THERAPEUTIC ADVANCES in
*Infectious Disease*

*Original Research*

# Profiling antimalarial drug-resistant haplotypes in *Pfcrt*, *Pfmdr*1, *Pfdhps* and *Pfdhfr* genes in *Plasmodium falciparum* causing malaria in the Central Region of Ghana: a multicentre cross-sectional study

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*Ther Adv Infect Dis*

2025, Vol. 12: 1–13

DOI: 10.1177/20499361251319665



***Abstract***

**Background:** The proliferation of *Plasmodium* parasites resistant to antimalarial drugs poses a serious threat to human life and remains an obstacle to managing and eradicating *Plasmodium falciparum*. The surveillance of molecular markers has become necessary to monitor the spread of resistant haplotypes and discover emerging mutations.

**Objective:** This molecular epidemiological study aimed to evaluate the prevalence of known mutations in the drug resistance genes *Pfcrt*, *Pfmdr*1, *Pfdhfr* and *Pfdhps* in the Central Region of Ghana.

**Design:** A multi-centre cross-sectional study.

**Methods:** This prospective study utilised dried blood spots from individuals with *P. falciparum-infection* from five districts in the Central Region of Ghana. Selective Whole Genome Amplification (sWGA) and Single Nucleotide Polymorphisms (SNPs) in *P. falciparum* chloroquine transporter genes (*Pfcrt*), *P. falciparum* multidrug resistance 1 (*Pfmdr*1), *P. falciparum* dihydropteroate synthase (*Pfdhps*) and *P. falciparum* dihydrofolate reductase (*Pfdhfr*) were analysed.

**Results:** Whole genome sequencing was carried out on 522 samples. Of these, 409 (78%) samples were successfully sequenced. Six (6) of the sequenced samples were of co-infection of other parasite species with *P. falciparum* and excluded from the analysis. Analysis of the *Pfcrt* gene revealed 0.5% were CVIET (C72, V73, M74I, N75E, K76T) while the *Pfcrt* CVMNK (C72, V73, M74, N75, K76) wild-type haplotypes were 97% with (2.5%) (CV[M/I][N/E][K/T]) being mixed haplotypes. In the *Pfmdr*1 gene, monoclonal haplotypes; NFD (N86, Y184F, D1246) and YFN (N86Y, Y184F, D1246N) occurred at 44% and 9.8%, respectively, whereas mixed- haplotypes (N[Y/F]D and [N/Y][Y/F]D) were 23.5% and 0.3%, respectively. Combined *Pfdhfr*/*Pfdhps* genes yielded about 88% *Pfdhfr* IRNI (N51I, C59R, S108N, I164) + *Pfdhps* A437G haplotypes (conferring partial resistance to Sulphadoxine-Pyrimethamine (SP)) while 9% of the parasites had *Pfhdfr* IRNI + *Pfdhps* A437G + K540E haplotypes (conferring full resistance to SP). The wild-type haplotype, *Pfdhfr* (N51, C59, S108, I164) and *Pfdhps* (S436, A437, K540, A581, A613) was not observed.

**Conclusion:** The findings show a low prevalence of CVIET and relatively higher rates for *Pfmdr*1 NFD and parasites with *Pfdhfr* IRNI (N51I, C59R, S108N, I164) + *Pfdhps* A437G haplotypes. These observations advocate for enhanced surveillance which is inimical to malaria management in an endemic area.

***Keywords:*** Central Region, Ghana, *P. falciparum* chloroquine transporter (*Pfcrt*), *P. falciparum* dihydrofolate reductase (*Pfdhfr*), *P. falciparum* dihydropteroate synthase (*Pfdhps*), *P. falciparum* multidrug resistance 1 (*Pfmdr*1)

Received: 23 April 2024; revised manuscript accepted: 20 January 2025.

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## Introduction

Resistance of *Plasmodium* parasites to chloroquine and fansidar (Sulphadoxine-Pyrimethamine (SP)) antimalarial medications has hampered malaria case treatment, leading to a global switch to artemisinin-based combination therapy (ACT) for the treatment of uncomplicated *falciparum* malaria.[1] In Sub-Saharan Africa, chloroquine and fansidar (SP) resistance were reported to have been imported from Southeast Asia.[2]

Currently, Artemether–lumefantrine (AL) and artesunate–amodiaquine (AS-AQ) are the most widely utilized ACTs in all African countries including Ghana.[3,4] Interestingly, in vitro and ex vivo susceptibility assays have documented elsewhere that these two drugs appear to exert conflicting selection pressures on single nucleotide polymorphisms (SNPs) in the *Plasmodium falciparum* multidrug resistance transporter 1 (*Pfmdr*1) gene.[5,6]

Genomic surveillance provides a rapid and efficient monitoring tool for studying drug resistance patterns. Through genetic surveillance, the K76T mutation in the chloroquine (CQ) transporter of *P. falciparum* (*Pfcrt*) was found to be associated with CQ resistance, while single nucleotide polymorphisms in the *Pfmdr*1 genes select for drug resistance of ACT partner drug resistance.[7–9]

In Africa, SNPs in codons N86, Y184 and D1246 of *Pfmdr*1 are the most prevalent, and various haplotype combinations result in decreased sensitivity to multiple drugs. For instance, AQ alone and AS-AQ have been shown to select for *Pfmdr*1 86Y, Y184 and 1246Y (the YYY haplotype) is associated with parasite recrudescence and reinfection following reduced sensitivity to amodiaquine while the AL combination selects for N86, 184F and D1246 (the NFD haplotype) associated with an increased tolerance of lumefantrine.[10,11] There are reports of other *Pfmdr*1 mutations, such as S1034C and N1042D, elsewhere but infrequent in Africa.[9,12]

Single nucleotide polymorphisms in the *P. falciparum* dihydropteroate synthase (*Pfdhps*) and *P. falciparum* dihydrofolate reductase (*Pfdhfr*) genes have been shown to cause resistance to the combination of SP drugs.[13] Key factors in the development of resistance to pyrimethamine in vitro are specific amino acid alterations at codon 108 (S108N), 51(N51I) and/or 59(C59R) and 164(I164L) on the *Pfdhfr* gene.[13–16] Regarding the *Pfdhps* gene, point mutations S436A/F, A437G, K540E, A581G and A613T/S provide resistance to Sulphadoxine in vitro.[17] Novel mutations such as K540T and K540N have been reported in *Pfdhps* in Indonesia, Cameroon, and India.[14] Even though K540T has not been explicitly implicated in resistance to Sulphadoxine,[14] K540N has previously been associated with lower levels of sulpha drug resistance.[18] Combinations of several mutations in both *Pfdhps* and *Pfdhfr* are used to distinguish between different levels of SP resistance.[19,20] Here, we use the nomenclature partial, complete/full and super resistance to describe the three levels of resistance, which are represented by *Pfdhfr* codons 51, 59, and 108, plus *Pfdhps* codons 437), (*Pfdhfr* codons 51, 59, and 108, plus *Pfdhps* codons 437 and 540) and (*Pfdhfr* codons 51, 59, and 108 and *Pfdhps* A437G K540E A581G) respectively.[20,21] The observed resistance in A437G is enhanced by the mutations *Pfdhps* K540E, *Pfdhps* A581G and *Pfdhps* A613S.[20]

In Ghana, malaria still persists in the southern (coastal) and middle (forest) belts and remains the leading cause of morbidity and mortality.[22,23] Studies on the molecular markers of resistance showed an increasing trend in the prevalence of haplotype *Pfmdr*1 N86-F184-D1246 from 2003 to 2010.[24] In another study in the Western region of Ghana, frequencies of the point mutations implicated in the emergence of antifolate resistance were reported to be very high at codons 108, 59 and 51 on the *Pfdhfr* gene and moderate prevalence at codons 540 and 437 on the *Pfdhps* gene. However, a synergistic analysis of mutations in both genes yielded about 13.0%, quadruple mutations in the sample set at codons I51, R59, N108 and G437 (IRNG) and 3.7% at codons I51, R59 N108 and E540 (IRNE).[13] Similarly, the percentages of the *Pfdhfr* 51I, 59R, 108N and *Pfdhps* 437G mutant alleles reported in selected regions in Ghana were reported to be high in several other studies.[25,26] In the central region, the percentage prevalence of mutations in *Pfcrt* and *pfmdr*1 stood at 29% for chloroquine resistance mutations in 2017, Cape Coast[27] and 66.36% prevalence of *Pfcrt* K76 in 2021.[8] Reports on the high prevalence ofY184F of the *Pfmdr*1 gene was also reported in 2017.[28]

Considering the high prevalence of malaria in the Central Region of Ghana[23,29] amidst all interventions against the disease as well as the paucity of genetic data on the spread of resistance to current chemotherapy in Ghana, it is imperative to

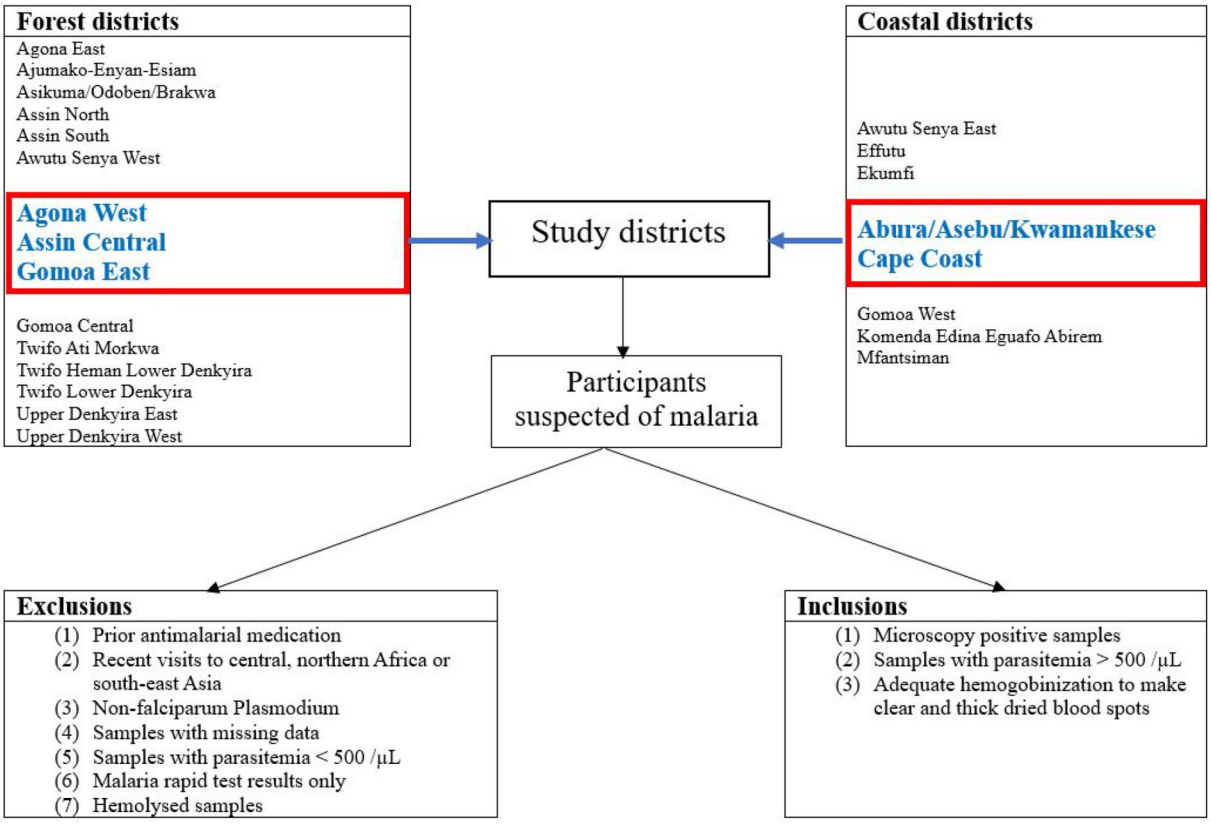


**Figure 1.** Flow chart for participant recruitment and sample selection for genotyping.
RDT- Rapid Diagnostic Tests.

determine the prevalence of known drug-resistance-related point mutations in *Pfcrt*, *pfmdr*1, *Pfdhfr* and *Pfdhps* in clinical isolates from the central region.

This study is meant to unearth the rate of parasites resistant circulating in the study sites and add to the *P. falciparum* genetic data reported within the region and throughout the country aiding in policy direction.

## Materials and methods

### *Selection of study areas and sampling period*

This multicentre cross-sectional study was done in Agona West (AW), Assin Central (AC), Gomoa East (GE), Abura-Asebu-Kwamankese (AAK), and Cape Coast (CC). The optimum allocation method was used to choose three districts at random from the wooded districts (AW, AC, and GE) and two districts from the coastal districts (AAK and CC). From these districts, study participants with malaria (RDT positive) were concurrently recruited from the respective district hospitals (Figure 1). The study participants were randomly selected by systematically selecting patients with malaria on each clinic day. Study participants were recruited from September 2020 to February 2021.

### *Study design*

This was a cross-sectional study conducted in the Central Region of Ghana. Malaria samples were obtained from study participants residing in randomly chosen districts within the region.

### *Criteria for study population selection*

In all districts, participants who started oral or parenteral antimalarial medication before receiving microscopy results were excluded. Furthermore, research participants who had ever left Ghana, to countries in Central and Northern Africa or South-East Asia, were equally excluded (Figure 1). Lastly,

the inclusion of laboratory-confirmed *Plasmodium* infection along with the participant and parental consent was crucial.

### *Sample size determination and blood sample sampling procedure*

According to our previous research, 38.2% of cases of malaria were confirmed by microscopy out of 2495 (62.5%) suspected malaria cases.[23] Thus, using Cochrane's formula; $n = z^2p(1-p)/d^2$, where $n$ = sample size, $z$ = confidence level at 95% (standard value of 1.96), $d$ = error margin at 5% (standard value of 0.05), a minimum sample size of 363 was reached calculated, setting the power of the study at 80%. At the end, 1525 participants were recruited. The large study participants used in this study were to cater for false negative microscopy results, non-*falciparum Plasmodium*, missing samples, samples with missing data and samples with very low parasitaemia (<500 parasites/μL) which do not yield enough genomic DNA for sequencing. At the end, 522 samples were eligible for sequencing.

### *Criteria for selecting samples for genotyping*

The criteria for selecting a sample for genotyping were parasitaemia > 500 parasites/μL. In addition, samples must not be haemolysed. Further, samples should be haemoglobinated enough to produce clear and thick blood spots (Figure 1).

### *Laboratory procedures*

*Blood sample collection, malaria screening and preparation of dried blood spots.* Blood samples were taken from study participants suspected of malaria. Four millilitres of whole blood were drawn into an Ethylenediaminetetraacetic acid (EDTA) tube and mixed uniformly. Initially, CareStart mRDT (Access Bio, Somerset, USA) was used to test the samples to identify malaria-positive cases of malaria parasites. Parasitaemia was determined as earlier published.[30,31] In summary, 6 μL of whole blood was used to prepare thick blood films, air dried, stained with 10% Giemsa for 10 min, and examined using the light microscope. Four dried blood spots were made from the detectable microscopy samples according to the Malaria Genome Laboratory protocols of the Wellcome Sanger Institute (WSI).

Plasmodium *DNA extraction and species identification.* Selective whole genome amplification (sWGA) was employed in this study to enrich to preferentially amplify *P. falciparum* DNA over human DNA background as described in Oyola et al.[32] A BDS 600PLUS robotic puncher (Microelectronic System, Brendale, Australia) punched 6–8 dried blood spots (DBS) into Eppendorf™ Deepwell™ plate of 96 wells (Fisher Scientific, UK). *P. falciparum* genomic DNA was extracted from DBS using the QIAamp DNA Investigator Kit (Qiagen, CA, USA) following the kit manufacturer's instructions. At least 5 ng of DNA were obtained per sample, for whole genome sequencing.

*Library preparation and sequencing of the genome of* P. falciparum. The reaction mix consisted of the following: template DNA, 1 × bovine serum albumin, 1 mM dNTP, 2.5 μM of each amplification primer, (primers used for this study are available at https://www.malariagen.net/wp-content/uploads/2023/10/GbS01_Tag_plate_preparation.pdf) 1 × Phi29 reaction buffer, and 30 units of Phi29 polymerase enzyme (New England Biolabs). Isothermal amplification conditions (35°C for 5 min, 34°C for 10 min, 33°C for 15 min, 32°C for 20 min, 31°C for 30 min, 30°C for 16 h before denaturing Phi29 polymerase enzyme at 65°C. After being cleansed with 200 μL of 80% ethanol, the purified XP amplicons were eluted using 50 μL of elution buffer. DNA libraries were created using New England Biolabs' NEBNext® Ultra™ DNA library preparation kit (New England Biolabs) before being sequenced on an Illumina HiSeq 2500 DNA sequencer. This protocol has been published.[32,33]

*Genetic analysis of gene alleles.* Standard Illumina QC was applied to the sequence data derived from every sample and each dataset was independently analysed by mapping sequence reads to the 3D7 reference genome using Burrows-Wheeler Aligner.[32] Prior to that, Torrent Suite pipeline software was used to de-multiplex and filter the raw reads using conventional quality filtering parameters. Read quality was assessed using the Torrent Suite FastQC plugin v0.10.1, and high - quality reads were aligned to the reference genome.[34] In the analysis of the genomic data, only successfully sequenced data obtained from each of the sample was analysed for *Pfcrt*, *Pfdhfr*, *Pfdhps*, *Pfmdr1* genes.

**Table 1.** Distribution of successfully sequenced genes by districts.

| District | *Pfdhps* | *Pfcrt* | *Pfdhfr* | *Pfmdr*1 |
|---|---|---|---|---|
| Abura-Asebu-Kwamankese | 39 (12.4) | 57 (14.1) | 48 (13.4) | 44 (13.1) |
| Agona Swedru Municipality | 21 (6.7) | 29 (7.2) | 24 (6.7) | 27 (8.0) |
| Cape Coast Metropolis | 61 (19.4) | 73 (18.1) | 67 (18.7) | 61 (18.2) |
| Assin Central Municipality | 128 (40.8) | 154 (38.2) | 139 (38.7) | 132 (39.3) |
| Gomoa East District | 65 (20.7) | 90 (22.3) | 81 (22.6) | 72 (21.4) |
| Total | 314 (77.9) | 403 (100) | 359 (89.1) | 336 (83.4) |

Source: Authors analysis.
Data are presented as numbers and proportions.
*n*, number of genes.

### *Data processing and statistical analysis*

The data was organised in Microsoft Excel 2016 and imported into SPSS Version 24 software (Chicago, IL, USA) for analysis. Descriptive statistics (numbers and corresponding percentages) were carried out.

### *Ethics approval and consent to participate*

This study received ethical approval from Ghana Health Service Ethics Review Committee (GHS-ERC Number: GHSERC017/03/20). In addition, the parent or legal guardian of each child provided a written informed consent and a child assent form. Written self-consent was obtained from study participants 18 years and over.

## Results

### *Sequencing success rates*

Out of 522 samples, 409 (78%) were successfully sequenced. Successful sequencing was where the entire amino acids denoting *Pfdhps*, *Pfcrt*, *Pfdhfr*, and *Pfmdr*1 haplotypes were present. Of the 409 samples that successfully sequenced parasites, six (6) were excluded from the analysis due to co-infection of other parasite species with *P. falciparum*. Therefore, analysis described in this publication is based on the successful sequencing of SNPs within 403 *Pfcrt*, *Pfmdr*1, *Pfdhps* and *Pfdhfr* genes. In this section, putative markers of antimalarial drug resistance focused on amino acid changes in *Pfcrt* M74, N75, K76; *Pfdhfr* N51, C59, S108 and I164; *Pfdhps* S436, A437, K540, A581 and A613; *Pfmdr*1 N86, Y184 and D1246. Different amino acid polymorphisms in the same genetic loci made it possible to find that 47 different parasite clones were circulating in the study sites. Table 1 Shows the distribution of successfully sequenced genes by districts.

### *Distribution of* Pfcrt *gene mutations*

The *Pfcrt* SNPs were successfully sequenced in 403 samples. Of this number, 391/403 (97%) had the wild-type CVMNK (C72, V73, M74, N75, K76) haplotype, 2/403 (0.5%) were of CVIET (C72, V73, M74I, N75E, K76T) haplotype, while the rest 10/403 (2.5%) had mixed haplotypes /infections (CV[M/I][N/E][K/T]). No haplotype of SVMNT was found.

### *Prevalence of mutant haplotypes in* Pfmdr*1 genes*

Regarding the *Pfmdr*1 gene, 336 parasites were successfully sequenced at all three loci, namely, amino acid positions N86, Y184 and D1246. Of the number of parasites successfully sequenced, 75 (22.3%) were of the NYD (N86, Y184, D1246) wild type, while the rest had mutations. The majority of the parasites (44.05%) had a single mutation NFD (N86, Y184F, D1246) haplotype while the YFN (N86Y, Y184F, D1246N) triple mutation was 9.8% (33/336). The rest (23.8%) were mixed haplotypes (N[Y/F]D and [N/Y][Y/F]D) (Figure 2).

### *Prevalence of* Pfdhfr *mutations*

Regarding the *Pfdhfr* gene, 359 parasites were successfully sequenced for all four amino acids that make up the *Pfdhfr* haplotype. The amino

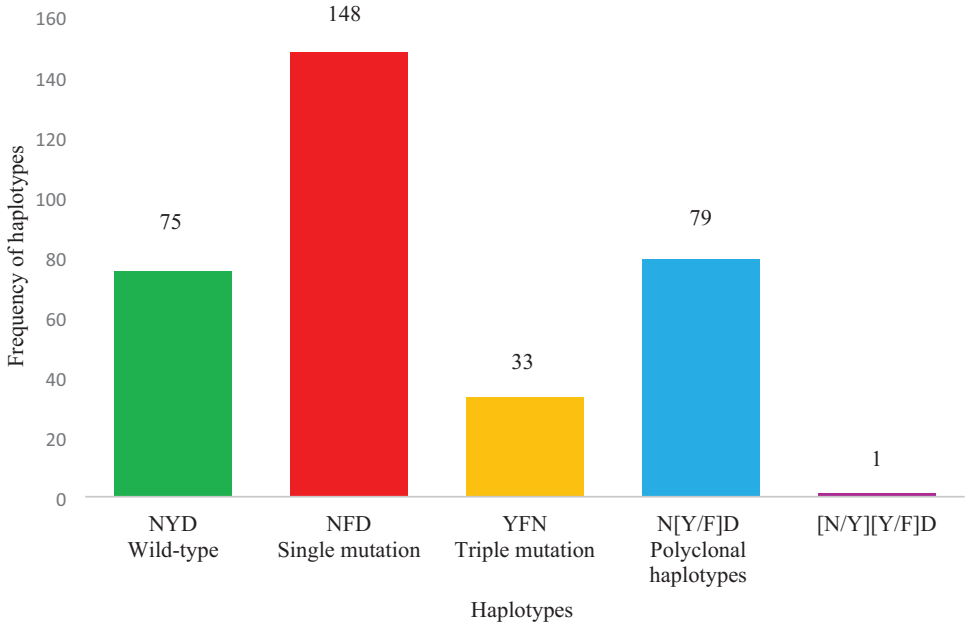


**Figure 2.** Distribution of the *Pfmdr*1 gene haplotypes at amino acid positions N86, Y184, D1246.

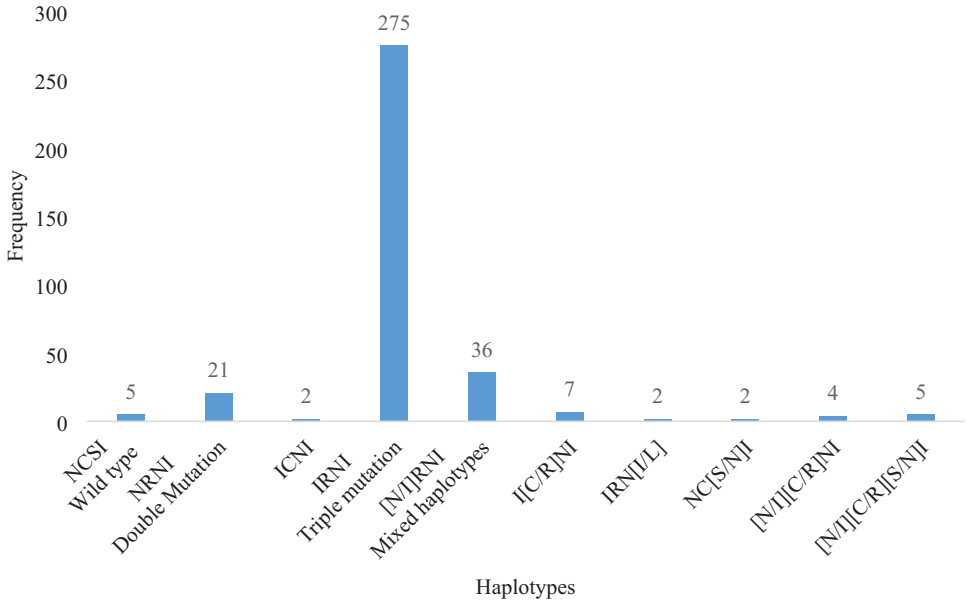


**Figure 3.** Distribution of point mutations in the *P. falciparum* dihydrofolate reductase (*Pfdhfr*) gene. The amino acid positions for the haplotypes are N51, C59, S108, I164.

acids are located at positions 51, 59, 108 and 164. Some parasites (1.4%, 5/359) had the wild-type haplotype (N51, C59, S108 and I164). The rest (98.6%) had various mutations. The triple mutant, IRNI, was in the majority (76.6%, 275/359). The dominant double mutant haplotype was NRNI (N51, C59R, S108N, I164) at a rate of 5.8% (21/359). Figure 3 contains the other mutant haplotypes in mixed haplotype infections.

### *Prevalence of* Pfdhps *mutations*

The haplotype for the *P. falciparum* dihydropteroate synthase gene (*Pfdhps*) has five amino acids

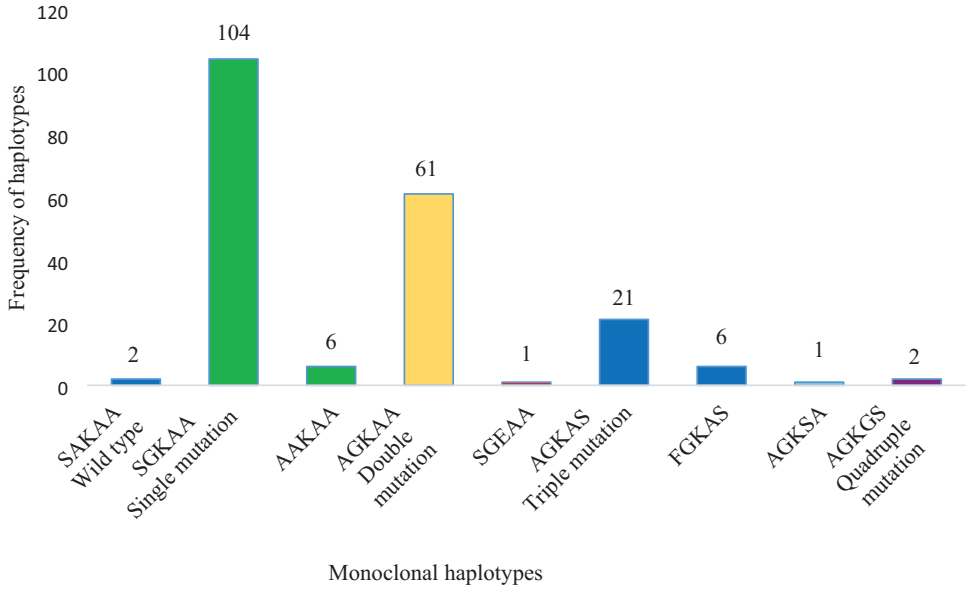


**Figure 4.** Distribution of monoclonal haplotypes in the *P. falciparum* dihydropteroate synthase gene (*Pfdhps*) at amino acid positions S436, A437, K540, A581 and A613.

at positions 436, 437, 540, 581 and 613. Of the total parasites sequenced, 314 were fully sequenced for all five amino acids. The wild-type *Pfdhps* haplotype is represented as S436, A437, K540, A581, and A613, denoted as SAKAA haplotype. The most prevalent haplotype was SGKAA (A437G) at a rate of 32.8% (104/314). The dominant double and triple mutant haplotypes AGKAA and AGKAS are prevalent in 19.4% (61/314) and 6.7% (21/314). The only quadruple mutant haplotype was AGKGS with a prevalence of 0.6% (2/314) (Figure 4). Figure 5 presents the distributions of mutant haplotypes in mixed haplotype infections.

### *Distribution of* the Pfdhfr/Pfdhps *haplotypes*

Analysing SP resistance, 294 parasites had both *Pfdhfr*/*Pfdhps* genes fully sequenced. In these combined genes, the wild-type haplotype (51N-59C-108S 164I-436S-437A-540K-581A-613A) was not identified. In monoclonal infections, the dominant *mutations of the Pfdhfr*/*Pfdhps* gene were the quadruple mutation – N51I-C59R-S108N- 164I-436S-A437G- 540K-581A-613A (27.9%) associated with partial resistance to fansidar. Only one double mutation in the *Pfdhfr* gene was found in 0.3% together with the wild-type *Pfdhps* gene (*Pfdhfr*/*Pfdhps* NRNI-SAKAA). The dominant triple, quintuple, and sextuple mutations were *Pfdhfr*/*Pfdhps* NRNI-SGKAA (2.0%), *Pfdhfr*/*Pfdhps* IRNI-AGKAA (15.7%) and *Pfdhfr*/*Pfdhps* IRNI-AGKAS (5.4%) respectively. Only two septuplet mutations (*Pfdhfr*/*Pfdhps* IRNI-AGKGS) were found at (0.7%) (Table 2). A total of 41 different mutations in the *Pfdhfr*/*Pfdhps* genes were found in mixed haplotype infections. Forty of the parasites harboured *Pfdhfr*/*Pfdhps* IRNI-[S/A]GKAA haplotypes, while 65% (26/40) of them were found at a single frequency (Supplemental File 1).

### *Elucidating the mixed haplotypes*

In the mixed haplotype infections, it was difficult to clarify the clones (wild-type or mutants) observed. It was rather easy to determine the clones present when the amino acid polymorphism occurred at only one gene locus. In *Pfmdr*1 N[Y/F]D, the likely clones were NYD (a wild-type clone) and NFD (a mutant clone). The same applied to *Pfdhps* [S/A]KAA, where SAKAA (wild type) and AAKAA (mutant), and *Pfdhfr* NC[S/N]I (NCSI – wild-type and NCNI – mutant), could be the case. However, in the case of *Pfcrt* CV[M/I][N/E][K/T], *Pfmdr*1 [N/Y][Y/F]D and *Pfdhps* [S/A][G/A]KAA, it was difficult to tell whether a wild-type haplotype existed.

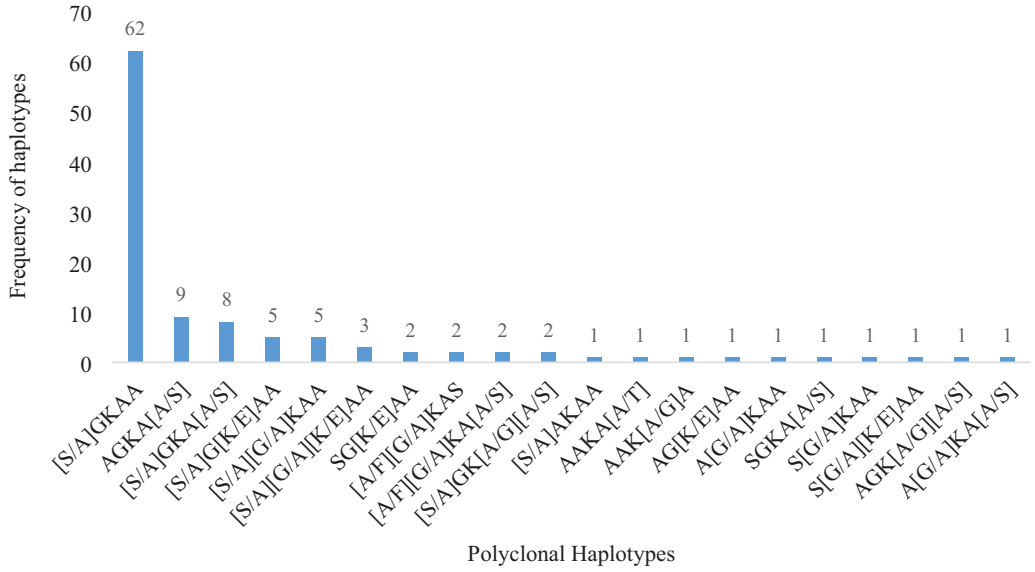


**Figure 5.** Mixed haplotype distribution in the *P. falciparum* dihydropteroate synthase gene (*Pfdhps*) at amino acid positions S436, A437, K540, A581 and A613.

## Discussion

The prevalence of malaria in the Central Region of Ghana has been found to be high.[23] For this reason, parasite mutations that have been associated with antimalarial drug resistance, known as putative antimalarial drug-resistant genes, were studied in the Central Region. The study focused on the *Pfcrt*, *Pfmdr*1, *Pfdhfr* and *Pfdhps* genes. Our findings revealed a low prevalence (3%) of *Pfcrt* mutations involving the chloroquine-resistant marker, K76T, as occurred in the CVIET (C72, V73, M74I, N75E K76T) haplotype. This finding is quite interesting, as an extensive reverse mutation in chloroquine-resistant parasites has increased in prevalence in Ghana[8,27] and elsewhere.[35] In 2020, the prevalence of chloroquine resistance in the Central and Eastern regions stood at 21% and 5%, respectively.[27] In the same year, the prevalence was 11.6% in the Greater Accra Region of Ghana,[7] an adjoining region to this study region. Also, Samples analysed from three other regions in Ghana (Greater Accra Region < Upper East Region-Navrongo < Bono East Region- Kintampo) produced a prevalence of 8% for *Pfcrt* K76T in 2018.[25] The decline in chloroquine-resistant parasites observed in this study could be attributed to total withdrawal of chloroquine and continuous adherence to ACT in Ghana. Despite the high prevalence of chloroquine-sensitive parasites (97%) identified in this study, the reintroduction of chloroquine for the treatment of malaria should be considered carefully. Elsewhere, the genetic makeup of the parasite and site-specific epidemiology may explain the differences between studies in Nigeria[36] and Angola[37] where the prevalence of chloroquine-resistant haplotype was recorded at 61.1% and 73%, respectively.

After chloroquine was withdrawn, and ACT was introduced in 2009,[38] certain mutations in the *Pfmdr*1 gene were found to confer resistance to artemisinin partner drugs. In our study area, the alleles of the Y184F mutant were the most prevalent and resulted in a relatively high prevalence of *Pfmdr*1 NFD (N86, Y184F D1246) with a relatively low prevalence of YFN (N86Y, Y184F, D1246N). Elsewhere, the Y184F mutation has been found to reduce the sensitivity of parasites to quinine, amodiaquine, chloroquine, mefloquine, and lumefantrine.[39] In Ghana, artemether-lumefantrine is the drug of choice for the treatment of malaria. However, more than 75% of the parasites analysed in this study bore the Y184F mutation in the YFN or NFD haplotypes. This observation is worrying for a country where antimalarial drug-resistant parasites have not yet been confirmed. The prevalence of *Pfmdr*1 Y184F in *P. falciparum* seems to be high, especially, in Africa. The prevalence was found to be 71.4% in Niger,[40] 66.4% in Cote d'Ivoire,[41] 68.7% in Burkina Faso[42] and elsewhere, 70% in India.[43]

The analysis for the *Pfhdfr*/*Pfdhps* genes was also carried out. About 77% of the samples had triple *Pfhdfr* IRNI haplotype. This rate was higher than the rate (>60%) reported by Amenga-Etego et al.[44] This study observed single to quadruple mutations, but Amenga-Etego et al. observed up to quintuple mutations. Mutations in the *Pfhdfr*/*Pfdhps* genes have been shown to render SP less effective against *P. falciparum* parasites. SP treatment was introduced in Ghana in 2003.[45,46] Since then, it has been used to prevent malaria in pregnant women[47] and children under 5 years.[48] To pregnant women, SP is administered at five different times during the course of the pregnancy.[49] Despite the use of SP to prevent malaria, the parasites are less sensitive to the drug to due to N51I, C59R, and S108N mutations in the *Pfdhfr* gene together with *Pfdhps* A437G (partial resistance), *Pfdhps* A437G + K540E (full resistance) and *Pfdhps* A437G + K540E + 581G/164L (super resistance), as published elsewhere.[21] The haplotype contributed by the *Pfdhfr* gene is IRNI, and that of *Pfdhps* A437G (partial), *Pfdhps* A437G + K540E (full), and *Pfdhps* A437G + K540E + A581G/A581L (super). This study observed IRNI in approximately 77% of the parasites, while in the case of the *Pfdhps* gene, A437G was observed in SGKAA, AGKAA, SGEAA, AGKAS, FGKAS, AGKSA, and AGKGS in 99% of the monoclonal infections. In monoclonal infections, one (0.5%) *Pfdhps* A437G + K540E was observed, while in mixed haplotype infections, 12 (11%) parasites were found to harbour the mutations *Pfdhps* A437G + K540E. Analysis of mutations in *Pfdhfr* and *Pfdhps* genes revealed that about 88% and 9% of the parasites had putative haplotypes that have been found elsewhere to confer partial or full resistance to SP, respectively.[21] No super-resistant haplotype was identified. In Africa, the full resistant haplotype (IRNI-SGEGAA) was first identified in Kenya,[50] therefore, this study will provide a recent report of the identification of this haplotype. SP failure in Kenya is attributable to this haplotype. Although these haplotypes have been found to confer resistance to SP,[21] this has not been proven in parasites in Ghana. Therefore, the clinical significance of these haplotypes must be determined, in a prospective chemotherapeutic efficacy study.

**Table 2.** Distribution of *Pfdhfr*/*Pfdhps* haplotypes associated with Sulphadoxine-pyrimethamine resistance in monoclonal infections.

| ***Pfdhfr*/*Pfdhps* gene mutations** | **Total number of *Pfdhfr*/*Pfdhps* genes successfully sequenced *N* = 294 (%)** |
|---|---|
| Wild types (*Pfdhfr* NCSI – *Pfdhps* SAKAA) | 0 |
| Single Mutation | |
| NCSI-AAKAA | 2 (0.68) |
| NCSI-SGKAA | 1 (0.34) |
| Double Mutation | |
| NRNI-SAKAA | 1 (0.34) |
| Triple Mutation | |
| NRNI-SGKAA | 6 (2.04) |
| IRNI-SAKAA | 1 (0.34) |
| Quadruple Mutation | |
| IRNI-SGKAA | 82 (27.89) |
| NRNI-AGKAA | 5 (1.70) |
| IRNI-AAKAA | 3 (1.02) |
| ICNI-AGKAA | 2 (0.68) |
| Quintuple Mutation | |
| IRNI-AGKAA | 46 (15.65) |
| IRNI-SGEAA | 1 (0.34) |
| NRNI-FGKAS | 1 (0.34) |
| Sextuple Mutation | |
| IRNI-AGKAS | 16 (5.44) |
| IRNI-FGKAS | 3 (1.02) |
| IRNI-AGKSA | 1 (0.34) |
| Septuplet Mutation | |
| IRNI-AGKGS | 2 (0.68) |

Distribution of *Pfdhfr*/*Pfdhps* haplotypes at amino acid positions 51N-59C-108S 164I-436S-437A-540K-581A-613A).

## Limitations

The major limitation of this study was our inability to expand the complex mixed haplotype into individual parasite clones. This is because only amino acids detected at each gene were available to the team at the time of this publication. Malaria Genome Laboratory of the WSI did not provide

the sequence reads but the amino acids detected at each gene loci of the gene target of interest. Secondly, the drug-resistant haplotypes reported herein are a result of a survey of molecular markers but not the result of therapeutic efficacy studies. Further, this publication does not have the *Kelch* 13 polymorphisms associated with antimalarial drug resistance. Finally, sequencing failure was observed among 113 (21.6%) of the 522 parasites. The reasons for the sequencing failure were not investigated.

## Conclusion

The study offers valuable information on the genetic alterations caused by the therapeutic and preventive interventions used in the Central Region of Ghana. The resurgence of parasites with CQ (Chloroquine) sensitivity confirms the reverse mutation of CQ-resistant genes that were seen in Ghana. However, reintroduction of CQ for malaria treatment should be done cautiously since the parasite could easily become resistant in the face of poor therapeutic practices. Significantly prevalent were haplotypes carrying the *Pfmdr*1 Y184F mutated allele commonly associated with decreased sensitivity to Artemether partner drug lumefantrine. *Pfmdr*1 D1246N were observed, which requires further investigations and will provide additional information for understanding their effect on susceptibility to artemisinin partner drugs. Furthermore, the very low prevalence of mutations *Pfdhfr* I164L, *Pfdhps* K540E and *Pfdhps* A581G must be monitored and contained, as these mutants can lead to the generation of full and super-resistant haplotypes, compromising the efficacy of SP use for intermittent preventive treatment during pregnancy (IPTp) and seasonal malaria chemotherapy in the region.

## Declarations

### *Ethics approval and consent to participate*

Ghana Health Service Ethics Review Committee (GHS-ERC Number: GHSERC017/03/20). The parent or legal guardian of each minor provided his or her written informed consent and an assent form from the child. Adults older than 18 years of age also completed written informed consent forms.

### *Consent for publication*

Not applicable.

### *Author contributions*

**Mavis Puopelle Dakorah:** Conceptualization; Data curation; Formal analysis; Methodology; Writing – original draft; Writing – review & editing.

**Enoch Aninagyei:** Conceptualization; Formal analysis; Methodology; Project administration; Supervision; Writing – original draft; Writing – review & editing.

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**Desmond Omane Acheampong:** Conceptualization; Project administration; Supervision; Validation; Writing – review & editing.

### *Acknowledgements*

We would like to thank the study participants, staff, and management of the following health facilities: St. Francis Xavier Catholic Hospital, St. Gregory Catholic Hospital, Abura Dunkwa Government Hospital, Ewim Polyclinic and Agona Swedru Municipal Hospital. We also appreciate the Sanger Malaria Research Unit for their support and genetic analysis of *Plasmodium* isolates.

### *Funding*

The authors received no financial support for the research, authorship and/or publication of this article.

### *Competing interests*

None to declare.

### *Availability of data and materials*

Datasets generated and analysed in this study are available in the Figshare data repository (10.6084/m9.figshare.25479547). However, a request for the data can be obtained from the corresponding author on a reasonable request.

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## Supplemental material

Supplemental material for this article is available online.

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## Appendix

### *Abbreviations*

| | |
|---|---|
| ACT | artemisinin-based combination therapy |
| DBS | dried blood spot |
| *Pfcrt* | *P. falciparum* chloroquine resistance transporter |
| *Pfdhfr* | *P. falciparum* dihydrofolate-reductase |
| *Pfdhps* | *P. falciparum* dihydropteroate synthetase |
| *Pfmdr* | *P. falciparum* multi-drug resistance |
| mRDT | Malaria rapid diagnostic test |
| SNPs | single nucleotide polymorphisms |
| sWGA | Selective Whole Genome Amplification |

### *Amino acid designations*

| | |
|---|---|
| A | alanine |
| C | cysteine |
| D | aspartic acid |
| E | glutamic acid |
| F | phenylalanine |
| G | glycine |
| I | isoleucine |
| K | lysine |
| L | leucine |
| M | methionine |
| N | asparagine |
| R | arginine |
| S | serine |
| T | threonine |
| V | valine |
| Y | tyrosine |