Prevalence of chloroquine and antifolate drug resistance alleles in *Plasmodium falciparum* clinical isolates from three areas in Ghana [version 1; referees: 1 approved, 1 approved with reservations]

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Abstract

**Background:** The emergence and spread of resistance in *Plasmodium falciparum* to chloroquine (CQ) and the antifolate drug sulfadoxine-pyrimethamine (SP) necessitated the change from CQ to artemisinin-based combination therapies (ACTs) as first-line drug for the management of uncomplicated malaria in Ghana in 2005.

**Methods:** To examine the prevalence of molecular markers associated with CQ and antifolate drug resistance in Ghana, we genotyped single nucleotide polymorphisms (SNPs) in the *P. falciparum* chloroquine resistance transporter (*pfcrt*, PF3D7_0709000), multidrug resistance (*pfmdr1*, PF3D7_0523000), bifunctional dihydrofolate reductase-thymidylate synthase (*pfdhfr*, PF3D7_0417200) and dihydropteroate synthase (*pfdhps*, PF3D7_0810800) genes in children with malaria reporting to hospitals in three different epidemiological areas of Ghana (Accra, Kintampo and Navrongo) between 2012 and 2017.

**Results:** The overall prevalence of the CQ resistance-associated *pfcrt* 76T allele was 8%, whereas *pfmdr1* 86Y and 184F alleles were present in 10% and 65% of infections respectively. Most of the isolates harboured the antifolate resistance-associated *pfdhfr* 51I, 59R and 108N alleles, including 68% of them with the triple mutant *pfdhfr* I51R/S59N/I108N combination. *Pfdhps* 437G and 540E were detected in 90.6% and 0.7% of infections, respectively. We observed no significant difference across the three study sites for all the polymorphisms except for *pfdhps* 437G, which was more common in Accra than at the other sites. Across both *pfdhfr* and *pfdhps* genes, a large proportion (61%) of the isolates harboured the quadruple mutant combination (I51R/S59N/I108N/G437).

**Conclusion:** Comparison of the present results to previously published data shows a significant decrease in the prevalence of CQ resistance alleles during the 12 years after CQ withdrawal, but an increase in the alleles that mediate SP...
resistance, which could be due to the continuous use of antifolate drugs for prophylaxis.

**Keywords**
Drug resistance, Malaria, Antifolates, Chloroquine, Plasmodium falciparum
Introduction

Malaria remains a major global health concern especially in Sub Saharan Africa. *P. falciparum* malaria is considered the most severe and also the leading cause of morbidity and mortality, especially among children under five years (Schumacher & Spinelli, 2012). In 2016 a global estimate of 216 million malaria cases was reported, which led to about 445,000 deaths (WHO, 2017). The global malaria mortality rate, however, has reduced by 29% since the year 2010, as a result of increased preventive and control measures (WHO, 2016).

The use of antimalarial drugs for malaria treatment and prevention has played an integral role in the control of the disease over the decades (Cui et al., 2015; Gosling et al., 2011; Greenwood, 2004; Schlitzer, 2007). Unfortunately, the emergence and the spread of drug resistant *P. falciparum* strains mitigated against the use of antimalarial drugs for the containment of the disease (Lin et al., 2010). *P. falciparum* chloroquine (CQ) resistant strains were first reported in the 1950s in Southeast Asia along the Cambodia–Thailand border (Young et al., 1963) and later reported in other countries globally. Currently, the parasite has been reported to have developed resistance to most available antimalarial monotherapies and this is exhibited by reduced parasite clearance rate and/or treatment failure (Dondorp et al., 2009).

ACTs are now the frontline drugs for treating uncomplicated *P. falciparum* malaria in almost all countries that are endemic with malaria, including Ghana (WHO, 2016).

Point mutations in specific genes in the parasite genome are implicated in resistance to specific antimalarial drugs (Cui et al., 2015; Fidock et al., 2000; Sidhu et al., 2002). A point mutation in the *P. falciparum* chloroquine resistance transporter gene (*pfcrt*, PF3D7_0709000) that replaces lysine with threonine at codon 76 had become a common single nucleotide polymorphism (SNP) allele in parasite populations as it is a critical mediator of resistance to CQ (Babiker et al., 2001). In addition, mutations in the *P. falciparum* multidrug resistance gene 1 (*pfmdr1*, PF3D7_0523000) that result in amino acid substitutions at positions N86Y and Y184F have been reported to confer parasite resistance to CQ, amodiaquine (AQ) and lumefantrine (L) (Duraisingh & Cowman, 2005). These mutations are believed to interfere with heme polymerization by preventing the accumulation of active drug within the food vacuole (Djimdé et al., 2001b).

On the other hand, sulfadoxine-pyrimethamine (SP) resistance has also been linked to point mutations in the bifunctional dihydrofolate reductase-thymidylate synthase (*pfdhfr*, PF3D7_0417000) and dihydropteroate synthase (*pfdhps*, PF3D7_0810000) genes. Resistance to antifolate drugs such as SP is known to be mediated by basal point mutations in these genes that result in amino acid substitutions at positions S108N and A437G in *pfdhfr* and *pfdhps* proteins respectively. Overall, studies have shown that additional point mutations in these drug resistant genes on top of the basal mutation makes parasites more refractory to the drug (Mita et al., 2014), and correlates with increased treatment failure (Plowe et al., 1997; Sibley et al., 2001). Therefore, parasites harbouring haplotypes that include the different SNP alleles in combination have been shown to confer higher resistance to the specific drugs. In this regard, the combined quintuple mutant haplotype (*pfdhfr* N51R_E540K_E540K_R363Q + *pfdhps* G412_A413) has been correlated with high SP treatment failure in East Africa (Kublin et al., 2002; Omar et al., 2001).

In Ghana, prior to the withdrawal of CQ a prevalence range of between 46%–98% of the mutant *pfcrt* K76T was reported across five sentinel sites (Duah et al., 2007). Interestingly, studies in other settings have shown that the replacement of CQ with ACTs resulted in a decline in the frequency of the mutant alleles and concomitant restoration of CQ susceptibility (Lauber et al., 2006; Mwai et al., 2009). In a study that was conducted in Tanzania, more than 90% recovery of the sensitive *pfcrt* K76 allele was reported after 10 years of CQ use being officially discontinued (Mohammed et al., 2013).

This study sought to ascertain the population trends in the prevalence of known drug-resistance-related point mutations in *pfcrt*, *pfmdr1*, *pfdhfr* and *pfdhps* in clinical isolates from three different malaria-endemic areas in Ghana a decade following the introduction of ACTs.

Methods

Ethical consideration

This study was approved by the Ethics Committees of the Ghana Health Service (GHS-ERC:12/05/12), the Kintampo Health Research Centre (KHRCIEC/FEA/2011-13), the Navrongo Health Research Centre (NHRC-IRB135/08/2012) and the Noguchi Memorial Institute for Medical Research (NMIMR) (NMIMR-IRB CPN 004/11-12). Informed consent of parents or guardians for all participants was obtained. An additional assent was also obtained from children aged 10–14 years prior to recruitment.

Study sites and sample collection

Parasite isolates were obtained from children aged 2–14 years, diagnosed with malaria at Municipal hospitals in Kintampo North Municipality (here after referred as Kintampo; 2012–2013 and 2016–2017), Accra (2016–2017) and Navrongo (2012–2013), in Ghana. Kintampo is a tropical zone in the Brong Ahafo region with all year round high malaria transmission, whereas Navrongo is a savannah zone in the Upper East region where malaria transmission is seasonal and rainfall-dependent (Owusu-Agyei et al., 2009) and Accra lies within the coastal savannah area with low seasonal malaria transmission (Klinkenberg et al., 2008). These three regions represent the different malaria transmission intensity zones in the country (Accra<Navrongo<Kintampo), and the study participants have been characterized in greater detail in our previous reports (Ademolue et al., 2017; Mensah-Brown et al., 2017). *P. falciparum* genomic DNA was analyzed for the prevalence of known antimalarial drug resistance-associated single nucleotide polymorphisms (SNPs) in *pfcrt* (K76T), *pfmdr1* (N86Y and Y184F), *pfdhfr* (N51I, C59R and S108N) and *pfdhps* (A437G and K540E) across the three study sites. Malaria was diagnosed using the first response ®malaria Ag. (HRP2) card test (Premier Medical Corporation, Ltd., Mumbai, India) and confirmed by microscopy. Venous blood samples were obtained and depleted of leucocytes
using lymphoprep gradient centrifugation, followed by passage through Plasmodipur filters (EuroProxima, Arnhem, Netherlands), the resulting infected red blood cells were stored at -20°C until DNA extraction.

**Extraction of genomic DNA and nested PCR**

*Plasmodium* gDNA was extracted from the samples using the QIAamp Blood Midi Kit (Qiagen, Manchester, UK) as per manufacturer’s instructions and stored at -20°C. Both outer and nested PCRs were carried out to amplify regions flanking known point mutations in *pfcr* (K76T), *pfmdr1* (N86Y and Y184F), *pfdfhfr* (N51I, C59R, and S108N) and *pfdfhps* (A437G and K540E) that mediate antimalarial drug resistance. All PCRs were carried out at final volume of 25 µL containing 1X of Maxima Hot Start Green PCR master mix (Thermo Scientific, Waltham, MA, USA) and 250 nM of each of the forward and the reverse primers. Five microlitres of the purified *P. falciparum* gDNA was used as template in the outer PCR and 1 µL of the resulting products was used as template DNA in the nested PCR. Previously reported primer sets and cycling conditions for both the outer and the nested PCRs were used (Djimdé et al., 2001a; Duraaisingh et al., 1998). Prior to the restriction digest, 5 µL of the nested PCR products were resolved on 2% agarose gel stained with ethidium bromide and images were resolved using the Amersham Imager 600 (General Electric Healthcare Life Sciences, Chicago, IL, USA).

**Restriction digestion of nested PCR amplicons**

The resulting nested PCR products for each of the four genes containing the SNP alleles of interest were analyzed by restriction fragment length polymorphism (RFLP). Each of the restriction digestion reactions was set at a final volume of 15 µL containing 5 µL of the nested PCR product, 1X FastDigest Green buffer and 0.3 µL of the appropriate restriction enzyme (Thermo Scientific). The restriction enzymes used, incubation temperature, incubation time as well as the expected band sizes for the wild-type and the mutant alleles of the point mutations were as reported in previous studies (Djimdé et al., 2001a; Duraaisingh et al., 1998). Ten microlitres of the restriction digestion fragments were resolved on 2% agarose gel stained with ethidium bromide and the resulting image resolved with the Amersham Imager 600 (GE, USA). Purified DNA obtained from laboratory strains of *P. falciparum* (Dd2, 3D7, FCR3, K1, 7G8 and W2) were used as controls for the sensitive and resistant alleles for each gene.

**Data analysis**

Data was analyzed using the Stata version 14.2 (Texas, USA), and the GraphPad Prism (Version 6.01). Analysis of contingency tables of frequency distribution of the point mutations between the study sites were analyzed by chi-square test. In addition, allele combination frequency distribution of the pooled 2012–2013 isolates was compared to the pooled 2016–2017 isolates utilizing the Fisher exact test for expected lower cell counts taking each marker as independent. All statistical tests were two-tailed and statistical significance was defined at P < 0.05.

**Results**

**Prevalence of alleles in *P. falciparum* genes that mediate chloroquine and antifolate drug resistance**

To survey known point mutations implicated in CQ and SP resistance we carried out RFLP using appropriate primer sets. We did not observe any significant differences in the distribution of isolates harbouring *pfcr* K76T, *pfmdr1* N86Y or *pfmdr1* Y184F point mutations associated with CQ and AQ resistance across the three transmission zones (P > 0.05 for all three SNPs), although all the three mutant alleles were found at a higher prevalence in Navrongo compared to Kintampo and Accra (Table 1). The total prevalence of *pfcr* 76T (8%) and *pfmdr1* 86Y (10%) mutant alleles were comparable (P = 0.39). Compared to CQ resistance-associated alleles, higher frequencies were observed in the three study sites for all the antifolate drug resistance-associated alleles, except *pfdfhps* K540E (Table 1 and Dataset 1). The frequency distribution of isolates harbouring the *pfdfhfr* 51I, 59R and 108N mutant alleles were comparable across the study sites (P > 0.05 for all the loci). The distribution of *pfdfhps* 437G, which is also associated with sulfadoxine resistance, was significantly different across the study sites (P=0.01).

**Trends in the prevalence of antimalarial drug resistance markers in the study populations**

To investigate the dynamics of the drug resistance alleles in the selected areas, we compiled data from previous studies that reported the frequencies of the various mutations in the same or near-by communities. Thus, the current data from Navrongo were compared to previous data from the same area, while data from Kintampo were compared to published data from Sunyani, which is a town located in the same region (Brong Ahafo) as Kintampo. Generally, a decreasing trend was observed from 2005 to 2017 in the proportions of the alleles associated with CQ resistance in both study sites except for *pfmdr1* 86Y in Navrongo (Figures 1A and B), which decreased from 2005/2006 to 2010 but appeared to plateau between 2012 and 2013. We, however, observed an increasing trend in the proportions of the pyrimethamine and sulfadoxine resistance alleles in *pfdfhfr* and *pfdfhps* respectively at both study sites from 2005 to 2013, with frequencies levelling off subsequently (Figures 1C–F).

**Analysis of *pfdfhfr* and *pfdfhps* haplotype combination distributions**

We used clinical isolates for which all the *pfdfhfr* and *pfdfhps* SNP alleles of interest were successfully genotyped to survey allele combinations and determine their distribution between study periods. The *pfdfhfr* triple mutant allele combination (*I*, *R*, *N*$_{180}$) was found at a prevalence of 53% in 2012/2013 and 79% in 2016/2017 (P<0.0001, Table 2). Across both genes, the prevalence...
of the quadruple allele combination (I, R, N, Y) increased from 43% to 73% from 2012/2013 to 2016/2017 study periods and the difference was statistically significant (P<0.0001). Double mutant allele combinations of pfdhfr (I, N, Y and I, R, N) had comparably low frequencies. In addition, other generally low prevalence (<10%) allele combinations in both pfdhfr and pfdhps included the triple mutant allele combination (R, N, G) and the double mutant allele combination (R, G, I) for both the 2012/2013 and the 2016/2017 periods (Table 2). A comparison of allele combination distribution by malaria transmission intensity revealed a significantly higher prevalence of the mutant allele combination in pfdhfr (I, R, N) and in both genes I, R, N, Y in the lowest transmission area, Accra, compared to Kintampo (P<0.002 and P=0.04, respectively).

**Discussion**

*P. falciparum* resistance to antimalarial drugs remains one of the biggest threats to the control and elimination of malaria globally. In Ghana, a change in the use of CQ to ACTs was implemented in 2005 as a result of high rate of malaria treatment failure (Duah et al., 2007). Mutant alleles in some key genes of the parasite are clearly linked to CQ and antifolate drug resistance. Recent reports have shown that some of these point mutations might also modulate the efficacy of the currently used ACTs (Gresty et al., 2014; Veiga et al., 2016). Thus, the availability of data on the prevalence of antimalarial drug resistance-associated alleles will be important for informing national policies on malaria treatment. Hence, in this study we determined the prevalence of alleles associated with CQ and antifolate resistance using clinical isolates from three malaria endemic regions in Ghana that have varying transmission intensities.

Both *in vitro* and molecular surveillance studies have associated CQ resistance mainly with the *pfcrt* 76T allele, but also with *pfmdr1* Y653F and 184F alleles. Data presented here show a gradual decline in frequency of the *pfcrt* 76T resistance-associated allele over the years since the official discontinuation of CQ as an antimalarial in Ghana, which is consistent with findings in other malaria endemic populations in east Africa such as Tanzania, Malawi and Kenya (Mohammed et al., 2013; Mwai et al., 2009). The fitness cost of harbouring the mutant alleles is thought to select against them in favour of the non-resistant background alleles (Babiker et al., 2009; Kiarie et al., 2015). Unlike *pfcrt* 76T and *pfmdr1* Y653F, the prevalence of *pfmdr1* 184F mutant allele (65%) appears to have not varied so much from 2005 to 2017 when compared to the 43% to 69% prevalence reported from 2005 to 2010 (Duah et al., 2013).

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### Table 1. Frequency distribution of pfcrt, pfmdr1, pfdhps and pfdhfr SNPs in *P. falciparum* isolates.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Amino acid position</th>
<th>Kintampo n (%)</th>
<th>Navrongo n (%)</th>
<th>Accra n (%)</th>
<th>Total n (%)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pfcrt</em></td>
<td>K76T</td>
<td>K 198 (93.0)</td>
<td>T 14 (7.0)</td>
<td>R 5 (16.7)</td>
<td>266 (83.4)</td>
<td>0.321</td>
</tr>
<tr>
<td></td>
<td>N86Y</td>
<td>N 153 (91)</td>
<td>Y 15 (9.0)</td>
<td>Y 198 (93.0)</td>
<td>368 (99.9)</td>
<td>0.124</td>
</tr>
<tr>
<td></td>
<td>S108N</td>
<td>S 117 (65.0)</td>
<td>F 117 (65.0)</td>
<td>S 153 (89.8)</td>
<td>487 (99.9)</td>
<td>0.418</td>
</tr>
<tr>
<td></td>
<td>G51N</td>
<td>G 151 (80.0)</td>
<td>I 151 (80.0)</td>
<td>I 266 (83.4)</td>
<td>477 (99.9)</td>
<td>0.058</td>
</tr>
<tr>
<td><em>Pfdhps</em></td>
<td>N51I</td>
<td>N 38 (20.0)</td>
<td>L 27 (15.0)</td>
<td>N 266 (83.4)</td>
<td>231 (99.9)</td>
<td>0.058</td>
</tr>
<tr>
<td></td>
<td>N86Y</td>
<td>N 153 (91)</td>
<td>Y 15 (9.0)</td>
<td>Y 198 (93.0)</td>
<td>368 (99.9)</td>
<td>0.124</td>
</tr>
<tr>
<td></td>
<td>S108N</td>
<td>S 21 (11.0)</td>
<td>S 117 (65.0)</td>
<td>S 153 (89.8)</td>
<td>487 (99.9)</td>
<td>0.418</td>
</tr>
<tr>
<td></td>
<td>G51N</td>
<td>G 151 (80.0)</td>
<td>I 151 (80.0)</td>
<td>I 266 (83.4)</td>
<td>477 (99.9)</td>
<td>0.058</td>
</tr>
</tbody>
</table>

*Mutated amino acid depicted in bold. **P-value based on Pearson chi-Square test or Exact chi-square test for categorical variables.*

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Note: The table provides a summary of the frequency distribution of SNPs in *P. falciparum* isolates from different regions in Ghana. The data shows the prevalence of different allele combinations and the statistical significance of these differences. The results indicate a significant increase in the prevalence of quadruple mutants from 2012/2013 to 2016/2017. The prevalence of mutations associated with CQ and antifolate resistance is lower in Accra compared to Kintampo.
Figure 1. Trends in the prevalence of antimalarial drug resistant alleles from 2005 to 2017. Summarized data from present study was compared to previous published data from Navrongo and Kintampo/Sunyani, Ghana, for pfmdr1 and pfcr7 point mutations (Duah et al., 2013) and for the antifolate resistance mutations (Duah et al., 2012). No data was available for the pfcr7 76T and pfmdr1 86Y in 2010 in Kintampo/ Sunyani (Figure 1A–B). Also, no data was available for Navrongo in 2016–2017 in all the analysis. Deep black represents 2005–2006, light black represents 2010, plain grey represents 2012–2013 and crossed grey represents 2016–2017.
Although high frequencies of the point mutations implicated in the development of resistance to antifolates were reported before the change in malaria treatment guidelines in 2005 in Ghana, the drug is still in use for intermittent preventive treatment of malaria in pregnancy (IPTp) and also recommended for seasonal malaria chemotherapy (SMC) among children under five in areas of high but seasonal malaria transmission. The percentages of the pfddhfr 51I (81%), 59R (82%), 108N (88%) and pfddhps 437G (88%) mutant alleles reported in this study are relatively higher when compared to the 71%, 42%, 64% and 80% prevalence reported in a recent study in a neighbouring country, Burkina Faso (Cisse et al., 2017), this could be due to differences in the uptake of IPTp in both countries. Unlike the mutations associated with CQ resistance which decreased, an increasing trend in the prevalence of the antifolate resistance-associated mutant alleles was observed from 2005 to 2017. This high prevalence may be due to SP intervention in groups such as pregnant women and young children acting as reservoirs of infections with resistance alleles as a direct consequence of continuous use of SP in IPTp and SMC campaigns that fuel transmission of these alleles in the general population. Another important factor may be the unauthorized use of SP for self-medication as it is readily available at health centres and pharmacy shops in the study areas (Abuaku et al., 2004), particularly because it is a single dose drug with very minimal to no adverse reactions. Higher SP treatment failure have been correlated with the pfddhfr/pfddhps quintuple (pfddhfr/pfddhps N51I/R59K/N108S/G437C) haplotypes (Kublin et al., 2002; Triglia et al., 1997). In this study, however, no isolate was observed to carry the quintuple mutant allele. This is consistent with other studies which show that though the variant quintuple mutant allele is almost fixed in east Africa, it is largely absent from West Africa (Naidoo & Roper, 2013; Roper et al., 2004). However, our data shows a significant increased prevalence of parasite isolates that harbour the quadruple mutant allele combination involving both genes (I_{N51I}R_{59K}N_{108S}/G_{437C}) in the 2012–2013 and 2016–2017 study periods. This suggests that selection by SP in our study settings is still continuing. These findings are corroborated by previous studies in Ghana (25%–69%) (Duah et al., 2012), Cameroon (47%) (Chauvin et al., 2015) and Equatorial Guinea (54%) (Berzosa et al., 2017), which suggests a high prevalence of variant quadruple mutant alleles in West to Central Africa. The pfddhps K540E point mutation, which is a surrogate for high level resistance to SP was found in a very low proportion of the clinical isolates (1 %) in this study. This is consistent with reports in other countries in the sub-region including Mali and Burkina Faso (Cisse et al., 2017; Coulibaly et al., 2014), and suggests that if selection is increased it might eventually lead to a higher level of SP resistance in West Africa.

The increasing trend in the prevalence of the SP resistance-associated alleles is of concern and highlights the need for monitoring in populations receiving IPTp, and SMC, and for trials of other drug combinations. The study indicates that CQ sensitive parasites have again become more common since the replacement of CQ with a variety of ACTs as first-line treatments of uncomplicated malaria in Ghana. This notwithstanding, our findings also show that between 5% to 14% of clinical infections may still carry CQ resistant parasites, which suggest that ACT partner drugs such as AQ that is widely used in Ghana

### Table 2. Temporal trends of SP drug resistance haplotypes from 2012 to 2017 by study period.

<table>
<thead>
<tr>
<th>pfddhfr</th>
<th>pfddhps</th>
<th>2012–2013 n=95 (%)</th>
<th>2016–2017 n=128 (%)</th>
<th>Total n=223 (%)</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>N51I</td>
<td>C59R</td>
<td>S108N</td>
<td>A437G</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>R</td>
<td>N</td>
<td>G/A</td>
<td>50 (52.6)</td>
<td>101 (79)</td>
</tr>
<tr>
<td>N</td>
<td>R</td>
<td>N</td>
<td>G</td>
<td>10 (10.5)</td>
<td>8 (6.3)</td>
</tr>
<tr>
<td>I</td>
<td>R</td>
<td>S</td>
<td>G</td>
<td>8 (8.4)</td>
<td>2 (1.6)</td>
</tr>
<tr>
<td>I</td>
<td>C</td>
<td>N</td>
<td>G</td>
<td>7 (7.4)</td>
<td>12 (9.4)</td>
</tr>
<tr>
<td>N</td>
<td>R</td>
<td>S</td>
<td>G</td>
<td>5 (5.3)</td>
<td>2 (1.6)</td>
</tr>
<tr>
<td>I</td>
<td>C</td>
<td>N</td>
<td>A</td>
<td>4 (4.2)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>N</td>
<td>C</td>
<td>N</td>
<td>G</td>
<td>3 (3.2)</td>
<td>1 (0.8)</td>
</tr>
<tr>
<td>N</td>
<td>R</td>
<td>N</td>
<td>A</td>
<td>3 (3.2)</td>
<td>1 (0.8)</td>
</tr>
</tbody>
</table>

Note: Numbers include only isolates that were successfully genotyped for all the four point mutations in pfddhfr and pfddhps.

Each haplotype has mutant amino acids shown in bold. *P-value based on Exact chi-square test.
may still be maintaining significant selection pressure on the pfcrd locus. In addition, the increasing prevalence of pfdrhr haplotypes known to correlate with SP treatment failure calls for continuous monitoring of parasite populations across the different eco-epidemiological areas of Ghana to gather data for assessing the public health implications.

Conclusion

This study reports an increasing prevalence of CQ sensitive clinical isolates after 12 years of CQ withdrawal at three diverse study sites in Ghana. On the contrary, if an increasing trend in the frequency of SP-resistance associated alleles continues, it may in the future undermine use of SP for prevention of malaria in pregnant women and children.

Data availability

The data supporting this article is available online at Open Data availability.

The use of anti-malarial drugs to prevent malaria in the pregnant women and children may in the future undermine use of SP for prevention of malaria in pregnant women and children. On the contrary, if an increasing trend in the frequency of SP-resistance associated alleles continues, it may in the future undermine use of SP for prevention of malaria in pregnant women and children. On the contrary, if an increasing trend in the frequency of SP-resistance associated alleles continues, it may in the future undermine use of SP for prevention of malaria in pregnant women and children. On the contrary, if an increasing trend in the frequency of SP-resistance associated alleles continues, it may in the future undermine use of SP for prevention of malaria in pregnant women and children. On the contrary, if an increasing trend in the frequency of SP-resistance associated alleles continues, it may in the future undermine use of SP for prevention of malaria in pregnant women and children. On the contrary, if an increasing trend in the frequency of SP-resistance associated alleles continues, it may in the future undermine use of SP for prevention of malaria in pregnant women and children. On the contrary, if an increasing trend in the frequency of SP-resistance associated alleles continues, it may in the future undermine use of SP for prevention of malaria in pregnant women and children.

Competing interests

No competing interests were disclosed.

Grant information

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Received

Page 8 of 16

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Abugri et al. analyzed the prevalence of chloroquine (CQ) and sulphadoxine-pyrimethamine (SP) resistance in clinical isolates in Ghana. The authors used known molecular markers of resistance in three genes that modulate sensitivities to these two different antimalarial drugs; pfcr and pfmdr1 for CQ resistance, and pfdhfr and pf dhps for SP resistance. Samples were collected from three different locations with different transmission intensities in two different time periods, 2012/2013 and 2016/2017. It is a well written manuscript but I have several comments.

Abstract:
1. In the background, the authors state the policy was changed from CQ (as the first-line treatment?) to ACT due to CQ resistance. Was SP ever used as a first- or second-line treatment?
2. According to the methods section (in the main text), the studies were conducted in 2012/2013 and 2016/2017, NOT between 2012 and 2017 because no data was collected in 2014 and 2015.
3. In the results section, the second line which begins with “most”, needs to be improved. The authors should consider re-writing.

Introduction:
1. In the first line, Sub Saharan Africa consider writing Sub-Saharan Africa
2. In the second paragraph, the parasites have developed resistance to antimalarial monotherapies?
   It might be important to specify. I am assuming the authors meant Artemisinin monotherapies.
3. In the paragraph that introduces SP, consider removing the term “On the other hand” and “also” since this is the first time you have introduced this concept in the introduction.
4. In the same paragraph (on SP), references needed for the first and second lines.
5. When N (108) I (51) and R (59) is mentioned, it makes sense to put everything in chronologic order I (51) R (59) and N (108) which the authors have done to do so in the rest of the manuscript.
6. The authors mention Duah et al 2007\(^1\) study which discussed the high prevalence of CQ resistance. However, they fail to mention the follow-up 2012 and 2013 studies although they mention the references later. To set the stage, I think the authors have to be upfront and mention what the follow-on studies by these authors (Duah et al. 2012\(^2\) and 2013[re-3]) found in Ghana.

Methods:
1. A map is needed to show the different locations where the samples were collected. It should be clear how far apart the distances are. Is Kintampo site at one district hospital or several hospitals?
2. Provide more information about the different field sites regardless of the fact that the authors have mentioned that more information is available elsewhere. The authors can be brief: what are the transmission rates, EIR etc.; what time of the year were the samples collected?
Results:
1. The first line is not important since it is stating the purpose of the study again which has already been mentioned. In addition, there is no point of repeating to mention that K76T, N86Y, Y184F associated with CQ, AQ and (not mentioned L) resistance because it is already mentioned in the introduction.
2. This is one of the major concerns of mine. First, samples were collected in two different time periods (2012/2013 in Kintampo and Navrongo; 2016/2017 in Kintampo and Accra). In a 5 year period, prevalence can change dramatically. Therefore, the frequency of distribution should be analyzed separately for the two time periods. If no differences are seen, then it should be stated as such but the analysis must be done separately.
3. This is follow-on to point #2 above. 437G was found to be higher in Accra and that’s why there was significant difference. This might because Accra samples were collected in 2016/2017 only. The authors need to breakdown the analysis per the period which the samples were collected such as comparing Navrongo 2016/2017 to Accra 2016/2017 only.
4. In malaria endemic regions, samples always contain mixed infections where both wildtype and mutant alleles are present. The authors have not mentioned anything about the presence of mixed infections and how such samples were analyzed.
5. Show Sunyani on the map so that the reader can get the sense of distance to Kintampo.
6. One of my other major concern with this study is the micro-epidemiology of malaria and the micro-heterogeneity in transmission. Malaria transmission can be highly heterogeneous over a small spatial scale not to mention dramatic change from season to season. Comparing data collected elsewhere (Sunyani vs. Kintampo) no matter the distance, and then treat it as if it is from the same location, this can be extremely problematic and misleading. The authors must make strong arguments and justify their point of view much more strongly than currently presented. See references below.
7. In the analysis of pfdfhr and pfdfsps haplotypes, only Kintampo has samples collected in 2012/2013 and 2016/2017, comparing three different locations which Navrongo only has 2012/2013 samples/data and Accra has only 2016/2017 samples/data is not completely accurate (as presented 2012/2013 vs. 2016/2017). This analysis must be revisited. The difference might be just because the samples were collected in different locations; it is important this is possibility is ruled out.

Discussion:
1. The first paragraph of the discussion needs to capture and highlight the key findings of the study, consider re-writing the first paragraph of the discussion to capture the key findings.
2. The discussion needs to compare more critically similar studies that have been conducted in Ghana (Duah et al. 2012 and 2013) and West Africa. What additional information does the current study contribute?
3. Compare data with some of the most recent studies conducted in East Africa (studies cited here are not the most recent). This should be in the context of the first-line treatment ACTs in East Africa (AL) vs. first-line treatment ACTs in West Africa which might be driving the changes in molecular epidemiology of malaria parasites.
4. In page 7, the authors mention that differences in Burkina Faso and Ghana maybe due to differences IPTp uptake. This is an important line which needs references. State clearly what the differences are.
5. The authors speculate that high prevalence may be due to use of SP in IPT and SMC. What other antifolate drugs are being used in these communities such as cotrimoxazole for treatment of opportunistic infections in HIV-infected population which might be contributing to the increased resistance over time?
6. IPTp have remained highly effective in locations where prevalence of SP resistance markers remain high and fixed in the parasite population. It is clear that from 2005 to 2017 that SP resistance markers are on the rise. The authors needs to discuss the implication of this trend in detail in the context of IPTp, are these mutations getting fixed in the population? How is monitoring of these markers going to influence policy makers if SP remains efficacious in IPTp not only in Ghana, but in other countries where prevalence of these markers remain high?

References

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

Is the study design appropriate and is the work technically sound?
Yes
Are sufficient details of methods and analysis provided to allow replication by others?  
Partly

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

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

Are the conclusions drawn adequately supported by the results?  
Yes

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

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

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**General comments:**  
The manuscript covers an important topic because of the following reasons.

1. Drug resistance has been one of the major problems that has significant impact on the WHO strategy of malaria control through case management, which entails prompt diagnosis and treatment with effective antimalarial drugs. Due to resistance to the drugs which were commonly used in the past, chloroquine (CQ) and sulphadoxine/pyrimethamine (SP), all endemic countries replaced them with artemisinin combination therapy (ACT). Thus, surveillance of molecular markers of resistance is critical to inform policy makers on the effectiveness of antimalarials currently used for treatment of malaria in the respective countries.

2. Amodiaquine (an aminoquinoline like CQ) and SP are still used in Ghana and other African countries. Whereas SP is used for intermittent preventive treatment in pregnant women (IPTp), amodiaquine-SP (which is an ACT) is used for seasonal mass chemoproprevention in the Sahel region of West Africa. Monitoring molecular markers of these drugs is very important in order to provide information on the performance of these antimalarials.

The manuscript is well written but it needs some improvements by working on the following areas:

**Specific comments:**

1. **Abstract:**
   1. Under the methods, it is important to mention the genotyping method used, and how the data was managed in order guide the reader. For instance, it is mentioned that the samples were collected between 2012 and 2017 but the results are presented as a single time point, without any mention on the different time points.
2. Results: Although it is mentioned in the methods that samples were collected between 2012 and 2017, this is not mentioned in the results and trends of the prevalence of the molecular markers are not shown.

3. Conclusion: This is based on the comparison of the current and previous data which is not shown in the results presented. Authors should either revise the conclusion or incorporate the data referred to in the abstract.

2. Methods:
   1. The authors should explain why sampling involved children aged 2 – 14 years. They should also give reasons as to why venous blood was collected for this type of study which could be done using samples collected as dried blood spots on filter papers (by just finger prick). And why were the samples were depleted of leucocytes?
   2. Under data analysis, they should give reasons for pooling the results of 2002-2003 and 2016 2017 instead of analysing each year separately.

3. Results:
   1. Authors should give a baseline table showing the number of children sampled at each of the study sites and time points to guide the reader in terms of distribution of samples. They should also show how many samples (with percentage) were successfully genotyped in this table to give an idea of the amplification success (proportion of samples with genotypes) for each of the markers analysed. This will be helpful in interpreting the results in subsequent sections.
   2. In the introduction, it was stated that, “This study sought to ascertain the population trends in the prevalence of known drug-resistance-related point mutations in pfcrt, pfmdr1, pfdhfr and pfdhps in clinical isolates from three different malaria-endemic areas in Ghana a decade following the introduction of ACTs” and it was also mentioned in the data analysis section that the data was pooled for 2002-2003 and 2016-2017. However, the section on the prevalence of alleles of CQ and antifolates as well as table 1 don’t show any data on the prevalence of the alleles at different time points (trends). Such data should be incorporated in the table and summarized in the text as well.
   3. While in the text authors refer to “prevalence of alleles”, the heading of table 1 talks about the “frequency of SNPs”. These are two different things and should be reconciled. The authors should also explain how they dealt with samples which had mixed infections and therefore mixed alleles.
   4. The prevalence of triple dhfr mutant alleles (I51R59N108) is not shown in table 2 and the figures mentioned (53% in 2002/2003 and 79% in 2016/2017) are for the quadruple mutations of both dhfr (I51R59N108) and dhps (G437) while the prevalence of 43% and 73% of the quadruple mutation mentioned in the text is not shown in table 2. Since table 2 presents combined dhfr and dhps mutations, all the reported haplotypes should cover both genes because the reported number and prevalence are basically considered to have combined parasites with/without mutations in the two genes (as reported in table 2). Thus, the following statement, “Double mutant allele combinations of pfdhfr (I51N108, R59N108 and I51R59) had comparably low frequencies” cannot be shown in the same table (as for the triple dhfr mutations) without including the allele on dhps. The double mutation I51G437 reported in text is not shown in table 2.

4. Discussion:
   1. The authors mentioned that, “The percentages of the pfdhfr 51I (81%), 59R (82%), 108N (88%) and pfdhps 437G (88%) mutant alleles reported in this study are relatively higher when compared to the 71%, 42%, 64% and 80% prevalence reported in a recent study in a neighbouring country, Burkina Faso (Cisse et al., 2017), this could be due to differences in
the uptake of IPTp in both countries.” However, they do not provide any data or evidence to show the differences in uptake of IPTp in the two countries.

2. At the end of the second paragraph, authors state that, “Unlike pfcr76T and pfmdr1 86Y, the prevalence of pfmdr1 184F mutant allele (65%) appears to have not varied so much from 2005 to 2017 when compared to the 43% to 69% prevalence reported from 2005 to 2010”. The role of ACTs in the selection of mdr1 mutations should be discussed, due to available evidence of increasing N86 and 184F after introduction of ACTs as reported in some countries.

3. This study covers molecular markers which are important for drugs used in both IPTp and SMC, the discussion should provide prominence on these interventions, in order to make a strong case for future surveillance and monitoring of these markers. This will provide an opportunity to monitor the impact of IPTp and SMC on the molecular markers of amodiaquine and SP resistance.

1. Conclusion:
The conclusion should be revised to align it to the objectives of the study which was stated at the end of the introduction; “This study sought to ascertain the population trends in the prevalence of known drug-resistance-related point mutations in pfcr7, pfmdr1, pfldhfr and pfldhps in clinical isolates from three different malaria-endemic areas in Ghana a decade following the introduction of ACTs”. Thus, together with a conclusion of the trends of the CQ markers, they should also summarise the trends of SP mutations before finishing with the possible implication of the findings.

1. Others comments.

1. Authors should revise the document to take care of some few typos.

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

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

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

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

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

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Referee Expertise: Antimalarial drug resistance, genomic epidemiology of malaria and national health laboratory systems.

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