RESEARCH ARTICLE

Prevalence of chloroquine and antifolate drug resistance alleles in *Plasmodium falciparum* clinical isolates from three areas in Ghana [version 1; peer review: 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 *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* I51R59N108 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 (I51R59N108/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.
resistance, which could be due to the continuous use of antifolate drugs for prophylaxis.

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

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Introducción

La malaria es una preocupación de salud global, especialmente en África Subsaariana. *P. falciparum* malaria es considerado el más grave y también el que más contribuye al morbimidad y mortalidad, especialmente entre niños menores de cinco años (Schumacher & Spinelli, 2012). En 2016, se estimó un total de 216 millones de casos de malaria, lo que corresponde a aproximadamente 445,000 muertes (WHO, 2017). Los casos de mortalidad global han disminuido en un 29% desde el año 2010, como resultado de medidas preventivas y de control (WHO, 2016).

El uso de antimaláricos para el tratamiento y prevención de la malaria ha jugado un papel crucial en el control de la enfermedad a lo largo de los decenios (Cui et al., 2015; Gosling et al., 2011; Greenwood, 2004; Schlitzer, 2007). Sin embargo, la emergencia y el aumento de resistencia a los antimaláricos en las especies de *P. falciparum* han sido un desafío para el control de la enfermedad (Lin et al., 2010). Los lineamientos en *P. falciparum* que resistían el cloroquina (CQ) fueron reportados por vez primera en el siglo XX en Asia oriental, en la frontera Camboya-Tailandia (Young et al., 1963) y luego reportados en otros países. Recientemente, la malaria ha sido identificada con una resistencia a la mayoría de los antimaláricos, lo cual se ha exhibido por una alta incidencia de anemia y fracción de parasitoide resistente a la CQ y otras drogas anti-malariales (Dondorp et al., 2015). Los ACTs ahora son los fármacos de primera línea para tratar la malaria no complicada, incluyendo Ghana (WHO, 2016).

Las mutaciones específicas en los genes del parasitoma son implicadas en la resistencia a los antimaláricos (Cui et al., 2015; Fidock et al., 2000; Sidhu et al., 2002). Una mutación en el gen *pfmdr1* del transportador de aminoácido por cadena de tRNA que sustituye la metionina de codón 76 por una alanina en los clonos 76T ha sido un criterio comprobado de resistencia a la CQ y diversos tratamientos antimaláricos (Babiker et al., 2001). Además, las mutaciones en el gen *pfdhfr* multi-resistance (SNP) gen 1 (pfdhfr, PF3D7_0709000) que reemplaza la lisina por triptófano en el codón 76 ha sido un marcador genético para la resistencia a la CQ (Babiker et al., 2001). En la muestra de 2013, se encontró una alta prevalencia de la mutación 76T en el área de investigación de Ghana. En este estudio, se evaluó la prevalencia de las mutaciones en los genes *pfmdr1*, *pfdhfr*, *pfcrt* y *pfdhps* en tres regiones de Ghana donde la malaria es un problema de salud prioritario.

**Métodos**

**Consideración ética**

Este estudio fue aprobado por el Comité de Ética de la Universidad de Ghana (GHS-ECR: 12/05/12), la Kintampo Health Research Centre (KHRCIEC/FEA/2011-13), el Hospital General de Navrongo (NHRC-IRB135/08/2012) y el Naguchii Memorial Institute for Medical Research (NMIMR) (NMIMR-IRB CPN 004/11-12). Se informó a los padres o tutores para todos los participantes. Se obtuvo el consentimiento informado de los padres o tutores, además del consentimiento informado de los menores de 10 años.

**Etapas de muestra y métodos**

Los pacientes de malaria se obtuvieron de niños de 2 a 14 años diagnosticados con malaria en hospitales municipales de Kintampo, North Municipal (antes llamado Kintampo; 2012–2013 y 2016–2017), Accra (2016–2017) y Navrongo (2012–2013), en Ghana. Kintampo es una región tropical en la región de Brong Ahafo, con una prevalencia de malaria alta y dependiente de la lluvia, mientras que Navrongo es una región donde la malaria es una amenaza en la región de Upper East, donde la transmisión de malaria es estacional y dependiente de la lluvia (Owusu-Agyei, 2009) y Accra está localizada al oeste de Gana, donde la transmisión de malaria es estacional y dependiente de la lluvia (Owusu-Agyei, 2009) y Accra está localizada al oeste de Gana, donde la transmisión de malaria es estacional y dependiente de la lluvia (Owusu-Agyei et al., 2009). Se analizó el genoma del *P. falciparum* y se encontró que en la región de Kintampo, la prevalencia de las mutaciones en los genes *pfmdr1*, *pfdhfr*, *pfcrt* y *pfdhps* es alta. Se obtuvieron muestras de sangre de 207 pacientes en total, de los cuales se analizaron 205 muestras, y se obtuvieron 199 muestras de éxito.

En este estudio, se encontró una alta prevalencia de mutaciones en los genes *pfmdr1*, *pfdhfr*, *pfcrt* y *pfdhps* en las muestras de pacientes en tres regiones de Ghana. Se encontró que la prevalencia de las mutaciones en los genes *pfmdr1*, *pfdhfr*, *pfcrt* y *pfdhps* es alta en la región de Kintampo, North Municipal (antes llamado Kintampo; 2012–2013 y 2016–2017), Accra (2016–2017) y Navrongo (2012–2013), en Ghana. Kintampo es una región tropical en la región de Brong Ahafo, con una prevalencia de malaria alta y dependiente de la lluvia, mientras que Navrongo es una región donde la malaria es una amenaza en la región de Upper East, donde la transmisión de malaria es estacional y dependiente de la lluvia (Owusu-Agyei et al., 2009). Se analizó el genoma del *P. falciparum* y se encontró que en la región de Kintampo, la prevalencia de las mutaciones en los genes *pfmdr1*, *pfdhfr*, *pfcrt* y *pfdhps* es alta. Se obtuvieron muestras de sangre de 207 pacientes en total, de los cuales se analizaron 205 muestras, y se obtuvieron 199 muestras de éxito.
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 pfcrt (K76T), pfmdr1 (N86Y and Y184F), pfdhfr (N51I, C59R, and S108N) and pfdhps (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 the outer and the nested PCRs were used (Djimdé et al., 2001a; Duraisingh 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; Duraisingh 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 pfcrt 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 pfcrt 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 pfdhps K540E (Table 1 and Dataset 1). The frequency distribution of isolates harbouring the pfdhfr 51I, 59R and 108N mutant alleles were comparable across the study sites (P > 0.05 for all the three loci). The distribution of pfdhps 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 pfdhfr and pfdhps respectively at both study sites from 2005 to 2013, with frequencies levelling off subsequently (Figures 1C–F).

Analysis of pfdhfr and pfdhps haplotype combination distributions
We used clinical isolates for which all the pfdhfr and pfdhps SNP alleles of interest were successfully genotyped to survey allele combinations and determine their distribution between study periods. The pfdhfr triple mutant allele combination (I1 R1 N2) 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_{9} R_{59} N_{108} G_{437}) 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_{9} N_{108} R_{59} N_{108} and I_{9} R_{59}) 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_{59} N_{108} G_{437}, I_{9} R_{59} G_{437}, and I_{9} N_{108} G_{437}) and the double mutant allele combination (R_{59} / G_{437}, N_{108} / G_{437} and I_{9} / G_{437}) 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_{9} R_{59} N_{108}) and in both genes I_{9} R_{59} N_{108} G_{437} 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 *pfcr* 76T allele, but also with *pfmdr1* 86Y and 184F alleles. Data presented here show a gradual decline in frequency of the *pfcr* 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 *pfcr* 76T 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 (Duah et al., 2013).

<p>| Table 1. Frequency distribution of <em>pfcr</em>, <em>pfmdr1</em>, <em>pfdhps</em> and <em>pfdhfr</em> SNPs in <em>P. falciparum</em> isolates. |
|---|---|---|---|---|---|---|---|</p>
<table>
<thead>
<tr>
<th>Gene</th>
<th>Amino acid position</th>
<th>Amino acid*</th>
<th>Kintampo n (%)</th>
<th>Navrongo n (%)</th>
<th>Accra n (%)</th>
<th>Total n (%)</th>
<th>P-value b</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pfcr</em></td>
<td>K76T</td>
<td>K</td>
<td>198 (93.0)</td>
<td>37 (88.1)</td>
<td>64 (88.9)</td>
<td>299 (91.7)</td>
<td>0.321</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>14 (7.0)</td>
<td>5 (11.9)</td>
<td>8 (11.1)</td>
<td>27 (8.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pfmdr1</em></td>
<td>N86Y</td>
<td>N</td>
<td>153 (91)</td>
<td>47 (82.5)</td>
<td>72 (92.3)</td>
<td>272 (89.8)</td>
<td>0.124</td>
</tr>
<tr>
<td></td>
<td>Y</td>
<td>15 (9)</td>
<td>10 (17.5)</td>
<td>6 (7.7)</td>
<td>31 (10.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Y184F</td>
<td>Y</td>
<td>64 (35)</td>
<td>15 (27.8)</td>
<td>31 (38.8)</td>
<td>110 (34.9)</td>
<td>0.418</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>117 (65)</td>
<td>39 (72.2)</td>
<td>49 (61.2)</td>
<td>205 (65.1)</td>
<td></td>
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<tr>
<td><em>Pfdhfr</em></td>
<td>N51I</td>
<td>N</td>
<td>38 (20)</td>
<td>9 (16.7)</td>
<td>6 (8.0)</td>
<td>53 (16.6)</td>
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<td></td>
<td>I</td>
<td>151 (80.0)</td>
<td>46 (83.3)</td>
<td>69 (92.0)</td>
<td>266 (83.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C59R</td>
<td>C</td>
<td>15 (13)</td>
<td>5 (16.7)</td>
<td>12 (15.4)</td>
<td>32 (14.1)</td>
<td>0.783</td>
</tr>
<tr>
<td></td>
<td>S108N</td>
<td>S</td>
<td>21 (11)</td>
<td>5 (9.1)</td>
<td>4 (5.1)</td>
<td>30 (9.5)</td>
<td>0.281</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>163 (89.0)</td>
<td>50 (90.9)</td>
<td>74 (94.9)</td>
<td>287 (90.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pfdhps</em></td>
<td>A437G</td>
<td>A</td>
<td>23 (13.0)</td>
<td>5 (9.1)</td>
<td>1 (1.3)</td>
<td>29 (9.4)</td>
<td>0.0112</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>153 (87.0)</td>
<td>50 (90.9)</td>
<td>77 (98.7)</td>
<td>280 (90.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>K540E</td>
<td>K</td>
<td>158 (99.9)</td>
<td>54 (98.2)</td>
<td>78 (100)</td>
<td>290 (99.3)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>1 (0.1)</td>
<td>1 (1.8)</td>
<td>0 (0)</td>
<td>2 (0.7)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

aMutated amino acid depicted in bold, bP-value based on Pearson chi-Square test or Exact chi-square test for categorical variables.
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 pfdhfr 511 (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. 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 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 pfdhfr/pfdhps quintuple (pfdhfr/pfdhps I51R,N59S,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 (I51R,N59S,N108G/G437C) 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%) (Bertzosa et al., 2017), which suggests a high prevalence of variant quadruple mutant alleles in West to Central Africa. The pfdhps 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

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

<table>
<thead>
<tr>
<th>pfdhfr</th>
<th>pfdhps</th>
<th>2012–2013</th>
<th>2016–2017</th>
<th>Total</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>n = 95 (%)</td>
<td>n = 128 (%)</td>
<td>n = 223 (%)</td>
<td></td>
</tr>
<tr>
<td>NS11</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 pfdhfr and pfdhps.
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 pfcr t locus. In addition, the increasing prevalence of pfhdhfr 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 in pregnant women and children.

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

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.

References


A map is needed to show the different locations where the samples were collected. It should be

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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 pfdhps 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 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 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 pfhdfr and pfhdps 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.

Reviewer Report 08 May 2018
https://doi.org/10.21956/aasopenres.13891.r26335

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Deus S. Ishengoma
National Institute for Medical Research, Tanga, Tanzania

**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 chemoprophylaxis 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 (I_{51}R_{59}N_{108}) 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 (I_{51}R_{59}N_{108}) and dhps (G_{437}) 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 (I_{51}N_{108}, R_{59}N_{108} and
I_{51} R_{59}) 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 I_{51} G_{437} reported in text is not shown in table 2.

4. Discussion:

1. The authors mentioned that, “The percentages of the pf dhfr 51I (81%), 59R (82%), 108N (88%) and pf dhps 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 pf crt 76T and pf mdr 1 86Y, the prevalence of pf mdr 1 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 mdr 1 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 pf crt, pf mdr 1, pf dhfr and pf dhps 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.

**Reviewer 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.