clinical isolates**

| | *P. falciparum* | number | % |
|---|---|---|---|
| | **no resistance alleles detected** | **127** | **65**% |
| *Pfcrt* | only C72 | 7 | 3.6% |
| | C72 and K76 | 5 | 2.6% |
| | only K76 | 31 | 15.9% |
| *pfcrt* and *pfmdr1* | K76 and N86 | 4 | 2.1% |
| *pfmdr1* | only N86 | 29 | 14.9% |
| | N86 and Y184 | 8 | 4.1% |
| | only Y184 | 10 | 5.1% |
| | Total | 195 | |
| | *P. vivax* | | |
| | **no resistance alleles detected** | **145** | **74**% |
| *pvmdr1* | only Y976 | 70 | 32.6% |
| | only F1076 | 0 | 0% |
| | Total | 145 | |

159) of the clinical isolates in Southern Ethiopia compared to 19.6% (11/56) in Eastern Ethiopia.

The *pfcrt* haplotypes were reconstructed for mutations at codons 72–76 in all *P. falciparum* isolates. The *pfcrt* wild type Cysteine-Valine-Methionine-Asparagine-Lysine (*pfcrt* CVMNK) mutates to either Cysteine-Valine-Isoleucine-Glutamate-Threonine (*pfcrt* CVIET) or Serine-Valine-Methionine-Asparagine-Threonine (*pfcrt* SVMNT) variants Table 5. The mutated *pfcrt* SVMNT haplotype was observed in 4.1% (8/195) of the isolates, whereas *pfcrt* CVMNK of wildtype was observed in 95.9% (187/195) Table 5. No *pfcrt* CVIET haplotypes were observed.

**Table 4 Distribution of *pfcrt*, *pfmdr1* and *pvmdr1* point mutations in the investigated clinical isolates in Southern and Eastern Ethiopia**

| | *P. falciparum* | SE no | SE (%) | EE no | EE (%) |
|---|---|---|---|---|---|
| *Pfcrt* | only C72 | 5 | 2.9% | 2 | 8.0% |
| | C72 and K76 | 4 | 2.4% | 1 | 4.0% |
| | only K76 | 23 | 13.5% | 8 | 32.0% |
| *pfcrt* and *pfmdr1* | K76 and N86 | 2 | 1.2% | 2 | 8.0% |
| *pfmdr1* | only N86 | 22 | 12.9% | 7 | 28.0% |
| | N86 and Y184 | 6 | 3.5% | 2 | 8.0% |
| | only Y184 | 7 | 4.1% | 3 | 12.0% |
| | *P. vivax* | | | | |
| *pvmdr1* | only Y976 | 59 | 37.1% | 11 | 19.6% |
| | only F1076 | 0 | | | 0.0% |

*pfmdr1*: *Plasmodium falciparum* multi-drug resistance 1; *pfcrt*: *Plasmodium falciparum* chloroquine resistance transporter; *pvmdr1*: *Plasmodium. vivax* multi-drug resistance 1; SE: Southern Ethiopia; EE: Eastern Ethiopia.

## Discussion

The aim of the current study was to investigate the prevalence of mutant SNPs in the loci *pfmdr1*, *pfcrt*, and *pvmdr1*, as well as copy number variations in *pfmdr1*, all of which are associated with anti-malarial drug resistance. A large proportion of *P. falciparum* clinical isolates in this study (84.1%) carried the chloroquine-susceptible *pfcrt* K76 allele. Previously it has been reported that the CQ-resistant mutation *pfcrt* K76T is a strong predictor of overall CQ resistance, and thus it is used as a biomarker of CQ resistance [16]. It appears that CQ-susceptible *Plasmodium falciparum* isolates are back in circulation in Ethiopia.

The finding of this study does not support a previous study conducted in Ethiopia that reported a 100% frequency of the *pfcrt* T76 mutation, and an 81% frequency of the mutation *pfmdr1* Y86 in *P. falciparum* [41]. However, the current finding is in line with studies conducted in two East African countries [27,42], where the frequency of the *pfcrt*-76 resistance alleles were repopulated with the sensitive ones after many years of CQ withdrawal. Studies showed that *pfcrt* mutations at codons 72 to 76 with non-synonymous substitutions result in wildtype *pfcrt* CVMNK or other *pfcrt* haplotypes CVIET or SVMNT that have been associated with CQ resistance[43]. The wild type *pfcrt* CVMNK haplotypes were observed in 187 (95.9%) of the *P. falciparum* isolates. The mutant *pfcrt* SVMNT was carried by 4.1% (8/195) of the investigated isolates, and it was identical to that of chloroquine resistant isolates identified in South-East Asia [44]. These are the first findings on the presence of the *pfcrt* SVMNT haplotype in clinical isolates in Ethiopia. This is an interesting finding and novel to the study as the SVMNT haplotype is still very rare in Africa. So far few studies reported the presence of this haplotype in African countries [45-48], of this haplotype mostly associated with amodiaquine resistance and lower level of chloroquine resistance compared to CVIET [49,50]. The *pfcrt* CVIET haplotypes were not detected here, although it is prevalent in Africa [51].

Concerning *pfmdr1*, while wild-type *pfmdr1* is thought to transport and accumulate CQ in the parasites' food vacuole, mutations N86Y, S1034C, N1042D, and D1246Y interfere with transportation of the anti-malarial drugs, leading to reduced CQ-sensitivity [14]. The percentage of wild-type alleles at codon 86 of *pfmdr1* was 85.1% (166/195), which is higher than 81% present in Schunk in 2006 and 84.5% in Eshetu in 2010 [41,52]. It should be clear that the study by Schunk in 2006 was conducted 7 years before the current study. This is clear that as the time difference between these two studies is large and with reduced drug pressure on CQ over time might have contributed to the return of CQ susceptibility.

**Table 5 Distribution of reconstructed *Pfcrt* haplotypes from clinical isolates from South and Eastern Ethiopia**

| Locus | haplotype | Investigated fragment (5′-3′) | Number (%) |
|---|---|---|---|
| *pfcrt* 74-76 | CVMNK (Sensitive) | 5′-TAATTGAAACAATTTTTG-3′ | 187 (95.9) |
| | SVMNT (Mutant) | 5′-TAAT***GA***A***T***A***A***AATTTTTG-3′ | 8 (4.1) |
| | CVIET (Mutant) | 5′-TAAT***GA***A***T***ACAATTTTTG-3′ | 0 |

*pfmdr1*: *Plasmodium falciparum* multi-drug resistance 1; *pfcrt*: *Plasmodium falciparum* chloroquine resistance transporter; *pvmdr1*: *Plasmodium vivax* multi-drug resistance 1.

The *pfcrt* K76T mutation in Southern Ethiopia was 13.5% (23/170) of the clinical isolates investigated compared to 86.5% (147/170) wild type allele that were sensitive to CQ. The *pfmdr1* locus N86Y was observed in 12.9% (22/170) compared to 87.1% (148/170) wild type allele that were sensitive to CQ. In Eastern Ethiopia, SNPs in the *pfmdr1* locus N86Y and *pfcrt* locus K76T were 28.0% (7/25) and 32% (8/25) respectively. There was no statistical difference between Southern and Eastern Ethiopia in SNPs in the *pfmdr1* locus N86Y and *pfcrt* locus K76T (P = 0.9).

Double mutations in *pvmdr1* at positions Y976F and F1076L have been suggested to be associated with reduced chloroquine susceptibility to *P. vivax* in Thailand and Indonesia, the two countries where *P. vivax* is prevalent. Other studies have addressed whether chloroquine or amodiaquine treatment failure might be associated with *P. vivax* isolates carrying the Y976F substitution [53]. *Pvmdr1* sequences from *P.vivax*, the orthologue of *pfmdr1*, were analyzed for the presence of mutations at positions 976 and 1076, and we found that 32.6% (70/215) of the isolates carried the F976 SNP. Mutations were not detected at codon 1076 in all *P. vivax* isolates studied here. On the other hand, for the investigated *pvmdr1* locus Y976 (Tyr976Phe), the mutation was observed in 37.1% (59/159) of the clinical isolates from Southern Ethiopia compared to 19.6% (11/56) from Eastern Ethiopia.

In line with the current study, studies conducted in Madagascar and Brazil showed that F1076L cannot be taken as an indicator of drug resistance, but rather it is a geographic variant [46,53,54]. Earlier, *in vivo* studies conducted in Ethiopia confirmed the presence of drug resistant *P. vivax* [8,35]. However, these studies were confounded by the inherent difficulties associated with *in vivo* tests for relapsing malaria [55] and the difficulties in differentiating between re-infection, recrudescence, and relapse after treatment failure [56].

A high proportion (97.9%, 191/195) of samples had chloroquine (CQ)-susceptible alleles at both codon 76 of *pfcrt* and codon 86 of *pfmdr1*. The rapid shift in *P. falciparum* from CQ-resistant to CQ-susceptible suggests that the removal of CQ for the treatment of *P. falciparum* or the pressure from AL that has been used since 2004 to treat *falciparum* malaria and mixed infection of *P.falciparum* and *P. vivax* or both may eventually lead to replacement of *pfmdr1* resistance genes by susceptible parasite populations [42,57]. Our finding was consistent with observations from Malawi [57], Kenya [42], and Tanzania [27], where the withdrawal of chloroquine resulted in the rapid spread of a chloroquine-susceptible *Pfcrt* K76 population. In Malawi, recovery of the susceptible *Pfcrt*-K76 from <15% to 100% within 13 years of CQ withdrawal has been reported [57]. Moreover, a study confirmed that the *pfmdr1* alleles 86 N, 184 F, and 1246D significantly increased in prevalence after AL treatment [58]. In another *in vivo* study, *P. falciparum* parasites carrying the chloroquine-susceptible *pfcrt* K76 allele was selected after treatment with AL [59].

It was difficult to determine whether the CQ-susceptible resurgence is due to back-mutations in the CQ-resistant allele or the expansion of surviving CQ-susceptible reservoir populations. Re-expansion appears to be more common in Africa, where transmission rates are higher and naturally immune individuals are more common than in Southeast Asia (where CQ-resistant alleles appear to have gone to fixation in many areas) [57]. It does not appear that CQ-resistant mutants in Ethiopia acquired mutations to compensate for the costs of being drug-resistant. It is believed that such compensatory mutations confer fitness levels greater than that of the original CQ-susceptible and CQ-resistant alleles [60]. CQ-susceptible alleles clearly have a selective advantage over resistant ones, *pfmdr1* Y86 having rebounded from 100% to 11% in 6 years (for samples collected on 2009 from East Ethiopia) to eight years time (for samples collected on 2011from South Ethiopia). On the other hand, N86Y mutation was rebounded from 81% to 9% [41,52].

In Ethiopia, due to resistance developed by *P. falciparum,* replacement of SP by artemether-lumefantrine has been mandatory. Since 2004, artemether-lumefantrine has been the drug of choice for the treatment of *P. falciparum* malaria. Unlike Malawi, in Ethiopia CQ was partially withdrawn and is still the drug of choice for the treatment of uncomplicated *P. vivax*. This finding shows that there has been strong selection for chloroquine-sensitive parasites after the nationwide replacement of chloroquine with SP. The return of chloroquine-susceptible alleles in a country like Ethiopia, which is endemic for malaria, can be considered a positive development toward replacing the expensive artemether-lumefantrine with a safe and cheap form of CQ in combination with other short-acting drugs. Likewise, in

malaria-endemic countries even partial returns of chloroquine sensitivity have a positive impact on public health [57].

## Conclusion

The return of CQ-sensitive parasites is likely due to either CQ withdrawal for the treatment of *P. falciparum* or the pressure from AL since 2004 or both. The return of CQ-susceptible *P. falciparum* following declining CQ resistance will have its own contribution in the battle against malaria. If chloroquine susceptibility does become widespread in Ethiopia,the possibility of using chloroquine in the future as a combination therapy with other short-acting drugs with different pharmacokinetic and pharmacodynamics profiles will be an additional anti-malarial option. This is a positive development, since CQ is cheap, safe, well-tolerated, long-lasting, and easy-to-prepare molecule. Additional surveillance studies to investigate resistance markers of *pfmdr1* and *Pfcrt* genes of *P. falciparum* in the same geographic area may help to assess the prevalence of anti-malarial resistance.

#### Competing interests

The authors have declared that they have no competing interests.

#### Authors' contributions

SKM designed and performed the field study and experiments, data analysis with drafting first draft. AA,NB, TG,RC, TT and GM contributed to the study design and study samples and revisions of MS. TPV designed the experiments, contributed to materials and tools, supervised the experiments, revision of the MS. All authors read and approved the final manuscript.

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