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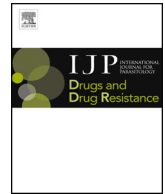
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Prevalence of *Plasmodium falciparum* *Pfcr* and *Pfmdr1* alleles in settings with different levels of *Plasmodium vivax* co-endemicity in Ethiopia

Elifaged Hailemeskel^{a,b,c,1}, Temesgen Menberu^{a,1}, Girma Shumie^a, Sinknesh Behaksra^a, Wakweya Chali^a, Migbaru Keffale^{a,d}, Mulualem Belachew^a, Getasew Shitaye^a, Hussien Mohammed^e, Daniel Abebe^{a,d}, Temesgen Ashine^a, Chris Drakeley^f, Hassen Mamo^b, Beyene Petros^b, Teun Bousema^{f,g}, Fitsum G. Tadesse^{a,d,g}, Endalamaw Gadisa^{a,*}

^a Malaria and Neglected Tropical Diseases Directorate, Armauer Hansen Research Institute, POBox, 1005, Addis Ababa, Ethiopia

^b Department of Biomedical Sciences, College of Natural and Computational Sciences, Addis Ababa University, POBox 1176, Addis Ababa, Ethiopia

^c Department of Biology, College of Natural and Computational Sciences, Wollo University, POBox, 1145, Dessie, Ethiopia

^d Institute of Biotechnology, Addis Ababa University, POBox, 1176, Addis Ababa, Ethiopia

^e Ethiopian Public Health Institute, POBox 1242, Addis Ababa, Ethiopia

^f Department of Immunology and Infection, London School of Hygiene & Tropical Medicine, WC1E 7HT, London, UK

^g Department of Medical Microbiology, Radboud University Medical Center, 6525, GA, Nijmegen, the Netherlands

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ABSTRACT

Plasmodium falciparum and *P. vivax* co-exist at different endemicity levels across Ethiopia. For over two decades Artemether-Lumefantrine (AL) is the first line treatment for uncomplicated *P. falciparum*, while chloroquine (CQ) is still used to treat *P. vivax*. It is currently unclear whether a shift from CQ to AL for *P. falciparum* treatment has implications for AL efficacy and results in a reversal of mutations in genes associated to CQ resistance, given the high co-endemicity of the two species and the continued availability of CQ for the treatment of *P. vivax*. This study thus assessed the prevalence of *Pfcr*-K76T and *Pfmdr1*-N86Y point mutations in *P. falciparum*. 18S RNA gene based nested PCR confirmed *P. falciparum* samples (N = 183) collected through community and health facility targeted cross-sectional surveys from settings with varying *P. vivax* and *P. falciparum* endemicity were used. The proportion of *Plasmodium* infections that were *P. vivax* was 62.2% in Adama, 41.4% in Babile, 30.0% in Benishangul-Gumuz to 6.9% in Gambella. The *Pfcr*-76T mutant haplotype was observed more from samples with higher endemicity of *P. vivax* as being 98.4% (61/62), 100% (31/31), 65.2% (15/23) and 41.5% (22/53) in samples from Adama, Babile, Benishangul-Gumuz and Gambella, respectively. However, a relatively higher proportion of *Pfmdr1*-N86 allele (77.3–100%) were maintained in all sites. The observed high level of the mutant *Pfcr*-76T allele in *P. vivax* co-endemic sites might require that utilization of CQ needs to be re-evaluated in settings co-endemic for the two species. A country-wide assessment is recommended to clarify the implication of the observed level of variation in drug resistance markers on the efficacy of AL-based treatment against uncomplicated *P. falciparum* malaria.

1. Introduction

Development and spread of anti-malaria drug resistance continued to be a stumbling-block in the fight against malaria. *Plasmodium falciparum* developed resistance to most of the antimalarials over the past 60 years (White, 2004). Resistance against Artemisinin combination therapies (ACT) (Carrara et al., 2013; Dondorp et al., 2009; Leang et al., 2013) is reported in Thai-Cambodian border, a historical ‘hotspot’ for

multi-drug resistance parasite evolution, emergence and spread. Although ACTs retain high efficacy in sub-Saharan African countries including Ethiopia (Barnes et al., 2009; Taffese et al., 2018), recent reports are suggestive of the emergence of ACT tolerant *P. falciparum* in African settings (Xu et al., 2018).

One of the mechanisms for emergence of drug resistance in *Plasmodium* is acquisition of mutations and duplication in target and/or transporter genes. Artemether-Lumefantrine (AL) selects for wild-type

* Corresponding author.

E-mail address: endalamaw.gadisa@ahri.gov.et (E. Gadisa).

¹ Equal contribution.

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codons at the *P. falciparum* chloroquine resistance transporter (*Pfcr*-K76) and *P. falciparum* multidrug resistance 1 (*Pfmdr*1) alleles (Baliraine and Rosenthal, 2011; Sisowath et al., 2005) and its use is associated with increases in *Pfmdr*1 gene copy number (Price et al., 2004). Variations were reported on the selective effects of different ACTs on single-nucleotide polymorphisms in *Pfcr* and *Pfmdr*1. A recent systemic review of 397 surveys in 30 countries in Africa documented that AL and artesunate-amodiaquine (ASAQ) exert opposing selective pressures and parasites under the selection pressure of one ACT tend to be more sensitive to the other (Okell et al., 2018). Higher proportion of *Pfmdr*1-86N and increases in its copy number have been reported in AL treated patients with parasite recrudescence (Venkatesan et al., 2014). It was noted that ASAQ selects the *Pfmdr*1 mutant haplotypes, 86Y, Y184, and 1246Y (Venkatesan et al., 2014). Chloroquine is known to select the mutant *Pfcr*-K76T and the wild type *Pfmdr*1-N86Y haplotypes (Gbotosho et al., 2012). Therefore, in areas where both *P. falciparum* and *P. vivax* are co-endemic the continued use of CQ might result in pronounced different selective pressure and, as a consequence, different drug resistance profiles in communities.

Unlike most of Africa, *P. falciparum* (60%) co-exists with *P. vivax* (40%) in most settings in Ethiopia. Driven by the widespread CQ resistant *P. falciparum*, Ethiopia changed its treatment policy for *P. falciparum* from CQ to sulphadoxine-pyrimethamine (SP) in 1998 (Jima et al., 2005; Taffese et al., 2018); shortly after SP was replaced by AL as first-line therapy for uncomplicated falciparum malaria in 2004 (MOH, 2018; Taffese et al., 2018). Throughout this period, CQ continued to be used as first line treatment for *P. vivax* (MOH, 2018). This may have implications for selection of drug resistance marker genes. Here, we examined the prevalence of *Pfcr*-K76T and *Pfmdr*1-N86Y alleles in *P. falciparum* after two decades of AL implementation as first line treatment in areas with different *P. vivax* co-endemicity levels in Ethiopia.

2. Materials and methods

2.1. Study sites and samples

Study sites were selected based on reported difference in *P. vivax* and *P. falciparum* co-endemicity levels (Ministry of Health, 2016). Six Woredas (districts) representing different epidemiological settings from three regional administrative states (Gambella, Benishangul-Gumuz and Oromia) were included. Gambella region, with Abobo and Lare districts, is a *P. falciparum* dominated area (97% of all *Plasmodium* infections are *P. falciparum*) (Tsegaye et al., 2014) with perennial transmission. Benishangul-Gumuz region, with Mao-Komo and Menge districts, is also *P. falciparum* dominated with relatively higher prevalence of *P. vivax* (10.1%) (Geleta and Ketema, 2016) and more seasonal transmission. The Oromia region study site districts of Babile and Adama are characterized by seasonal malaria transmission with co-endemicity for *P. falciparum* and *P. vivax* where *P. vivax* infections attributed to be 41.4% (Keffale et al., 2019) in the first and 62.2% (Tadesse et al., 2018) in the later district, respectively (Fig. 1 and Table 1). Oromia regional state has wider variation in the *P. vivax* and *P. falciparum* infection proportions due to its huge landmass and sampling was done in separate years i.e. Adama in 2016 and Babile in 2017, hence we have made separate treatment of the data from the study sites.

Study participants were recruited in community and health facility based cross-sectional surveys. Community based cross-sectional surveys were conducted in Babile district from July to November 2017 and from October to December 2016 in all other sites. In Adama passively-detected clinical malaria infected patients (n = 36) were recruited in addition to the community survey samples (n = 33). Finger prick blood samples (~300 µL) were collected from the study participants for malaria diagnosis using rapid diagnostic test (RDT) (First Response Malaria Ag (pLDH/HRP2, Combo RDT, Premier Medical Corporation Ltd., India) or microscopy and to prepare dried blood spots (DBS) on Whatman 3MM filter paper (Whatman, Maidstone, UK). Socio-

demographic and malariometric data were captured using pre-tested structured questionnaire.

2.2. Detection of *Pfcr* and *Pfmdr*1 polymorphisms

Study participants were diagnosed for malaria using thick blood smears and thin smears were used for species identification following Giemsa-staining by two experienced microscopists who were independent and blind for the participants' clinical status and RDT results. A third World health organization certified microscopist was consulted in case of discordant results. Blood film slides were considered negative if no parasite was detected after examining 100 microscopic fields (Tadesse et al., 2018).

Furthermore, DNA was extracted from a 6 mm diameter punch of DBS using Chelex-Saponin extraction method as described elsewhere (Baidjoe et al., 2013); eluted DNA was stored at -20 °C until further use. Malaria species was confirmed with 18S based nested polymerase chain reaction (nPCR) as described elsewhere (Snounou et al., 1993). Samples that were confirmed to be *P. falciparum* mon-species infection using the 18S RNA nPCR (Snounou et al., 1993) were further amplified for the *Pfcr*-76 and *Pfmdr*1-86 genes using nPCR with outer (N1) and inner primer pairs (N2) (Supplement 1). The N2 products were assessed using restriction fragment length polymorphism (RFLP) to detect mutations at each locus (Djimde et al., 2001; Veiga et al., 2006). Restriction enzymes used and conditions of digestion were as described elsewhere (UMSM, 2012). For *Pfcr*-76 codon, a known positive control of 3D7 (wild-type, *Pfcr*-76K allele) and negative control of Dd2 (mutant) were included in each reaction (kindly provided by the Ethiopian Public Health Institute). Restriction enzyme digested products were visualized with UV transilluminator after electrophoresis using 2% Agarose gel (SIGMA-ALDRICH) with a no enzyme digest control run alongside positive controls, negative controls and test samples (Bio Rad, USA). Mixed haplotypes having both wild and mutant type were interpreted as combined band patterns of digested and undigested products identified per sample of DNA.

2.3. Ethics statement

The study protocol was approved by the National Research Ethical Review Committee (3.10\016\20), Institutional Ethical Review Board of the College of Natural Science at Addis Ababa University, and AHRI/ALERT (Ref.No.SOM/DRERC/BCH005/2009, PO24/17). Written informed consent was obtained from each adult and parent/legal guardians for children younger than 18 years. Symptomatic patients were treated as per the national treatment guideline (MOH, 2018).

2.4. Statistical analysis

Both laboratory and field data were double entered. The cleaned data was analyzed using STATA 13 (StataCorp, TX, USA). The difference in prevalence among study sites was compared using Fisher's exact test. Pairwise comparison, after Bonferroni correction, was run to test differences in distribution of the wild and mutant alleles between the study sites for both *Pfcr*-76 and *Pfmdr*1-86 codons. A *P*-value ≤ 0.05 was considered statistically significant.

3. Results

3.1. Characteristics of the study participants

A total of 183 samples from 148 asymptomatic and 35 symptomatic participants with *P. falciparum* infection by 18S nPCR were included in the study. Out of the 183 samples, 169 (92.3%) and 168 (91.8%) were successfully amplified for *Pfcr*-76 and *Pfmdr*1-86 codons, respectively. Adama was the only site where asymptomatic and symptomatic study participants were considered. There was no significant difference

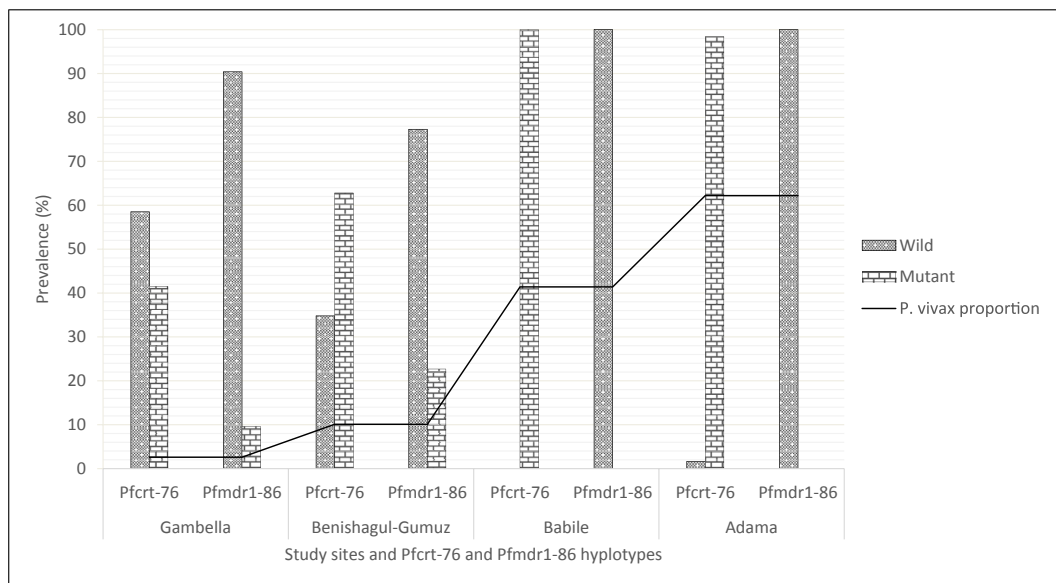


Fig. 1. The prevalence of *Pfcr-76K/T* and *Pfmdr1-86N/Y* point mutations among samples from four study sites with different *P. vivax* co-endemicity level compared to *P. falciparum*. Where; wild = wild type alleles *Pfmdr1-86N* and *Pfcr-76K* and Mutant = mutant type alleles *Pfmdr1-86Y* and *Pfcr-76T*.

among wild type and mutant haplotypes of the *Pfcr-76* codons between the asymptomatic (7.4%, 2/27) and symptomatic (8.6%, 3/35; $P = 0.125$) samples of Adama hence considered together in the subsequent analysis. Only wild type form of *Pfmdr1-86* was detected in both groups.

3.2. Prevalence of *Pfcr-76* haplotypes varies between study sites and *P. vivax* co-endemicity

Overall, the wild type *Pfcr-76K* haplotype was detected in 23.7% (40/169) and the mutant *Pfcr-76T* in 76.3% (129/169) of samples across sites. We observed a statistically significant difference in the prevalence of *Pfcr-76* codons among the study sites ($P < 0.001$): mutant (*Pfcr-76T*) type was virtually fixed in Babile (100%, 31/31) and with near fixation at Adama (98.4%, 61/62) districts of Oromia region where *P. vivax* is highly endemic (41.4% and 62.2% of cases, respectively) (Keffale et al., 2019; Tadesse et al., 2018). On the other hand, a higher prevalence of the wild type (*Pfcr-76K*) codon was found in Abobo and Lare districts of Gambella (58.5%, 31/53) and in Mao-Komo and Menge districts of Benishangul-Gumuz (34.8%, 8/23) (Table 1).

Table 1

Characteristics of study participants and frequency point mutations of *P. falciparum* chloroquine resistance transporter (*Pfcr-76*) and multi drug resistance 1 (*Pfmdr1-86*) genes among samples from four sites in Ethiopia from 2016 to 2017.

| Characteristics | Gambella N = 55 ^e | Benishangul-Gumuz N = 26 ^e | Babile N = 33 ^e | Adama N = 69 ⁺ | Total N = 183 | P-value |
|-------------------------------------------------------|---------------------------------|------------------------------------------|-------------------------------|------------------------------|------------------|---------|
| Age (years), Median (IQR) | 13(8–18) | 12(9–18) | 15(8–35) | 20 (8–32) | 14(8–25) | |
| Female sex, n (%) | 24(43.6) | 11(42.0) | 13(39.4) | 27(39.1) | 75(41.0) | |
| Proportion of infections that are <i>P. vivax</i> , % | 2.6 ^a | 10.1 ^b | 41.4 ^c | 62.2 ^d | | |
| Amplified samples for <i>Pfcr-76</i> codon, n/N | 53/55 | 23/26 | 31/33 | 62/69 | 169/183 | |
| <i>Pfcr-76K</i> (wild type), % (n/N) | 58.5 (31/53) | 34.8 (8/23) | 0.0 (0/31) | 1.6 (1/62) | 23.7(40/169) | |
| <i>Pfcr-76T</i> (Mutant), % (n/N) | 41.5 (22/53) | 65.2 (15/23) | 100.0(31/31) | 98.4(61/62) | 76.3(129/169) | < 0.001 |
| Amplified samples for <i>Pfmdr1-86</i> codon, n/N | 52/55 | 22/26 | 32/33 | 62/69 | 168/183 | |
| <i>Pfmdr1-86N</i> (wild type), % (n/N) | 90.4(47/52) | 77.3(17/22) | 100.0(32/32) | 100.0(62/62) | 94.1(158/168) | |
| <i>Pfmdr1-86Y</i> (Mutant), % (n/N) | 9.6(5/52) | 22.7(5/22) | 0.0(0/32) | 0.0(0/62) | 5.9 (10/168) | < 0.001 |

IQR, interquartile range.

⁺ asymptomatic cases (n = 33) & passively recruited clinical cases (n = 36).

^a Tsegaye et al., Mal J 2014.

^b Geleta and Ketema Malar Res Treat (2016).

^c Keffale et al. TRSTMH 2019.

^d Tadesse et al. CID 2019.

^e Asymptomatic cases.

The proportion of infections that is *P. vivax* is very low (< 10%) in these settings (Geleta and Ketema, 2016; Tsegaye et al., 2014) (Table 1). After Bonferroni correction, differences in the proportion of *Pfcr-76T* among the study sites was observed ($P = 0.0342$).

3.3. High prevalence of *Pfmdr1-86* wild haplotype across the study sites

The overall prevalence of the mutant codon, *Pfmdr1-86Y*, was 5.9% (10/168). The wild type codon, *Pfmdr1-86N*, was found fixed (100%) in Babile and Adama districts with similar higher proportions in Abobo and Lare districts of Gambella (90.4%, 47/52) and Mao-Komo and Menge districts of Benishangul-Gumuz (77.3%, 17/22). The prevalence of the mutant type (*Pfmdr1-86Y*) was only 9.6% (5/52) and 22.7% (5/22) in the districts from Gambella and Benishangul-Gumuz, respectively. Moreover, the observed prevalence of the different haplotypes was significantly different among the study sites ($P = 0.001$) (Table 1). No difference was observed in the proportion of *Pfmdr1-86N* in between study sites after Bonferroni correction ($P = 1.0$).

4. Discussion

The return of wild type alleles of CQ resistance marker genes has been reported across Africa following replacement of CQ by ACTs (Baraka et al., 2018; Kavishe et al., 2014; Okell et al., 2018; Venkatesan et al., 2014). The possible associations of these markers with ACT drug tolerance underline the need for continued surveillance for the success of malaria control program. In this study we assessed the prevalence of *Pfcr*-K76T and *Pfmdr*1-N86Y codons from different *P. falciparum* and *P. vivax* co-endemicity settings in Ethiopia.

The current study has documented a higher frequency of *Pfcr*-76T mutant haplotypes proportional to the endemicity level of *P. vivax* as being 98.4%, 100%, 65.2% and 41.5% in samples from Adama, Babile, Benishangul-Gumuz and Gambella, respectively. Lower proportion of parasites with the mutant allele (*Pfcr*-76T) was detected in samples from Gambella and Benishangul-Gumuz where *P. falciparum* is the dominant species. The nearly approaching fixation of *Pfcr*-76T mutant haplotype in Adama and its fixation in Babile districts of Oromia region was in agreement with previous reports in Ethiopia (Golassa et al., 2014; Mula et al., 2011). This might be partly due to the high prevalence of *P. vivax* co-endemicity that probably lead to the continued utilization and easy access of CQ in these areas. Similar findings associating resistance markers with CQ use have been reported elsewhere: persisting high levels of mutant *Pfcr* haplotypes in infections from Brazil and, in contrast, the reappearance of wild type forms in Nigeria have been attributed to differences in the selective pressure by CQ use (Gbotosho et al., 2012). Moreover, the slow reappearance of CQ sensitive parasites harboring *Pfcr*-K76 haplotype in low *P. vivax* prevalence areas of Gambella and Benishangul-Gumuz corroborates observations from other African countries such as Malawi (Kublin et al., 2003), Côte d'Ivoire (Dagnogo et al., 2018), Zambia (Mwanza et al., 2016), Tanzania (Mohammed et al., 2013) and Kenya (Achieng et al., 2015).

Higher proportion of the wild type allele, *Pfmdr*1-N86, (77.3–100%), was detected in all sites; fixed in Babile and Adama. This finding was in line with previous reports from different parts of Ethiopia (Lo et al., 2017; Mekonnen et al., 2014a; Mula et al., 2011). Earlier studies conducted more than a decade ago indicated a non-negligible level of the mutant form in Ethiopia (Eshetu et al., 2010; Schunk et al., 2006). This is probably due to the AL-regimen rolled out as first line treatment for *P. falciparum* infection across the study sites. In African settings, after decades of AL use a rise in the “NFD” *Pfmdr*1 haplotypes at codons-86,184 and 1246 have been observed with a parallel decline in the “YYY” haplotypes of these codons (Duah et al., 2013; Okell et al., 2018; Sondo et al., 2016; Venkatesan et al., 2014). These studies found that the “NFD” haplotypes were associated with the reduced sensitivity of the parasite to AL treatment.

The observed difference across the study sites is plausibly associated with antimalarial drug pressure as observed in other co-endemic settings. The re-emergence of genotypes such as *Pfcr*-K76 and *Pfmdr*-1 codons-N86, 184F and D1246 after CQ withdrawal has been associated with selection of parasites with reduced sensitivity to AL (Achieng et al., 2015; Conrad et al., 2014; Dokomajilar et al., 2006; Sisowath et al., 2009) which may eventually lead to resistance (Ljolje et al., 2018; Sisowath et al., 2005; Venkatesan et al., 2014). Moreover, in settings where *P. falciparum* and *P. vivax* are sympatric where mixed species infections are common, the detection of *P. falciparum* is frequent following treatment of *P. vivax* with CQ (Heuchert et al., 2015; Mekonnen et al., 2014b; Tajebe et al., 2014). Thus, the low CQ level to which the *P. falciparum* will be exposed might favor selective pressure.

The strength of this study is that it considered samples from different levels of *P. vivax* and *P. falciparum* co-endemicity (with varied CQ pressure) and both symptomatic and asymptomatic individuals. It is less likely to have a shift in proportion of infections due to *P. vivax* in the two years of sampling (20016 and 2017); the over decade national malaria control program data summary (2001–2016), documented no

considerable change in the proportion of infections due to *P. vivax* in Ethiopia (Taffese et al., 2018). The almost complete reversal of the wild haplotype, *Pfmdr*1-N86 codon, in all our sites reinforce the claim that such phenomenon might be due to ACT based treatment. Thus, we suggest parallel surveillance of drug resistance markers to monitor the effects of the antimalarial-drugs deployed in areas where *P. vivax* and *P. falciparum* are co-endemic. The similarity in the drug resistance profile among asymptomatic individuals and clinical patients is suggestive of asymptomatic infections could serve as hidden reservoirs. Therefore, our finding also underpin the importance of involving asymptomatic reservoirs in the evaluation of drug resistance especially in areas approaching elimination (Nyunt et al., 2017).

This study would have been more comprehensive if clonality of infections and copy number variations (CNVs) of target genes were assessed. We reported mixed haplotypes for *Pfcr*-76 (8.0%, 13/169) and *Pfmdr*1-86 (4.2%, 7/168). As the study was based on PCR-RFLP method alone ascertaining whether any of the two haplotypes were coming from the same genome or multi-clonal infections was difficult; hence we reported the mixed haplotypes together with the mutant proportions. Also, *Pfmdr*1-86 CNV is implicated for the emergence of *P. falciparum* diminished susceptibility to anti-malarial drugs, such as mefloquine, AS-MQ and AL combinations (Duah et al., 2013; Venkatesan et al., 2014).

In conclusion, after two decades of the replacement of CQ with AL for the treatment of uncomplicated falciparum malaria in Ethiopia, there is slow but site-specific reversal of the *Pfcr*-K76 haplotype while the *Pfmdr*1-N86 wild type was almost fixed across study sites, a preliminary finding that calls the need for monitoring and responding to emerging signs of drug resistance to preserve the efficacy of anti-malarial drugs. Thus, we recommend country-wide assessments to clarify the implication of the observed level of variation in drug resistance markers on the efficacy of AL-based treatment against uncomplicated *P. falciparum* malaria.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijpddr.2019.09.002>.

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Conflicts of interest

None declared.

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