



RESEARCH ARTICLE

REVISED Prevalence of chloroquine and antifolate drug resistance alleles in *Plasmodium falciparum* clinical isolates from three areas in Ghana [version 2; peer review: 2 approved]

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v2 First published: 18 Apr 2018, 1:1 (<https://doi.org/10.12688/aasopenres.12825.1>)

Latest published: 03 Dec 2018, 1:1 (<https://doi.org/10.12688/aasopenres.12825.2>)

Abstract

Background: The emergence and spread of resistance in *Plasmodium falciparum* to chloroquine (CQ) 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.

Sulphadoxine-pyrimethamine (SP) which was the second line antimalarial drug in Ghana, was now adopted for intermittent preventive treatment of malaria in pregnancy (IPTp).

Methods: To examine the prevalence of molecular markers associated with CQ and antifolate drug resistance in Ghana, we employed restriction fragment length polymorphism polymerase chain reaction to genotype and compare single nucleotide polymorphisms (SNPs) in the *P. falciparum* chloroquine resistance transporter (*pfcr*t, PF3D7_0709000), multidrug resistance (*pfmdr*1, PF3D7_0523000), bifunctional dihydrofolate reductase-thymidylate synthase (*pfdhfr*, PF3D7_0417200) and dihydropteroate synthase (*pfdhps*, PF3D7_0810800) genes. Parasites were collected from children with malaria reporting to hospitals in three different epidemiological areas of Ghana (Accra, Kintampo and Navrongo) in 2012-2013 and 2016-2017.

Results: The overall prevalence of the CQ resistance-associated *pfcr*t 76T allele was 8%, whereas *pfmdr*1 86Y and 184F alleles were present in 10.2% and 65.1% of infections, respectively. The majority of the isolates harboured the antifolate resistance-associated *pfdhfr* alleles 51I (83.4%), 59R (85.9 %) and 108N (90.5%). *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 compared to Kintampo for the 2016-2017 isolates. Across both *pfdhfr* and *pfdhps* genes, a large proportion (61%) of the isolates harboured the quadruple mutant

Open Peer Review

Reviewer Status

	Invited Reviewers	
	1	2
version 2 (revision) 03 Dec 2018		 report
version 1 18 Apr 2018	 report	 report

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combination (I₅₁ R₅₉ N₁₀₈/ G₄₃₇).

CQ resistance alleles decreased during the 12 years after CQ withdrawal, but an mediate SP resistance alleles increased.

Conclusion: Surveillance of the prevalence of resistance alleles is necessary in monitoring the efficacy of antimalarial drugs.

Keywords

Drug resistance, Malaria, Antifolates, Chloroquine, Plasmodium falciparum

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Author roles: **Abugri J:** Conceptualization, Data Curation, Formal Analysis, Investigation, Methodology, Writing – Original Draft Preparation, Writing – Review & Editing; **Ansah F:** Data Curation, Investigation, Methodology, Writing – Original Draft Preparation, Writing – Review & Editing; **Asante KP:** Investigation, Supervision, Writing – Review & Editing; **Opoku CN:** Project Administration, Resources, Writing – Review & Editing; **Amenga-Etego LA:** Conceptualization, Data Curation, Formal Analysis, Investigation, Methodology, Supervision, Writing – Review & Editing; **Awandare GA:** Conceptualization, Formal Analysis, Funding Acquisition, Investigation, Methodology, Resources, Supervision, Writing – Original Draft Preparation, Writing – Review & Editing

Competing interests: No competing interests were disclosed.

Grant information: This work was supported by the African Academy of Science through the Developing Excellence in Leadership, Training and Science (DELTA) programme [DEL-15-007] (GA). This work was also supported by the Wellcome Trust [107755/Z/15/Z] (GA), and World Bank Group [ACE02: WACCBIP] (GA).

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

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How to cite this article: Abugri J, Ansah F, Asante KP *et al.* **Prevalence of chloroquine and antifolate drug resistance alleles in *Plasmodium falciparum* clinical isolates from three areas in Ghana [version 2; peer review: 2 approved]** AAS Open Research 2018, 1:1 (<https://doi.org/10.12688/aasopenres.12825.2>)

First published: 18 Apr 2018, 1:1 (<https://doi.org/10.12688/aasopenres.12825.1>)

REVISED Amendments from Version 1

The majority of reviewers' recommendations have been effected, and for the others, appropriate explanations/rebuttals have been provided. Details of the cross-sectional sampling in 2012–2013 and 2016–2017 and the genotyping methods used have been included. In addition, an explanation has been provided for the choice of the 2–14 year age group in the study. The data have been disaggregated and reanalyzed separately for the 2012–2013 and 2016–2017 time points. This study depended on archived samples from an erythrocyte invasion study so only samples that were successfully genotyped for any number of mutations were presented in Table 1. Table 1 now compares data from the same time points across study sites while Table 2 now captures frequencies of allele combinations in both *pfdhfr* and *pfdhps*. In this study mixed infections were not examined and we have provided an explanation for that in the revised manuscript. A map of Ghana has been included as Figure 1 to show the distance between Sunyani and Kintampo and the use of data for Sunyani to represent Kintampo has been corroborated with relevant literature. The graph on trend analyses is now designated Figure 2. References have been updated to capture extant literature in Ghana and other West and Eastern African countries. The discussion on IPTp and SMC uptake in Ghana and Burkina Faso has been expanded. Further details on the role of ACTs in the selection of *mdr1* mutations has been included and relevant references provided therein. The major findings of the study have now been captured in the discussion as recommended. In addition, the use of antifolate drugs for the management of opportunistic infections in HIV has been discussed. The conclusion has been revised to align with the study objectives.

See referee reports

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 militated 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 subsequently reported in other countries globally. Currently, the parasite has been reported to have developed resistance to most available artemisinin monotherapies and this is exhibited by reduced parasite clearance rates and/or treatment failures (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 (*pfcr*, PF3D7_0709000) that replaces lysine with threonine at codon 76 had become a common single nucleotide polymorphism (SNP) 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 (Djimé *et al.*, 2001b).

P. falciparum resistance to Sulfadoxine-pyrimethamine (SP) has been linked to point mutations in the bifunctional dihydrofolate reductase-thymidylate synthase (*pfdhfr*, PF3D7_0417200) and dihydropteroate synthase (*pfdhps*, PF3D7_0810800) genes (Cowman *et al.*, 1988; Triglia *et al.*, 1997; Wang *et al.*, 1997). 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 (Mita *et al.*, 2014). 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* I₅₁ R₅₉ N₁₀₈ + *pfdhps* G₄₃₇ E₅₄₀) 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 *pfcr* 76T 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 (Laufer *et al.*, 2006; Mwai *et al.*, 2009). In a study that was conducted in Tanzania, more than 90% recovery of the sensitive *pfcr* K76 allele was reported after 10 years of CQ use being officially discontinued (Mohammed *et al.*, 2013). Follow-up studies in Ghana have reported a decline in the prevalence of *pfcr* 76T and *pfmdr1* 86Y but an increasing prevalence *pfdhfr* I 51, R 59, N 108 and 437G resistant alleles from 2003 to 2010 (Duah *et al.*, 2013; Duah *et al.*, 2012)

This study sought to ascertain the population trends in the prevalence of known drug-resistance-related point mutations in *pfcr*, *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

This study leveraged the availability of samples from a concurrent study at the time on erythrocyte invasion mechanisms and whole genome sequencing of the malaria parasites. The appropriate sample collection at the time to meet the erythrocyte invasion studies, the whole genome sequencing of the malaria parasites was adopted. We used the samples so gotten to carry out the drug resistance study. The choice of 2–14 years was premised on development of immunity that was key in the erythrocyte invasion study.

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). Accra lies within the coastal savannah area with low seasonal malaria transmission (Klinkenberg *et al.*, 2008). Malaria transmission in Accra peaks during the June to August rainy season. The entomological inoculation rate (EIR) in Kintampo, Navrongo and Accra are >250, >100 and <19.2 infectious bites per person per year, respectively (Kasasa *et al.*, 2013; 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). Samples were obtained from participants during the rainy seasons at the respective study sites. *P. falciparum* genomic DNA was analyzed for the prevalence of known antimalarial drug resistance SNPs in *pfcr* (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), and 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), *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 both the outer and the nested PCRs were used (Djimé *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 (Djimé *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 2012–2013 isolates were compared to the 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

The prevalence of antimalarial drug resistance alleles in three different transmission zones were determined and compared across sites and sampling time points.

. We did not observe any significant differences in the distribution of isolates harbouring *pfcr* K76T, *pfmdr1* N86Y or *pfmdr1* Y184F point mutations 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 (2012–2013) compared to Kintampo (2012–2013 and 2017) and Accra (2016–2017) (Table 1). The total prevalence of *pfprt* 76T (8%) and *pfmdr1* 86Y (10.2%) 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 also comparable across the study sites ($P > 0.05$ for all the three loci). However, the distribution of *pfdhps* 437 G mutant allele, was significantly different across the study sites ($P = 0.01$). *Pfdhps* 437G was significantly higher in Accra (2016–2017) compared to Navrongo (2012–2013) ($P = 0.03$), Kintampo (2012–2013) ($P = 0.004$) and Kintampo (2016–2017) ($P = 0.004$). The

frequency of *pfdhps* 437G in Navrongo (2012–2013), Kintampo (2012–2013) and Kintampo (2016–2017) were comparable ($P > 0.05$).

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. Kintampo and Sunyani are located in the same region (Brong Ahafo) but approximately 122 Km apart (Figure 1) Sunyani lies in the Forest Zone whilst Kintampo lies within the Forest Savannah transition zone, however, both sites have similar

Table 1. The prevalence of *pfprt*, *pfmdr1*, *pfdhps* and *pfdhfr* mutant alleles in *P. falciparum* isolates.

Gene	Amino acid ^a Position	Amino Acid ^b	Kintampo (2012-2013) n (%)	Navrongo (2012-2013) n (%)	Accra (2016-2017) n (%)	Kintampo (2016-2017) n (%)	Total ^c n (%)	P-value ^d
<i>Pfprt</i>	K76T	K	148 (86.3)	37 (88.1)	64 (88.9)	50 (98.0)	299 (91.7)	0.243
		T	13 (13.7)	5 (11.9)	8 (11.1)	1 (2.0)	27 (8.3)	
<i>Pfmdr1</i>	N86Y	N	103 (89.6)	47 (82.5)	72 (92.3)	50 (94.3)	272 (89.8)	0.166
		Y	12 (10.4)	10 (17.5)	6 (7.7)	3 (5.7)	31 (10.2)	
	Y184F	Y	42 (34.1)	15 (27.8)	31 (38.8)	22 (37.9)	110 (34.9)	0.574
		F	81 (65.9)	39 (72.2)	49 (61.2)	36 (62.1)	205 (65.1)	
<i>Pfdhfr</i>	N51I	N	28 (20.6)	9 (16.7)	6 (8.0)	10 (18.9)	53 (16.6)	0.124
		I	108 (79.4)	46 (83.3)	69 (92.0)	43 (81.1)	266 (83.4)	
	C59R	C	12 (17.9)	5 (16.7)	12 (15.4)	3 (5.8)	32 (14.1)	0.256
		R	55 (82.1)	25 (83.3)	66 (84.6)	49 (94.2)	195 (85.9)	
S108N	S	17 (13.0)	5 (9.1)	4 (5.1)	4 (7.5)	30 (9.5)	0.280	
	N	114 (87.0)	50 (90.9)	74 (94.9)	49 (92.5)	287 (90.5)		
<i>Pfdhps</i>	A437G	A	16 (12.7)	5 (9.1)	1 (1.3)	7 (14.0)	29 (9.4)	0.031
		G	110 (87.3)	50 (90.9)	77 (98.7)	43 (86.0)	280 (90.6)	
	K540E	K	106 (99.1)	54 (98.2)	78 (100)	52 (100)	290 (99.3)	-
E		1 (0.9)	1 (1.8)	0 (0)	0 (0)	2 (0.7)		

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

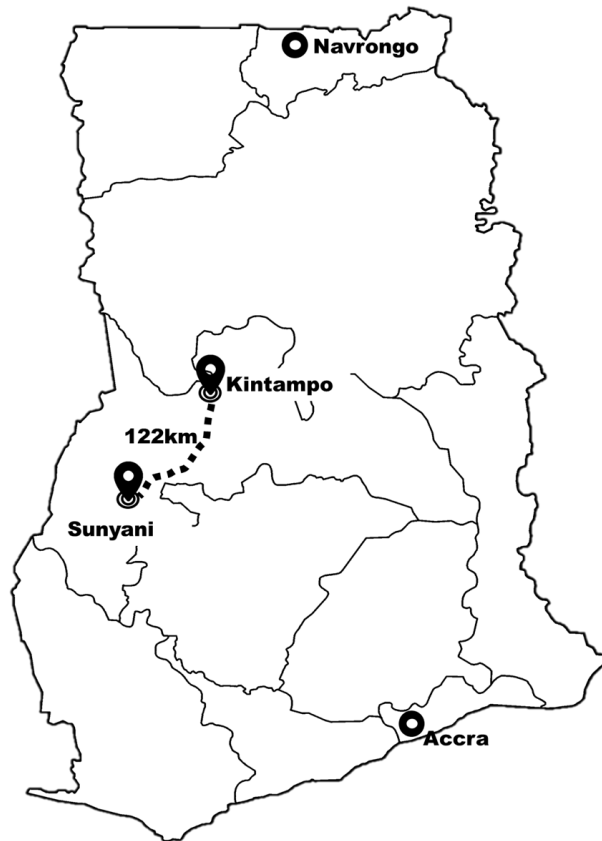


Figure 1. A map of Ghana showing our study sites. The distance between Sunyani and Kintampo is approximately 122 Km. Housing structures in both localities are largely similar with a slight difference in the vegetation but almost the same geospatial characteristics.

agricultural practices, housing structure, and land geology, all of which have been reported to influence malaria epidemiology (Baidjoe *et al.*, 2016; Hu *et al.*, 2016). 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* 86 Y in Navrongo (Figures 2A 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 2C–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 prevalence of the quadruple allele combination ($I_{51} R_{59} N_{108}/G_{437}$) in Navrongo (2012–2013), Kintampo (2012–2013), Kintampo (2016–2017), and Accra (2016–2017) were 17/25 (68.0), 37/51 (72.6) 43/53 (81.1) and 58/71 (81.7), respectively (Table 2). The frequency of $I_{51} R_{59} N_{108}/G_{437}$ was comparable across the study sites ($P > 0.05$ for all comparisons). Low prevalence (<10%) allele combinations in both *pfdhfr*

and *pfdhps* were observed for the triple mutant allele combination ($R_{59} N_{108}/G_{437} I_{51} R_{59}/G_{437}$ and $I_{51} N_{108}/G_{437}$) and these were also comparable across the study sites ($P > 0.05$ for all haplotypes) (Table 2).

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). In this study, we determined the prevalence of alleles associated with CQ and antifolate resistance using clinical isolates from three malaria endemic regions with varying transmission intensities in Ghana. We observed a decreasing prevalence of CQ resistance-associated alleles but an increasing prevalence of SP resistance-associated alleles. The distribution of the alleles across the three study sites were not significant, except for *pfdhps* 437G which was significantly higher in Accra compared to Navrongo and Kintampo. The frequency of *pfdhfr/pfdhps* haplotypes in 2012–2013 and 2016–2017 were not significantly different across the three study sites. Both *in vitro* and molecular surveillance studies have associated CQ resistance mainly with the *pfcr1* 76T allele, but also with *pfmdr1* 86Y and 184F alleles. *Pfcr1* 76T and *pfmdr1* 86Y mutant alleles have also been reported to decrease *P. falciparum* susceptibility to amodiaquine

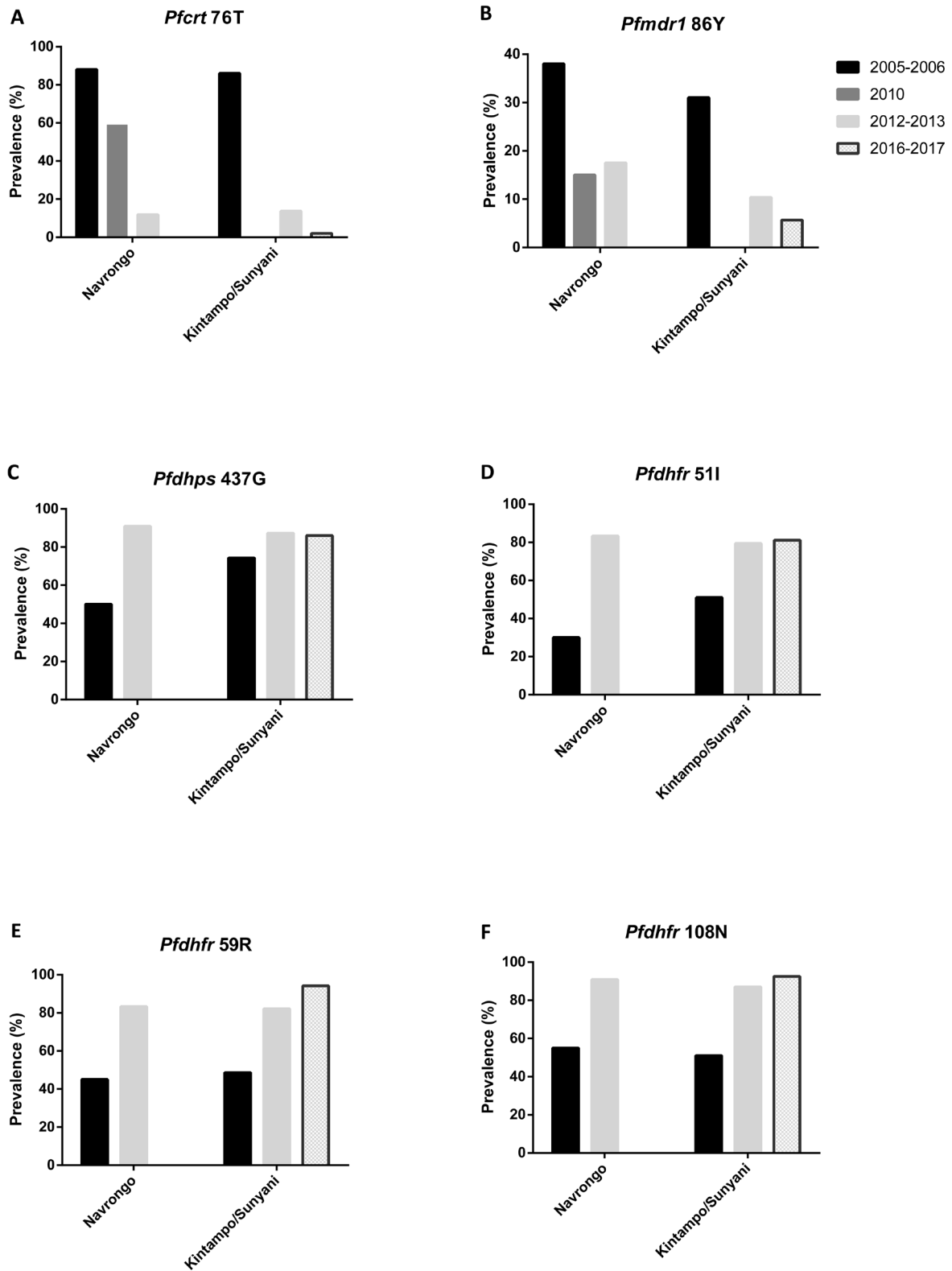


Figure 2. Trends in the prevalence of antimalarial drug resistant alleles from 2005 to 2017. Summarized data from present study was compared to previous published data from Navrongo and Kintampo/Sunyani, Ghana, for *pfmdr1* and *pfcrt* point mutations (Duah *et al.*, 2013) and for the antifolate resistance mutations (Duah *et al.*, 2012). No data was available for the *pfcrt* 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.

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

Haplotype	Kintampo 2012-2013, n = 51 (%)	Navrongo 2012-2013, n = 25 (%)	P-value
IRNG	37 (72.6)	17 (68.0)	0.789
RNG	6 (11.8)	2 (8.0)	0.714
IRG	4 (7.8)	4 (16.0)	0.427
ING	4 (7.8)	2 (8.0)	1
	Kintampo 2012-2013, n = 51 (%)	Kintampo 2016-2017, n = 53 (%)	
IRNG	37 (72.6)	43 (81.1)	0.356
RNG	6 (11.8)	6 (11.3)	1
IRG	4 (7.8)	1 (1.9)	0.201
ING	4 (7.8)	3 (5.7)	0.713
	Kintampo 2016-2017, n = 53 (%)	Accra 2016-2017, n = 71	
IRNG	43 (81.1)	58 (81.7)	1
RNG	6 (11.3)	2 (2.8)	0.0722
IRG	1 (1.9)	1 (1.4)	1
ING	3 (5.7)	10 (14.1)	0.151

Note: Numbers include only isolates that were successfully genotyped for all the four point mutations in *pfhfr* and *pfhps*. Each haplotype has mutant amino acids shown in bold. *P-value based on Exact chi-square test

but increase parasite sensitivity to dihydroartemisinin, lumenfantine and mefloquine (Gresty *et al.*, 2014; Veiga *et al.*, 2016). Despite the use of ACTs (artemether-lumefantrine, artesunate-amodiaquine, and dihydroartemisinin-piperazine) in Ghana since 2005, decreasing prevalence of *pfert* 76T and *pfmdr1* 86Y mutant alleles were observed in this study when compared to study by Duah and colleagues in 2013 (Duah *et al.*, 2013). This shows a gradual decline in the frequencies of these alleles since the discontinuation of CQ as an antimalarial in Ghana, this observation is consistent with findings in other malaria endemic populations in east Africa such as Tanzania, Malawi, Kenya and Zambia where artemether lumefantrine is the first-line drug for uncomplicated malaria (Mohammed *et al.*, 2013; Mwai *et al.*, 2009; Mwanza *et al.*, 2016). A study in Kenya posits that the K76 is preferential selection by Artemeter Lumefantrine(AL) (Achieng *et al.*, 2015) 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 *pfert* 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). Contrary to this observation, a study in Tanzania reported an increasing prevalence of *pfmdr1* N86 and 184F following the introduction of artemether-lumefantrine (Thomsen *et al.*, 2011). Notably, parasites that have a combination of *pfmdr1* mutant alleles (N86, 184F and D1246) are reported to have reduced sensitivity to artemether-lumefantrine treatment (Baliraine & Rosenthal, 2011; Happi *et al.*, 2009; Kavishe *et al.*, 2014). Other studies have also linked duplication of *pfmdr1* to resistance to partner drugs of ACTs

(Rodrigues, Henriques et (Borges *et al.*, 2011; Rodrigues *et al.*, 2010).

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 *pfhfr* 51I (81%), 59R (82%), 108N (88%) and *pfhps* 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, using samples obtained in 2010 (Cisse *et al.*, 2017). SP was used as a second-line treatment for uncomplicated malaria in both countries until 2005 when its usage was restricted for IPTp (Koram *et al.*, 2005; Tahita *et al.*, 2015). In Burkina Faso, resource persons are engaged at the community level to promote IPTp uptake and referrals to antenatal clinics (ANCs) whereas IPTp in Ghana is taken at the ANCs and health care centres (Gies *et al.*, 2009; Hill *et al.*, 2013). With the aforementioned strategies there is likely increased compliance in Burkina Faso compared to Ghana and this may explain the low resistance in the former. Furthermore, since drug resistance evolution is spatiotemporal the differences in periods of sampling could also account for the differences observed. The 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. Co-trimoxazole is used in Ghana (Fadeyi *et al.*, 2015), however, there is limited data on its usage in the three study sites. Besides the prevalence of HIV in Ghana is only 3 % (Ghana AIDs Commission, 2017) and therefore the use of antifolate drugs such as cotrimazole for the management of opportunistic infections is not as widespread as the use of antifolate antimalarial drugs. Higher SP treatment failure has been correlated with the *pfdhfr/pfdhps* quintuple (*pfdhfr/pfdhps* I₅₁R₅₉N₁₀₈/G₄₃₇E₅₄₀) haplotypes (Kublin *et al.*, 2002; Triglia *et al.*, 1997). Parasites harbouring *pfdhfr/pfdhps* I₅₁R₅₉N₁₀₈/G₄₃₇ and I₅₁R₅₉N₁₀₈/G₄₃₇E₅₄₀ haplotypes have been described as “partial” and “full” SP resistance, respectively (Naidoo & Roper, 2013). In this study, no isolate was observed to carry the full resistance allele combination. 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 show an increased prevalence of parasite isolates that harbour other SP resistance haplotypes 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 *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 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 are widely used in Ghana may still be maintaining significant selection pressure on the *pfcr* locus. In addition, the increasing prevalence of the *pfdhfr/pfdhps* partial SP resistance haplotypes could result in the fixation of these alleles within the parasite population. The continuous use of SP for IPTp and SMC may result in emergence of the “full” SP resistance haplotype and compromise the use of SP IPTp and SMC are the two significant

sources of SP drug pressure on the parasite population in pregnant women and young children, respectively. Recent studies have shown that these interventions have contributed to reduction in maternal and child morbidity and mortality (Coldiron *et al.*, 2017). Undoubtedly, these interventions are critical (York, 2017), but could easily be undermined by rising resistance in these populations. Therefore, it is very important to closely monitor the prevalence of molecular markers of resistance associated with antifolate antimalarial drugs to guide policies on the continuous use of these drugs in Ghana and other African countries. There are probably other factors that contribute to the evolution of resistance markers as SP has been shown to be efficacious even in the face of fixation of SP resistant alleles (Iriemenam *et al.*, 2012).

Conclusion

This study reports an increasing prevalence of CQ sensitive clinical isolates after 12 years of CQ withdrawal at three different study sites that capture the eco-epidemiology of malaria in Ghana. The prevalence of the antifolate drug resistant alleles remain relatively high across the study sites. Besides, there is an increasing trend in the frequency of SP-resistance associated alleles at all sites. Taken together, these observations point to the need for a robust antimalarial drug discovery strategy to provide a vast array of alternatives for chemotherapy in readiness for the likelihood of future poor parasite response to the use of SP for prevention of malaria in pregnant women and for SMC in children. However, it is pre-mature to recommend the discontinuation of SP use due to the high prevalence of antifolate drug resistance alleles since the drug can be efficacious where there is fixation of these alleles.

Data availability

The data supporting this article is available online at Open Science Framework: Dataset 1. Prevalence of chloroquine and antifolate drug resistance alleles in *Plasmodium falciparum* clinical isolates from three areas in Ghana. <http://dx.doi.org/10.17605/OSF.IO/N2GZF> (Abugri *et al.*, 2018) under a CC0 1.0 Universal licence.

Grant information

This work was supported by the African Academy of Science through the Developing Excellence in Leadership, Training and Science (DELTAS) programme (DEL-15-007 to GA) and Wellcome Trust (107755/Z/15/Z to GA). This work was also supported by the World Bank Group (ACE02: WACCBIP to GA).

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

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Version 2

Reviewer Report 11 January 2019

<https://doi.org/10.21956/aasopenres.13998.r26676>

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Edwin Kamau

Walter Reed Army Institute of Research, Silver Spring, MD, USA

The authors have adequately responded to all my comments, the manuscript is now sufficient for approval.

Competing Interests: No competing interests were disclosed.

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

Version 1

Reviewer Report 01 June 2018

<https://doi.org/10.21956/aasopenres.13891.r26410>

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Edwin Kamau

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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; *pfprt* 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* 2007¹ 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.* 2012² 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 be 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 pfdhfr and pfdhps 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 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?

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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 confirm that I have read this submission and 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 Centre, 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 chemoprevention 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 *pfcr1*, *pfmdr1*, *pfdhfr* and *pf dhps* 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 *pf dhfr* (**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 *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 IPT_p in both countries.” However, they do not provide any data or evidence to show the differences in uptake of IPT_p in the two countries.
2. At the end of the second paragraph, authors state that, “Unlike *pfcr1* 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”. 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 IPT_p 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 IPT_p 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 *pfcr1*, *pfmdr1*, *pfdhfr* and *pfdhps* 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 confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.
