Assessment of antimalarial drug resistant markers in asymptomatic *Plasmodium falciparum* infections after 4 years of indoor residual spraying in Northern Ghana

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Abstract

Background
Drug resistance remains a concern for malaria control and elimination. The effect of interventions on its prevalence needs to be monitored to preempt further selection. We assessed the prevalence of *Plasmodium falciparum* gene mutations associated with resistance to the antimalarial drugs: sulfadoxine-pyrimethamine (SP), chloroquine (CQ) and artemisinin combination therapy (ACTs) after the scale-up of a vector control activity that reduced transmission.

Methods
A total of 400 *P. falciparum* isolates from children under five years were genotyped for seventeen single nucleotide polymorphisms (SNPs) in *pfcrt*, *pfmdr1*, *pfdhfr*, *pfdhps* and *pfk13* genes using polymerase chain reaction (PCR) and high resolution melting (HRM) analysis. These included 80 isolates, each randomly selected from cross-sectional surveys of asymptomatic infections across 2010 (baseline), 2011, 2012, 2013 (midline: post-IRS) and 2014 (endline: post-IRS) during the peak transmission season, when IRS intervention was rolled out in Bunkpurugu Yunyoo (BY) District, Ghana. The proportions of isolates with drug resistant alleles were assessed over this period.

Results
There were significant decreases in the prevalence of *pfdfhr*- L51R R59N108 haplotype from 2010 to 2014, while the decline in *pfdfhr/pfdhps*- L51R S59 N108 G437 during the same period was not significant. The prevalence of lumefantrine (LM), mefloquine (MQ) and amodiaquine (AQ) resistance-associated haplotypes *pfmdr1*- N86 F184 D1246 and *pfmdr1*- Y86 Y184 Y1246 showed decreasing trends (z = -2.86, P = 0.004 and z = -2.71, P = 0.007,
respectively). Each of \textit{pfcr-T76} and \textit{pfmdr1-Y86} mutant alleles also showed a declining trend in the asymptomatic reservoir, after the IRS rollout in 2014 ($z = -2.87, P = 0.004$ and $z = -2.65, P = 0.008$, respectively). Similarly, Pyrimethamine resistance mediating polymorphisms \textit{pfdhfr-N108}, \textit{pfdhfr-I51} and \textit{pfdhfr-R59} also declined ($z = -2.03, P = 0.042$, $z = -3.54, P < 0.001$ and $z = -4.63, P < 0.001$, respectively), but not the sulphadoxine resistance mediating \textit{pfdhps-G437} and \textit{pfdhps-F436} ($z = -0.36, P = 0.715$ and $z = 0.41, P = 0.684$, respectively). No mutant \textit{pfk13-Y580} were detected during the study period.

**Conclusion**

The study demonstrated declining trends in the prevalence of drug resistant mutations in asymptomatic \textit{P. falciparum} infections following transmission reduction after an enhanced IRS intervention in Northern Ghana.

**Introduction**

In spite of all the gains made in global malaria control over the past fifteen years, progress stalled between 2015–2018 [1], resulting in 228 million cases and 405,000 deaths in 2018 alone [1]. The disease burden is highest in sub-Saharan Africa where children under five years are the most affected [1] and \textit{Plasmodium falciparum} resistance to antimalarial treatment continues to threaten the efforts at elimination of malaria [2–5].

In Ghana, the disease is the leading cause of morbidity and mortality and is transmitted perennially in the southern (coastal) and middle (forest) belts, and seasonally in the northern (savanna) belt [6]. In 2015, the country recorded approximately 10 million suspected malaria cases with 31% in children under five years. An estimated 2100 malaria-related deaths were also recorded in 2015 of which children under five formed about 50% [7]. The primary interventions for malaria control in Ghana are: early diagnosis with prompt and effective treatment using artemisinin based combination therapies (ACTs); scaling-up of vector control measures that emphasize, universal Insecticide-treated nets (ITN) coverage; targeted Indoor Residual Spraying (IRS) in selected areas; seasonal malaria chemoprevention (SMC); and Intermittent Preventive Treatment in pregnancy (IPTp). As in other sub-Saharan African countries, chloroquine (CQ) use in Ghana was discontinued and replaced with ACTs, due to antimalarial resistance. Specifically, artesunate-amodiaquine (ASAQ) was introduced in 2005 and subsequently artemether-lumefantrine (AL) and dihydroartemisinin-piperazine (DHAP) were included as additional first-line treatments for uncomplicated malaria in 2007 and 2009, respectively [8]. Sulphadoxine Pyrimethamine (SP) was deployed as prophylaxis in IPTp in 2004 and SP+AQ for SMC among children under five years in the northern regions of the country since 2015. The 2014 Ghana Demographic and Health Survey (DHS) [9] and the 2016 Ghana Malaria Indicator Survey (MIS) [10] reported the use of ACTs by 78.2% and 53% of Ghanaian children under age 5 years, respectively. ITN use has increased from 3% in 2003 to over 60% of children by 2011, following mass free distribution campaigns in many parts of the country [11].

With support from the U.S President’s Malaria Initiative (PMI), the U.S Centers for Disease Control (CDC) and ABT Associates, the Ghana Health Service (GHS) in collaboration with the Noguchi Memorial Institute for Medical Research (NMIMR) in 2010 implemented extensive IRS surveys in the Bunkpurugu Yunyoo (BY) District of the Northern Region of Ghana. The aim was to significantly reduce malaria transmission intensity and parasitaemia, as part of...
the country’s malaria control strategies. As a result of the program, the prevalence of parasitaemia in children under age five declined from 52.4% in 2010 to 22.2% in 2014 [12] and entomological inoculation rate (ie. transmission intensity) dropped from 0.350 to 0.010 infectious bites/person/night during the same period (Dadzie et al., manuscript under review).

Despite these achievements, Ghana was one of the 12 most malaria-burdened African countries according to the WHO, representing 3% of the global malaria burden with an increase in malaria cases in 2018 compared with 2017 [1]. This raises questions about the sustainability of transmission reduction as a result of these vector control interventions and calls for a renewed drive in malaria control.

Treatment, prophylaxis and anti-vector interventions reduce parasite prevalence, transmission, morbidity, and mortality, but how the interactions between these interventions and the resultant change in epidemiology affect the prevalence of drug resistant markers is not fully understood. There remains a reservoir of asymptomatic P. falciparum infection in circulation predominantly in older children and adults, that sustains transmission and thus contributes to the spread of antimalarial resistance even after highly successful vector control measures. This asymptomatic reservoir represents infections in individuals with varying parasite densities, but with no fever or other acute symptoms that would warrant them to seek treatment [13, 14]. A proportion of asymptomatic parasitaemia is submicroscopic and only identifiable with molecular methods, the other is detectable using microscopy and rapid diagnostic tests (RDT) [14,15]. The ability to detect and manage these infections thus remains vital to the success of elimination. Ascertaining how the remaining reservoir would respond to anti-malarial therapy after an intervention has reduced transmission intensity would also have implications for future malaria control activities in a locale.

While some studies have found an association between transmission reduction and a decline in the drug resistance evolution [16–18], others have reported a reverse association [19,20] and some others have found no clear association [21–23]. Other studies have also found a mixed association [24]. Transmission reduction may indeed suppress the development of drug resistance [16–18], however, at very low transmission intensity, it may likely increase the evolution of drug resistance [24]. At very low transmission intensities, parasite populations tend to inbreed significantly and sexual recombination is reduced. As such, drug resistant gene combinations in the population stabilize and rapidly increase in frequency [20,25].

Resistance to antimalarial drugs is attributed to single nucleotide polymorphisms (SNPs) in different P. falciparum genes, including: the chloroquine resistance transporter (pfcr) gene on chromosome 7 (associated with chloroquine resistance) [26,27]; the multidrug resistant (pfmdr1) gene on chromosome 5 (associated with chloroquine, mefloquine, amodiaquine, lumefantrine and artesiminin resistance) [28–30]; the dihydropteroate synthetase (pfdsps) gene on chromosome 8 (associated with sulphadoxine resistance) [31,32]; the dihydrofolate reductase (pfhfr) gene on chromosome 4 (associated with pyrimethamine resistance) [33,34]; and the Kelch 13 (pfk13) gene on chromosome 13 (associated with artesiminin resistance) [35–37]. The use of these molecular markers to detect and monitor drug resistant parasites comprise one of the most valuable methods in assessing antimalarial drug efficacy.

In this study, we assessed temporal trends in the prevalence of mutations in pfcr, pfmdr1, pfdsps, pfhfr and pfk13 genes in P. falciparum isolates from cross-sectional surveys of the most malaria vulnerable group during the peak transmission season in the BY district. Multi-drug resistance and linkage between drug resistance genes were also evaluated with clonal infections. Ultimately, this study will provide information that will guide subsequent targeted malaria control strategies in Ghana.
Materials and methods

Study site and samples

This study was part of a yearly IRS intervention carried out between 2010 and 2014 in Bunkpurugu Yunyoo District of the Northern Region of Ghana [Fig 1], where malaria transmission is high and seasonal. The comprehensive study design and features for the IRS intervention have been described elsewhere [12]. In the course of the IRS intervention, cross-sectional surveys were conducted in 50 communities in March/April (end of the dry season) and October/November (end of the rainy season) to determine the impact of IRS on *P. falciparum* parasitaemia and severe anaemia in children under the age of five. For each survey, Dried blood blots (DBS) (one per child) were obtained from all study participants following rapid malaria testing [CareStart™Malaria HRP2/pLDH (pf/PAN) Combo (AccessBio, New Jersey, USA)], light microscopy, and anaemia testing (Hemocue AB, Ängelholm, Sweden). Parasite quantification and species identification were done using thick and thin smears, respectively. Briefly, parasites were quantified against 200 white blood cells (WBC) as counts per microliter of blood, assuming 8000 WBC per microliter of blood. Two senior microscopists independently read all blood slides and a third senior microscopist read the discordant slides pertaining to species identification and sexual/sexual parasite presence. The third microscopist’s reading was considered the decider for all discordant slides [12]. Mixed infections of *P. falciparum* with *P. malariae* accounted for 1.5–4.9% and *P. falciparum* with *P. ovale* was 0.2–2.3% [12], and these were excluded from our analysis. Information based on demographic, clinical, parasitological and entomological assessments were also obtained. For this study, we generated random...
numbers for sample selection from the sample list captured in an excel spreadsheet. We randomly selected 80 \textit{P. falciparum} mono-infected smear-positive blood samples each from 2010 (baseline), 2011, 2012, 2013 (midline: post-IRS) and 2014 (endline: post-IRS), from the peak transmission (end of rainy season) season and the same sub-districts were used for downstream analysis.

**Ethical statement**

All aspects of this study were approved by the Institutional Review Board of the Noguchi Memorial Institute for Medical Research (NMIMR IRB; FWA Number: 00001824), Accra, Ghana (Study Number: 009/10-11). Details of the study were explained in local dialect to the legal parents or guardians of the participants and each signed a written consent form.

**SNP genotyping of drug resistance markers**

Genomic DNA was extracted from slide positive DBSs using QiaAmp DNA Blood Mini Kit (Qiagen GMBH, Hilden, Germany), following the manufacturer’s protocol and stored at -20°C before use. Asymmetrical PCR and high resolution melting (HRM) curve analysis were carried out to genotype alleles in \textit{pfcrt} (C72S, M74I, N75E, K76T), \textit{pfmdr1} (N86Y, Y184F, D1246Y), \textit{pfdhps} (S436F, A437G, K540E, A581G, A613S), \textit{pfhdfr} (N51I, C59R, S108N, I164L) and \textit{pfk13} C580Y that are associated with antimalarial drug resistance. All PCRs were performed in a final volume of 5μL containing 1X LightScanner Master mix (BioFire Defense, Salt Lake City, UT, USA), 0.5μM excess primer (reverse), 0.1μM limiting primer (forward), 0.4 μM 3’-blocked probe and 0.01–10 ng/μl template DNA. Previously reported primer sets and cycling conditions were used \cite{38,39}. Following PCR, the amplified products were analysed using HRM from 40°C to 90°C on the Roche LightCycler96 instrument (Roche Diagnostics, Germany). The LightCycler 96 SW 1.1 software was used for the analyses of melting profiles and allele calling based on the changes in fluorescence. Purified genomic DNA from laboratory-adapted \textit{P. falciparum} strains (3D7, Dd2, HB3, TM90, 1241) with known genotype profiles for wildtype and mutant alleles were used as standards to discriminate sensitive and resistant alleles in samples.

**Data interpretation**

Melting curves/peaks representative of each allele was used to ascertain the SNP genotype of each sample. For a sample containing both wildtype and mutant alleles for a particular SNP, the melting profile showed two distinct melting peaks/curves representative of each allele.

The \textit{pfcrt}-C72S, M74I, N75E, K76T and \textit{pfmdr1}-N86Y, Y184F, D1246Y SNPs were analysed individually and defined as either wildtype, “infection with no mutation detected” or mutant, “infection with mutation detected”. Similarly, the \textit{pfhdfr}-N51I, C59R, S108N, I164L; \textit{pfdhps}-S436F, A437G, K540E, A581G, A613S; and \textit{pfk13}-C580Y point mutations were also classified as wildtype or mutant.

Single gene haplotypes were categorized as: wildtype- “infection with no mutation detected” or either single, double, triple, quadruple, quintuple, sextuple and septuple mutants for infections with 1, 2, 3, 4, 5, 6 and 7 mutant alleles respectively. Thus \textit{pfcrt} (C72S, M74I, N75E, K76T) haplotypes were either wildtype or triple, whereas \textit{pfmdr1} (N86Y, Y184F, D1246Y) haplotypes were wildtype, single, double or triple. Similarly, \textit{pfhdfr} (N51I, C59R, S108N, I164L) haplotypes were defined as wildtype, single, double, triple or quadruple, whereas that of the \textit{pfdhps} (S436F, A437G, K540E, A581G, A613S) were categorized as wildtype, single, double, triple, quadruple or quintuple. Multi-gene haplotypes were constructed for \textit{pfcrt/pfmdr1} (C72S, M74I, N75E, K76T, N86Y, Y184F, D1246Y), \textit{pfhdfr/pfdhps} (N51I, C59R, S108N, A437G,
K540E, A581G) and pfcrtpfmdr1pfdhfrpfdhps (K76T, N86Y, N51I, C59R, S108N, A437G, K540E).

The prevalence of mutations, were determined as the proportion of samples carrying the target non-wildtype variant among the total number of successfully analysed samples. In estimating the prevalence of mutations and for haplotype analysis, all infections with mixed alleles (wildtype and mutant) were excluded.

Data analysis
The demographic data of study participants (proportion of males and females, mean age and mean parasitaemia) and prevalence of alleles in pfcrtpfcrtpfmdr1 (N86Y, Y184F, D1246Y), pfdhps (S436F, A437G, K540E, A613S), pfdhfr (N51I, C59R, S108N, I164L) and pfk13 C580Y were consolidated for each survey year. Differences in mean age and parasitaemia among the survey years were analysed using ANOVA. The Chi-square ($\chi^2$) and Fisher exact tests were used to determine the significance of observed differences in the proportion of sexes (male and female) and the prevalence of key drug resistance alleles. The trends in the prevalence of drug resistance alleles over the study period, from 2010 to 2014 were analysed using the Cuzick’s (nptrend) test. Statistical analysis was performed using STATA software package version 12 (College Station, TX: StataCorp LP. USA). P values <0.05 were considered to indicate statistical significance.

Results
Study population
The distribution of sex and age of study participants were comparable across the study period ($\chi^2 = 2.66, P = 0.616 and F = 0.09, P = 0.986, respectively$). The proportion of males and females were 53.8 and 46.2% for 2010, 47.5 and 52.5% for 2011, 42.5 and 57.5% for 2012, 51.2 and 48.8% for 2013, and 52.5 and 47.5% for 2014. The mean ages (in months) were 32.74, 33.56, 33.26, 32.64 and 32.20 for 2010 to 2014 respectively. The geometric mean parasitaemia (in parasites/μL) however differed significantly across the study period (6077, 15099, 2602, 4317 and 3563, F = 4.74, P = 0.001), with an overall decline of 58.6% (Table 1).

Genotyping success
For the 400 (80 each for 5 years) asymptomatic P. falciparum isolates selected from 2010 to 2014, the genotyping success ranged between 73.8% (59/80) to 93.8% (75/80) for pfcrtpfmdr1; 86.3% (69/80) to 97.5% (78/80) for pfmdr1; 85.0% (68/80) to 100% (80/80) for pfdhfr; 86.3% (69/80) to 98.8% (79/80) for pfdhps; 70.0% (56/80) to 91.3% (73/80) for pfk13 as shown in S1 Table. Samples that were not successfully genotyped for each of the five genes did not differ in gender ($P = 0.225$ to 0.728) and age ($P = 0.1439$ to 0.9924) compared to those that were successfully genotyped.

Table 1. Background characteristics of study participants in Bunkpurugu Yunyoo District across survey years.

| Characteristic          | 2010        | 2011        | 2012        | 2013        | 2014        | P-value |
|------------------------|-------------|-------------|-------------|-------------|-------------|---------|
| Gender [% (n/N)]       |             |             |             |             |             |         |
| Male                   | 53.8 (43/80) | 47.5 (38/80) | 42.5 (34/80) | 51.3 (41/80) | 52.5 (42/80) | 0.616   |
| Female                 | 46.2 (37/80) | 52.5 (42/80) | 57.5 (46/80) | 48.7 (39/80) | 47.5 (38/80) |         |
| Age [mean ± SD] (months) | 32.74 ± 16.56 | 33.56 ± 16.08 | 32.64 ± 16.43 | 32.64 ± 16.43 | 32.20 ± 15.61 | 0.986   |
| GMPD [Range] (parasites/μL) | 6077 [520–85960] | 15099 [800–294400] | 2602 [120–232673] | 4317 [197–489216] | 3563 [123–133714] | 0.001   |

GMPD, geometric mean parasite density (in parasites/μL). Statistical analysis performed using Pearson’s chi-square and ANOVA tests.
genotyped. The success of genotyping was independent of parasitaemia, as there was no significant difference in geometric mean parasitaemia between successfully genotyped and failed samples for most of the genes except for \( \text{pfcrt} \) and \( \text{pfk13} \) genes \( \text{pfcrt}:1318 \) and 6479 parasites/\( \mu \)L, \( \text{pfk13}:2091 \) and 6214 parasites/\( \mu \)L for nonsuccessful and successful respectively, \( F = 7.11, P = 0.008 \) and \( F = 5.95, P = 0.015 \)\].

**Prevalence and temporal trends of key \( \text{pfcrt}, \text{pfmdr1}, \text{pfdhfr}, \text{pfdhps}, \text{pfk13} \) drug resistant point mutations**

The prevalence of key chloroquine resistant alleles: \( \text{pfcrt}\cdot \text{T76} \) and \( \text{pfmdr1}\cdot \text{Y86} \) were lower than the wildtype alleles and fluctuated during the study period: 37.5% and 34.2% in 2010, 40.0% and 39.0% in 2011, 32.2% and 19.2% in 2012, 16.4% and 21.3% in 2013, and 23.1% and 21.7% in 2014, respectively \( (\chi^2 = 12.76, P = 0.013 \) and \( \chi^2 = 11.91, P = 0.018 \)\). There was a significant decline in the prevalence of these alleles from baseline, during and post-IRS intervention \( (z = -2.87, P = 0.004 \) and \( z = -2.65, P = 0.008 \) respectively) \[Fig 2\].

Similarly, the prevalence of \( \text{pfdhfr}\cdot \text{N108} \) and \( \text{pfdhps}\cdot \text{G437} \) mutations wavered during the period. For 2010, 2011, 2012, 2013 and 2014 these were 95.0% and 46.8%, 94.9% and 53.8%, 83.8% and 52.2%, 91.8% and 48.7%, and 85.5% and 49.8% respectively \( (\chi^2 = 9.37, P = 0.057 \) and \( \chi^2 = 1.42, P = 0.841 \)\). Only the pyrimethamine resistance mediating mutant \( \text{pfdhfr}\cdot \text{N108} \) showed a trend in decline in prevalence after the intervention \( (z = -2.03, P = 0.042 \)\) \[Fig 2\].

The core Kelch-13 artemisinin resistant associated allele \( \text{pfk13}\cdot \text{Y580} \) was not found throughout the survey years.

**Prevalence and temporal trends of other \( \text{pfcrt}, \text{pfmdr1}, \text{pfdhfr} \) and \( \text{pfdhps} \) drug resistant point mutations**

No mutant \( \text{pfcrt}\cdot \text{S72} \) was identified across the survey years, while \( \text{pfcrt}\cdot \text{I74} \) and \( \text{pfcrt}\cdot \text{E75} \) mutations were detected in 37.5% of the asymptomatic \( P. \) falciparum isolates surveyed for 2010,
The prevalence of the pyrimethamine resistance mediating polymorphisms in \textit{pfldhfr} gene was generally higher than the sulphadoxine resistance mediating polymorphisms in \textit{pfldhps}. There was a significant decline in the trend of \textit{pfldhfr}-151 and \textit{pfldhfr}-R59 after the intense IRS was rolled out in BY (z = -3.54, P < 0.001 and z = -4.63, P < 0.001, respectively). There was no \textit{pfldhfr}-L164 mutation found throughout the years of survey. The prevalence of the least abundant \textit{pfldhps}-F436 and G581 non-significantly increased from 6.3% to 9.7% (z = 0.41, P = 0.684) and 0.0% to 2.8% (z = 1.52, P = 0.129), respectively between baseline and post-intervention surveys. Within that same period, the least frequent \textit{pfldhps}-S613 mutant decreased non-significantly in prevalence from 29.1% in 2010 to 15.3% in 2014, (z = -1.86, P = 0.063).

The mutant \textit{pfldhps}-E540 was however absent across all time points. The trends in the prevalence of the \textit{pfcrt}, \textit{pfmdr1}, \textit{pfldhfr} and \textit{pfldhps} mutations are summarized in Table 2 below.

\textbf{Pfcr\textit{t} and \textit{pfmdr1} haplotype frequencies and trends across survey years}

To determine the effect of transmission reduction on the observed \textit{pfcrt} and \textit{pfmdr1} haplotype frequencies and their trends across survey years, combinations of alleles per isolate were considered as haplotypes across loci and frequencies compared across temporal populations. Overall, two unique \textit{pfcrt} haplotypes were detected across the study period and in each year of survey, with the wildtype C$_{72}$M$_{74}$N$_{75}$K$_{76}$ being the most abundant haplotype, ranging from 60.0–83.6%. The predominant African/South East Asian triple mutant, \textit{pfcrt}- C$_{72}$I$_{74}$E$_{75}$T$_{76}$ haplotype associated with chloroquine resistance showed a statistically significant trend in decline (z = -2.87, P = 0.004), between baseline and post-intervention. On the other hand, eight unique \textit{pfmdr1} haplotypes were found across the survey time points, with six in 2010 and 2011, seven in 2012, five in 2013 and 2014. The \textit{pfmdr1} single mutant N$_{86}$F$_{184}$D$_{1246}$ haplotype that is associated with low lumefantrine response persisted from baseline, during and post-IRS intervention and the frequency ranged from 50.0–66.7%. There was a decline in the frequency of the single and double \textit{pfmdr1} mutant haplotypes N$_{86}$F$_{184}$D$_{1246}$ and Y$_{86}$Y$_{184}$Y$_{1246}$ associated with lumefantrine and amodiaquine resistance, respectively (z = -2.86, P = 0.004 and z = -2.71, P = 0.007), with fluctuating frequencies between baseline and post-intervention surveys. The temporal trends in \textit{pfcrt} and \textit{pfmdr1} haplotype frequencies across survey years are presented in Fig 3.

\textbf{Pfldhfr and Pfldhps haplotype frequencies and trends across survey years}

Overall, five unique \textit{pfldhfr} haplotypes were found across all time points, with four in 2010, 2011, 2012 and 2014 and five in 2013. The most prevalent \textit{pfldhfr} mutant haplotype observed across all time points was I$_{51}$R$_{59}$N$_{108}$I$_{164}$, occurring at frequencies ranging from 44.8–73.3%. The frequency of the triple mutant I$_{51}$R$_{59}$N$_{108}$ associated with pyrimethamine resistance reduced significantly (z = -2.61, P = 0.009) between baseline and post-intervention surveys, during the peak transmission seasons in BY. Twelve unique \textit{pfldhps} haplotypes were found across time points, with six in 2010, seven in 2011 and eight in 2012, 2013 and 2014. The main mutant haplotype observed across all time points was S$_{436}$G$_{437}$K$_{540}$A$_{581}$A$_{613}$, with frequency between 26.6–38.5%. The sulphadoxine resistance-conferring double mutant G$_{437}$E$_{540}$ was absent across all time points, while the frequency of G$_{437}$G$_{581}$ also associated with sulphadoxine resistance showed no trend during the study period (z = 1.39, P = 0.165). The temporal trends in \textit{pfldhfr} and \textit{pfldhps} haplotype frequencies across survey years are presented in Fig 4.
Frequency of combined pfcrt and pfmdr1 haplotypes

The CQ and AQ resistant pfcrt/pfmdr1 haplotype, C72Y74E75T76Y84Y184Y1246 was observed only in two isolates (3%) in 2010 (pre-IRS) and one (1.5%) in 2011, while undetected in 2012, 2013 and 2014. Most isolates genotyped had the C72M74N75K76N84F184D1246 haplotype, associated with reduced response to lumefantrine (the partner drug in AL). This haplotype had frequencies of 39.4% in 2010, 36.4% in 2011, 48.3% in 2012, 54.1% in 2013 and 53.3% in 2014. There was however no consistent temporal trend in the haplotype frequency with the IRS intervention in BY (z = -1.32, P = 0.186).

Table 2. Trends in the prevalence of pfcrt, pfmdr1, pfdhfr and pfdhps alleles across survey years.

| Gene   | Mutation | Base change | Amino acid | 2010% (n/N) | 2011% (n/N) | 2012% (n/N) | 2013% (n/N) | 2014% (n/N) | P-value |
|--------|----------|-------------|------------|-------------|-------------|-------------|-------------|-------------|---------|
| Pfcrt  | C72S     | T           | C          | 100 (75/75) | 100 (73/73) | 100 (59/59) | 100 (70/70) | 100 (66/66) |         |
|        | A        | S           |            | 0.0 (0/75)  | 0.0 (0/73)  | 0.0 (0/59)  | 0.0 (0/70)  | 0.0 (0/66)  |         |
|        | M74I     | A           | M          | 62.5 (45/72)| 60.0 (42/70)| 67.8 (40/59)| 83.6 (56/67)| 76.9 (50/65)| 0.004   |
|        | G        | I           |            | 37.5 (27/72)| 40.0 (28/70)| 32.2 (19/59)| 16.4 (11/67)| 23.1 (15/65)|         |
|        | N75E     | T           | N          | 62.5 (45/72)| 60.0 (42/70)| 67.8 (40/59)| 83.6 (56/67)| 76.9 (50/65)| 0.004   |
|        | A        | E           |            | 37.5 (27/72)| 40.0 (28/70)| 32.2 (19/59)| 16.4 (11/67)| 23.1 (15/65)|         |
|        | K76T     | A           | K          | 62.5 (45/72)| 60.0 (42/70)| 67.8 (40/59)| 83.6 (56/67)| 76.9 (50/65)| 0.004   |
|        | C        | T           |            | 37.5 (27/72)| 40.0 (28/70)| 32.2 (19/59)| 16.4 (11/67)| 23.1 (15/65)|         |
| Pfmdr1 | N86Y     | A           | N          | 65.8 (48/73)| 61.0 (47/70)| 80.8 (59/73)| 78.3 (59/75)| 78.3 (59/73)| 0.008   |
|        | T        | Y           |            | 34.2 (25/73)| 39.0 (30/77)| 19.2 (14/73)| 21.3 (16/75)| 21.7 (15/75)|         |
|        | Y184F    | A           | Y          | 9.7 (7/72)  | 13.7 (10/73)| 13.9 (10/72)| 21.7 (15/69)| 29.8 (20/67)| 0.001   |
|        | T        | F           |            | 90.3 (65/72)| 86.3 (63/70)| 86.1 (62/70)| 78.3 (54/69)| 70.2 (47/67)|         |
|        | D1246Y   | G           | D          | 90.4 (66/73)| 94.9 (74/78)| 95.9 (70/73)| 96.0 (72/75)| 98.6 (68/69)| 0.035   |
|        | T        | Y           |            | 9.6 (7/72)  | 5.1 (4/78)  | 4.1 (3/73)  | 4.0 (3/75)  | 1.4 (1/69)  |         |
| Pfdhfr | N51I     | A           | N          | 32.5 (25/77)| 26.7 (20/75)| 44.8 (30/67)| 47.9 (34/71)| 55.2 (37/67)| <0.001  |
|        | C59R     | A           | C          | 67.5 (52/77)| 73.3 (55/75)| 55.2 (37/67)| 52.1 (37/71)| 44.8 (30/67)|         |
|        | S108N    | G           | S          | 5.0 (4/80)  | 5.1 (4/79)  | 16.2 (11/68)| 8.2 (6/73)  | 14.5 (10/69)| 0.042   |
|        | I164L    | A           | I          | 100 (80/80)| 100 (79/79)| 100 (68/68)| 100 (73/73)| 100 (69/69)|         |
|        | T        | L           |            | 0.0 (0/80)  | 0.0 (0/79)  | 0.0 (0/68)  | 0.0 (0/73)  | 0.0 (0/69)  |         |
| Pfdhps | S436F    | C           | S          | 93.7 (74/79)| 88.8 (69/78)| 92.8 (64/69)| 91.0 (71/78)| 90.3 (65/72)| 0.684   |
|        | T        | F           |            | 6.3 (5/79) | 11.6 (9/78) | 7.2 (5/69) | 9.0 (7/78) | 9.7 (7/72) |         |
|        | A343G    | C           | A          | 53.2 (42/79)| 46.2 (36/78)| 47.8 (33/67)| 51.3 (40/78)| 54.2 (39/72)| 0.715   |
|        | K540E    | A           | K          | 46.8 (37/79)| 53.8 (42/78)| 52.2 (36/69)| 48.7 (38/78)| 45.8 (33/72)|         |
|        | A581G    | C           | A          | 100 (79/79)| 100 (78/78)| 100 (69/69)| 100 (78/78)| 100 (72/72)|         |
|        | G        | G           |            | 0.0 (0/79)  | 0.0 (0/78)  | 0.0 (0/69)  | 0.0 (0/78)  | 0.0 (0/72)  |         |
|        | A613S    | G           | A          | 70.9 (56/79)| 87.2 (68/78)| 88.4 (61/69)| 84.6 (66/78)| 84.7 (61/72)| 0.063   |
|        | T        | S           |            | 29.1 (23/79)| 12.8 (10/78)| 11.6 (8/69) | 5.4 (12/78)| 15.3 (11/72)|         |
| Pfk13  | C580Y    | G           | C          | 100 (70/70)| 100 (65/65)| 100 (56/56)| 100 (68/66)| 100 (73/73)|         |
|        | A        | Y           |            | 0.0 (0/70)  | 0.0 (0/65)  | 0.0 (0/65)  | 0.0 (0/68)  | 0.0 (0/73)  |         |

Note. Wild type amino acids are depicted in normal font, while mutant amino acids are in bold and underlined. Statistical analysis performed using Cuzick’s test for trends.

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Fig 3. Trends in the frequency of pfcrt and pfmdr1 haplotypes at baseline, during and after intervention. (A) pfcrt. (B) pfmdr1. Statistical analysis performed using Cuzick’s test for trends.

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Fig 4. Trends in the frequency of pfdhfr and pfdhps haplotypes at baseline, during and after intervention. (A) pfdhfr. (B) pfdhps. Statistical analysis performed using Cuzick’s test for trends.

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Frequency of combined pfdhfr and pfdhps haplotypes

The frequency of combined pfdhfr and pfdhps haplotypes were also compared to determine the effect of transmission reduction on the synergy between the two genes in conferring resistance to SP after the IRS intervention in BY. The frequency of quadruple I\textsuperscript{51}R\textsuperscript{59}N\textsuperscript{108}G\textsuperscript{437} haplotype associated with partial SP resistance decreased overall across time points. It was prevalent at 29.0% in 2010, 36.5% in 2011, 19.7% in 2012, 26.8% in 2013 and 23.8% in 2014 although this decrease was not significant (z = -1.55, P = 0.120). No quintuple I\textsuperscript{51}R\textsuperscript{59}N\textsuperscript{108}G\textsuperscript{437}E\textsuperscript{540} or sextuple I\textsuperscript{51}R\textsuperscript{59}N\textsuperscript{108}G\textsuperscript{437}E\textsuperscript{540}G\textsuperscript{581} haplotypes associated with full and super resistance to SP respectively were found across survey years in the circulating BY asymptomatic P. falciparum.

Frequency of multilocus pfcr\textsubscript{T}, pfmdr\textsubscript{1}, pfdhfr and pfdhps haplotypes

The linkage between CQ and SP resistant alleles within isolates between 2010 and 2014 in BY (pre-IRS and endline: post-IRS) were assessed based on combined pfcr\textsubscript{T}-T76, pfmdr\textsubscript{1}-Y86, pfdhfr-I51, R59 and N108 and pfdhps-G437, E540 alleles. Only isolates that were successfully genotyped for all seven markers were included. In all, 30 distinct haplotypes were detected across all time points, with 20 haplotypes in 2010, while 2011, 2012, 2013 and 2014 had 18, 17, 16 and 16 distinct haplotypes, respectively. No septuple mutant haplotypes were detected during the study period, whereas only 1 sextuple haplotype was observed. The number of quintuple, quadruple, triple and double-mutant haplotypes detected over time were 4, 7, 9 and 6, respectively. The highest frequency of all sextuple haplotypes in a particular year was 7.6% (5/66) and this was in 2011; quintuple was 21.8% (14/64) in 2010; quadruple, 33.3% (22/66) in 2011. Similarly, the frequency of the triple and double mutant haplotypes were highest in 2012 and 2013 with values of 40.7% (22/54) and 18.6% (11/59), respectively. Of the most abundant triple mutant haplotypes in a particular year [40.7% (22/54)], majority [77.3% (17/22)] were made up of the pyrimethamine resistance-associated haplotype I\textsuperscript{51}R\textsuperscript{59}N\textsuperscript{108} and so was the most frequent quadruple haplotypes [33.3% (22/66)], which also had [59.1% (13/22)] carrying the partial SP resistance-conferring haplotype, I\textsuperscript{51}R\textsuperscript{59}N\textsuperscript{108}G\textsuperscript{437}. The frequency of the haplotype constructs observed during the study period is shown in S2 Table.

Discussion

In the light of the emergence of artemisinin resistance in West Cambodia and spread in parts of South-East Asia, surveillance of antimalarial drug resistance in sub-Saharan Africa is important for early detection and containment of emerging resistant strains. Although vector control interventions do not directly shape the malaria parasite population, there remains a large and persistent but mostly neglected asymptomatic P. falciparum reservoir after these interventions reduce transmission. This circulating reservoir is dominated by infection in partially immune older children and adults [40,41]. Again, although drug pressure is the major driving force for the evolution and or spread of drug resistant parasites, and is often greater in symptomatic infections than in asymptomatic cases [42], the asymptomatic reservoir drives transmission after the interventions and therefore plays a role in the evolution and/or spread of antimalarial resistance.

Our study focused on asymptomatic infections that existed in children under five years in an area of seasonal transmission in Ghana where chloroquine was withdrawn and ACTs implemented in 2005. IPTp coverage with SP during the time period ranged between 54.1–70.7% (IPTp1), 38.7–58.3% (IPTp2), 24.6–41.7% (IPTp3) [43], and an IRS intervention was rolled out in 2011 resulting in a reduction in malaria transmission. By utilizing a repeated cross-sectional study design, we analyzed the prevalence and their trends, if any, in the key polymorphisms of the genes that mediate or augment antimalarial resistance (pfcr\textsubscript{T}, pfmdr1,
pf dhfr, pf dhps, and pf k13). The study was conducted at the end of the rainy seasons in 2010 (pre-IRS) through to 2014 (endline: post-IRS) in BY District, Ghana and thus, the results reflect the trends in the evolution of drug resistance during the peak transmission seasons. In addition, we analyzed the frequencies of P. falciparum haplotypes and linked genotypes to determine if there were combinations of genotypes or haplotypes that predominated the area after the IRS rollout that could be used to predict the emergence and/or spread of multidrug resistance in this reservoir. Albeit our study design is limited by the lack of samples from the older children and adults who contribute mostly to the reservoir of infection, children under 5 years old largely contribute to drug resistance as a result of the drug pressure exerted on them. As drug resistance in the asymptomatic group is much lower in comparison with the symptomatic groups [42], children under 5 will be the major source of transmissible resistant parasites especially when transmission intensity has reduced as a result of an enhanced intervention such as IRS as seen in the BY district of Ghana.

In general, there were fluctuations in the prevalence of all the alleles tested and this corroborates with previous studies conducted in Sudan and The Gambia that showed that the prevalence of drug-resistant parasites fluctuate in areas with seasonal malaria transmission [44,45]. Some of the fluctuations nonetheless showed trends over the five-year study period.

Our surveys of the asymptomatic P. falciparum reservoir in BY observed a decline in the prevalence of key mutants pfcr-T76 and pfmdr1-Y86 that mediates CQ resistance [30,46,47] and an increase in the wildtype alleles, following the withdrawal of CQ and IRS rollout. These findings are consistent with observations made in children with clinical infections from Ghana, particularly in the same region [48], and in the Brong Ahafo Region in 2012/2013 [49]. Similarly, other African studies observed a resurgence of the CQ-sensitive alleles following the withdrawal of the drug [50–55]. The return of CQ sensitive parasites may be due to a combination of factors. These include, the fitness cost on the resistant strains in the absence of the drug pressure, the selection of wild-type alleles at both pfcr and pfmdr1 by the ACT partner drug, lumefantrine, as opposed to the 4-aminoquinolines (CQ and amodiaquine (AQ)) [56–59]. In addition, AQ in ASAQ is still maintaining a sustained pressure on the parasite population in BY as well [60] by selecting for mutant pfcr and pfmdr1 alleles in circulation. Also there have been sporadic anecdotal evidence of continued private sector sale and use of chloroquine after its removal, however our study was not designed to capture that data.

Drug pressure due to LM and MQ have been associated with the selection of pfcr-K76, pfmdr1-N86, F184 and D1246 [61–63]. On the other hand, the selection of pfcr-T76, pfmdr1-Y86, Y184 and Y1246 have been attributed to decreased AQ sensitivity [64–66]. A trend in lower prevalence of pfcr-T76 and pfmdr1-Y86, Y184 and Y1246 as compared to pfcr-K76 and pfmdr1-N86,F184,D1246 were observed in BY with the enhanced IRS rollout over the study period. This is consistent with prevalence (35–53% and 4–6%, respectively) recorded in clinical settings in Ghana, between 2003 to 2010 [48]. These findings suggest that LM resistant parasites are evolving faster than AQ resistance parasites in Ghana. This is not surprising because, since its introduction in 2007 as an additional first-line treatment for uncomplicated malaria for patients who adversely reacted to ASAQ, [67], AL has become the preferred ACT of the two [68,69].

Contrary to previous studies in Ghana (with no IRS intervention) where the pfmdr1-N86,F184,D1246 associated with LM resistance maintained a high prevalence over time [70] or saw a trend in increasing prevalence [48], we observed a trend in the reduction of pfmdr1-N86,F184,D1246 and pfmdr1-Y86,Y184,Y1246 frequencies, although most parasites isolates carried pfmdr1-N86,F184,D1246 than the pfmdr1-Y86,Y184,Y1246. Our findings indicate that with the IRS intervention that culminated in a reduction in malaria transmission in BY, there was a delay in the selection of mutant alleles associated with LM and AQ treatment failure in the presence of
the drug pressures. This could be explained by reductions in: the population size of the mosquito vectors, circulating mutant parasites [17], the number of parasite clones per host and the level of drug use in the population [17,23] as transmission intensity reduced in BY district.

Multidrug resistance has predominated South East Asia where numerous strategies for *P. falciparum* elimination have been implemented [71–77]. This is because inbreeding in the clonal parasite populations has prevailed and drug resistant gene combinations in the population have become stable and rapidly increase in frequency [20,25]. We observed only one sextuple mutant parasite and no septuple, an indication that there was very little linkage between mutant alleles associated with the ACT partner drugs and anti-folate drugs currently deployed, and no immediate threat of multidrug resistance in BY nine years after ACTs were deployed as first line treatment of uncomplicated malaria and the enhanced IRS rollout in 2014.

The observed higher prevalence of *pfdfhfr* mutations as compared to the *pfldhps* in this study is not surprising, as the mechanism of SP resistance in *P. falciparum* is a continuous stepwise process occurring first in *pfdfhfr* gene, after which *pfldhps* mutation is next selected for, but only when most parasite population carry at least a double but usually triple *pfdfhfr* mutant alleles [34,78,79]. We observed a high prevalence of S108N (83.8% - 95.0%), C59R (49.3% - 81.3%) and N51I (44.8% - 73.3%) followed by the A437G (46.2% - 54.2%) mutations, and eventually A613S, S436F and A581G were implicated. These findings are consistent with results reported in other settings in Africa [53,80–83]. Similar to the report by Mockenhaupt and colleagues, we detected *pfldhps*-G581 and S613 among parasites from the same region in Ghana (Northern Region) [84]. Like studies conducted in other regions of Ghana, Kenya and Senegal [53,85,86], we have reported the F436 in the BY district after the intense IRS rollout. This thus emphasizes the need for continuous monitoring of these previously rare markers for timely response, as they have been shown to confer high level resistance to SP treatment [87].

This study also found *pfdfhfr* - I51R,N108I,A164 (44.8–73.3%) and *pfldhps*-S436G,A437K,E540A,S581A,A613 (26.6–38.5%) as the most prevalent SP resistant haplotypes across all time points, and this corroborates with finding from Cameroon and Gabon [88,89]. Ultimately, the absence of SP super resistant parasites bearing the quintuple mutants I51R,N108I,A164,E540A haplotype as a consequence of the absence of *pfldhps*-E540 gives strong justification for the continued use of SP for IPTp in Ghana.

Furthermore, the critical *pfk13* gene mutation C580Y, which is associated with delayed clearance of ART in Southeast Asia [35,90–92], was not detected across any of the time points. Similarly, the other 8 validated Southeast Asian K13 alleles associated with ART resistance (F446I, N458Y/I, M476I/V, Y493H, R539T, I543T, P553L, R561H), are also rare in Africa [93,94]. The African K13 mutant alleles (including G449A/D, V520A, S522C, C542Y, G544R, G545E, A557S, R561C, A578S, A617V/T, V637A/I/D, G638R) either do not show evidence of selection in the parasite population or have not yet been associated with clinical artemisinin resistance [37,90,94,95]. This thus corroborates with findings that resistance to ART has not been established in Africa [95–97]. Continuous surveillance on the continent is however essential for early detection of resistance to ART.

A limitation to the current study is that, our findings are from one geographical site with a common transmission setting. However, to truly appreciate how transmission intensity changes influence drug resistance, it would be prudent to have obtained and compared data from different geographical areas of varying transmission intensities (preferably from low, moderate and high transmission areas) to that of data from intervention studies like the present study and to have allowed more time to elapse.
Conclusion

In summary, the validated K13 resistance mutation Y580, was not detected in the study district. The low prevalence of SP super-resistant parasites bearing the quintuple mutants I51, R59, N108, G437, E540 haplotype supports the continued use of SP for IPTp in Ghana. This study demonstrated that the use of effective drug treatment policy, coupled with reduction in transmission intensity as a result of vector control (IRS scale-up) led to the reduction in the prevalence of drug resistant markers associated with ACT partner drugs: AQ and LM. Further empirical evidence from different geographical settings of varying transmission intensities and in older children and adults is needed to enhance the understanding of the effect of transmission reduction on the evolution of drug resistance in asymptomatic populations.

Supporting information

S1 Table. Alleles proportions across survey years.
(DOCX)

S2 Table. Multilocus pfcr1, pfmdr1, pf dhfr and pf dhps haplotypes across survey years.
(DOCX)

S1 Data.
(XLS)

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