



Chloroquine resistance transporter drives divergent multi-locus drug resistance genetic backgrounds and susceptibility in Gambian *Plasmodium falciparum*

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ARTICLE INFO

Article history:

Received 6 July 2025

Revised 19 February 2026

Accepted 4 April 2026

Available online 14 April 2026

Editor: Fabrizio Bruschi

Keywords:

Plasmodium falciparum

Drug resistance

Artemisinin-based combination therapy (ACT)

Antimalarial susceptibility

Molecular resistance markers

Genetic polymorphisms

ABSTRACT

Objective: Sustained antimalarial drug use against *Plasmodium falciparum* has often resulted in drug resistance and the selection of associated genetic markers in malaria endemic populations. The genetic markers of resistance to legacy drugs like chloroquine persist in The Gambia. This study assesses the effect of these legacy drug-resistance genomic backgrounds on in vitro responses to antimalarial drugs in The Gambia.

Methods: *P. falciparum* drug-resistance genotypes at *pfprt*, *pfmdr1*, *pfdhfr*, *pfdhps*, and *pfkelch13* genes was determined from amplicon sequencing of 364 isolates. In vitro 50% growth inhibitory concentrations (IC₅₀s) were determined for 68 isolates to chloroquine, dihydroartemisinin, lumefantrine, mefloquine, piperaquine, and amodiaquine. In vitro response to drugs was assessed for parasite groups with different major multi-locus drug-resistance genetic backgrounds.

Results: Two main multi-locus drug-resistance backgrounds driven by *pfprt* haplotypes (CVMNK/CVIET) were observed. CVIET (62%) isolates were resistant to chloroquine, whereas CVMNK was less sensitive to mefloquine and dihydroartemisinin. The *pfmdr1* NFD haplotype was 60% prevalent, with 184F at 67%. Infections with the CVIET-NFD combination (31.7%) had the highest chloroquine IC₅₀s. *Pfdhfr* S108N (98%) and *pfdhps* A437G (85%) mutants were highly prevalent. No validated *pfkelch13* resistance mutations were detected.

Conclusions: Artemisinin-based combination therapies remain effective in The Gambia, supported by the absence of *pfkelch13* mutations. However, reversion to chloroquine sensitivity tends to result in reduced sensitivity to artemisinin-based combination therapy drugs. These genotype-phenotype dynamics need continuous monitoring to guide drug interventions towards malaria elimination.

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1. Introduction

Extensive combined use of antimalarial drugs and vector interventions significantly reduced malaria morbidity and mortality in endemic regions from 262 million and 839,000 in 2000 to 214 million and 438,000 in 2015, respectively [1]. However, esti-

mated malaria cases and deaths have stalled in the last decade and increased by 23 million and 24,000, respectively, between 2019 and 2023 [2]. The advent of artemisinin and its derivatives (artemether, artesunate, and dihydroartemisinin [DHA]) as essential components of artemisinin-based combination therapies (ACTs) contributed to reducing malaria mortality [3]. ACTs include a fast-acting artemisinin derivative combined with longer-lasting partner drugs (amodiaquine [AQ], mefloquine [MQ], lumefantrine [LUM], piperaquine [PPQ], or sulphadoxine-pyrimethamine [SP]) to enhance efficacy and prevent resistance [4]. Beyond ACTs, chemother-

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apy, chemoprophylaxis, intermittent preventive therapy, and other interventions with SP in combination with a quinoline have also been deployed, together exerting significant evolutionary pressure on parasite populations, that could lead to new multidrug-resistant phenotypes.

Widespread use of the historical drug chloroquine (CQ) led to the emergence of CQ resistance, marked by mutations in the *pfcr*t and *pfmdr*1 genes of *Plasmodium falciparum*. These also significantly reduced the efficacy of other quinolines, posing a major challenge to malaria control efforts [5]. When CQ was replaced by SP at the dawn of the millennium, resistance also rapidly emerged, marked by mutations in the *pfdhfr* and *pfdhps* genes. With ACT use, *P. falciparum* has developed partial artemisinin resistance, mostly associated with single-point mutations in the *pfkelch*13 gene (*pfk*13) [5,6]. These mutations lead to reduced artemisinin susceptibility in young ring-stage parasites and delayed parasite clearance from the blood after treatment with an artemisinin derivative. This necessitates improved understanding of malaria drug-resistance mechanisms and continuous surveillance towards innovative drug strategies for malaria treatment and elimination [7].

Drug resistance has historically been monitored through therapeutic efficacy studies every 2 years in symptomatic malaria patients following a World Health Organisation recommended protocol [8]. This alongside in vitro and ex vivo drug testing and genomic surveillance can comprehensively evaluate the performance of antimalarial medications and identify emerging tolerance and associated genetic markers [9,10]. This is particularly important in sub-Saharan Africa, where the malaria burden and the prevalence of historical and the emergence of markers of drug resistance in *P. falciparum* are heterogeneous, and partial resistance to artemisinins threatens the efficacy of ACTs [11–15].

Multiple antimalarial drug approaches in Africa (SP with or without amodiaquine, respectively, for seasonal malaria chemoprevention [SMC] and intermittent preventive therapy, and different first and second line ACTs, especially artemether-lumefantrine) mount variable selective pressure on the parasite. In the far-west Atlantic coast, low malaria prevalence in The Gambia and Senegal is ushering in hopes of elimination. However, more than half of the infections retain a CQ-resistance-associated genetic background in *pfcr*t [16,17]. This is relevant because *pfcr*t mutations also modulate susceptibility to other quinolines including ACT partner drugs. It is in an interactive epistatic relation with other resistance loci that increases parasite fitness in the absence of CQ, thereby positioning *pfcr*t as a central genetic scaffold that could enable the emergence of multidrug resistance [17]. Pyrimethamine resistance is already fixed, while various *pfdhps* haplotypes of sulphadoxine resistance are in circulation [3,18]. Partial artemisinin resistance variants in *pfkelch*13 have not been described in the region, and the effect of various combinations of legacy resistance loci on ACT partner drug susceptibility and parasite evolutionary trajectories remains unclear. This study determined the distribution of linked polymorphisms across known drug-resistance markers and measured the impact on the responses of the Gambian isolates of *P. falciparum* to artemisinin derivatives and ACT partner drugs.

2. Materials and methods

2.1. Sample collection

Ethical clearance was obtained from The Gambia Government/Medical Research Council Unit, The Gambia (MRCG) Joint Ethics Committee and approval from the Gambian Ministry of Health. Patients with suspected malaria, which was confirmed with rapid diagnostic tests, consented to the study and were recruited during routine annual seasonal cross-sectional malaria surveillance

at four health facilities in the Coastal Western region (Brikama and Fajikunda) and 350 km to the east in the semi-urban upper river region (Sotuma Serre and Sabi) between September and December 2022. Two specimen types were collected per patient: dried blood spots for molecular assays and 3 mL of venous whole blood for parasite culture adaptation and in vitro testing. A total of 68 patients aged ≥ 12 years who presented to the health centre with a history of fever and symptoms of uncomplicated malaria within the previous 24 h and no presentation of complicated/severe malaria at the time of rapid diagnostic test diagnosis. All samples were transported in biological hazard cooler boxes at 4 °C to the MRCG malaria laboratories. Chelex-extracted DNA from each dried blood spot was used for molecular diagnosis with varATS PCR [19], speciation and *P. falciparum* genotyping. Whole blood samples with *P. falciparum* isolates underwent in vitro drug susceptibility testing (IC_{50}). Extracted genomic DNA was stored at -20 °C for subsequent library preparation for targeted sequencing.

2.2. *P. falciparum* parasite genotyping for diversity and drug-resistance markers

Plasmodium falciparum drug-resistance genes (*pfcr*t, *pfmdr*1, *pfdhfr*, *pfdhps*, and *pfkelch*13) were amplified from each sample DNA in a multiplexed PCR next-generation sequencing primer mix, which also targets several genomic regions for genetic diversity determination in a modular panel developed by Paragon Genomics [20]. The multiplexed PCR reaction mix was prepared with the *Plasmodium* CleanPlex kit that consisted of 5X Master Mix, primer pools for the different modules, nuclease-free water, and 6 μ L of genomic DNA followed by bead clean-up for size selection. Each sample was barcoded and sequenced as a multiplex on MiSeq. Short-read sequences (fastq) generated were analysed for quality, variant calling, and microhaplotypes using the MAD4HaTTeR pipeline v0.2.1 available on the GitHub repository [21]. MAD4HATTER was used at default settings, including adapter/primer trimming (Cutadapt), quality filtering, and DADA2-based denoising to generate high-confidence *P. falciparum* inferred exact amplicon sequence variants, retaining only sequencing pools with adequate target coverage ($100\times$). Microhaplotypes derived from the final matrix counts were summarised to estimate allele frequencies, complexity of infection, and pairwise relatedness (identity-by-descent) using MOIRE and DECIPHER R packages (RStudio version 12.1 + 402) [21]. Key mutations in the antimalarial-resistance genes *pfcr*t C72/V73/M74/N75/K76, *pfmdr*1 N86/Y184/D1246, *pfdhps* S436/A437/K540/A581/A613, *pfdhfr* N51/C59/S108/I164, and *pfk*13 were characterised for all isolates. To determine isolates clustering by multi-locus haplotype groups across drug-resistant genes, a neighbour joining tree from pairwise genetic distance was constructed in MEGAX [22] and annotated and visualised with iTOL [23].

2.3. In vitro *P. falciparum* culture

Following microscopy of Giemsa-stained samples, those confirmed as *P. falciparum* with predominantly young ring-stage parasites were analysed for drug susceptibility. For parasitaemia determination, an aliquot of 2% cell suspension in SYBR green I DNA was incubated for 30 min at ambient temperature, and parasite density was estimated by counting 100 000 erythrocytes of SYBR green-stained red blood cells on the Cytex Guava easyCyte Flow Cytometer. For samples with at least 0.5% parasitaemia, serum was removed and packed cells suspended in incomplete RPMI medium at 50% haematocrit followed by layering on lymphoprep and centrifuged for 20 mins at 1500 rpm with no brake, for phase separation. The peripheral blood mononuclear cell and buffy coat layers predominantly containing white blood cells were removed to

reduce leukocyte interference in drug tests and cell acquisition targeting only malaria parasite-infected erythrocytes. The infected red blood cell pellet was washed thrice in incomplete media and resuspended at 2% haematocrit in complete RPMI-1640 growth medium supplemented with 0.5% Albumax, 0.5 M HEPES, and 25 µg/mL gentamicin. Each isolate was cultured in vitro at 37 °C in a gas chamber made of 96% nitrogen, 3% carbon dioxide, and 1% oxygen mixture.

2.4. *P. falciparum* in vitro drug susceptibility testing

The IC₅₀ of each *P. falciparum* isolate was determined from growth inhibition for each of the following antimalarial drugs: DHA (100–0.39 nM), CQ (750–2.93 nM), AQ (250–0.98 nM), MQ (600–2.3 nM), PPQ (6000–0.92 nM), and LUM (18 000–2.74 nM) following modified previously published protocols [24]. Briefly, two- or threefold serial dilutions (ensuring a minimum of nine dilutions) of each drug were prepared in 96-well tissue culture plates using complete media. Parasites were synchronised using 5% sorbitol treatment, at ≥80% ring forms (0–12 h post-invasion) and added to duplicates of each drug dilution to obtain 2% haematocrit and a parasitaemia of 0.5%, to ensure adequate parasite growth over 72 h, reduce growth competition, and improve compatibility across isolates. Each culture was stained with SYBR Green I, and parasite growth was determined from DNA fluorescence at 450 nM using a Fluoroscan microplate reader. Drug-free controls were included to monitor baseline growth. IC₅₀ values were calculated by growth curve fitting in In vitro Analysis and Reporting tool IVART [25], using the average of replicate wells at each drug concentration for where the coefficient of variation was below 30% [26]. Drug susceptibility of laboratory reference strains (CQ-sensitive Pf3D7 and CQ-resistant PfDd2) were additional internal standards to monitor batch variability and consistency with previous studies [12]. Some IC₅₀ values were also determined from four parameter growth curves fitted in GraphPad Prism software both to validate IVART-derived IC₅₀ estimates and to obtain values for samples that could not be resolved by IVART model. GraphPad Prism and IVART have been shown to yield highly concordant IC₅₀ estimates [27].

2.5. Statistical analyses

The distribution, median, and interquartile range (IQR) for IC₅₀ data were analysed on GraphPad Prism for the in vitro susceptibility of isolates against each drug. Differences in drug responses of multi-locus haplotypes across drug-resistance genes were assessed using Mann-Whitney *U* and Kruskal-Wallis tests. Pearson χ^2 test of independence was used to evaluate the correlation between drug responses. A *P*-value of less than 0.05 was considered statistically significant. Cut-off values used to define resistance phenotypes were based on previously published thresholds [28–30].

3. Results

3.1. High frequencies of CQ- and antifolate-resistance-associated haplotypes

A total of 364 isolates were collected during the 2022 malaria season, which were all genotyped (Supplementary Fig. S1; Supplementary Table S1). Of these, in vitro assays were established for 68 isolates, and the genotypic distribution within this subset did not significantly differ from the larger sample group (Table 1). The mutant *pfprt* haplotype CVIET occurred at a frequency of 0.62, while the wild *pfprt* haplotype CVMNK occurred at 0.33 frequency (Table 1). Multiclonal infections with more than a single *pfprt* haplotype were found at 5%. For *pfmdr1*, the wild-type NYD haplo-

type at codons 86, 184, and 1246 occurred at a low frequency of 0.30, with 0.60 infections harbouring the single mutant NFD haplotype. The frequency of the Y184F allele was 0.67, whereas the N86Y occurred at frequencies of 0.03. For all infections sampled, the D1246Y allele was detected at a frequency of 0.01 (Supplementary Table S1). The most frequent mutations in the antifolate-resistance genes were 108 N (0.98) and 437 G (0.86) for *Pfdhfr* and *Pfdhps*, respectively. Gambian isolates predominantly harboured the *pfdhfr* triple mutant haplotype IRNI (0.95) and the *Pfdhps* single mutant SGKAA (0.70; Table 1). No validated artemisinin-resistance variant was observed for the *pfkelch13* gene. The remaining set of the 296 isolates had inadequate parasitaemia for in vitro assays (Supplementary Fig. S1).

3.2. Two major multi-locus *P. falciparum* antimalarial-drug-resistance genetic backbones in The Gambia

The level of *P. falciparum* within infection complexity was modest, with 15.1% of infections having more than a single strain. Based on genetic distance clustering, 11 pairs of infections had highly similar genetic barcodes, and these were predominantly infections with the *pfprt* CVMNK genotype from the coastal urban health centres of Brikama and Fajikunda (Supplementary Fig. S2). Multi-locus haplotype combinations across *pfprt*, *pfmdr1*, *pfdhfr*, and *pfdhps* genes classified the isolates into two major clusters that were further divided into five drug-resistance genetic backbones with a membership of at least five isolates (Fig. 1A). The two major clusters were determined primarily by *pfprt* CVMNK (Pf3D7 wild type haplotype) and CVIET (PfDd2 mutant haplotype), with the sub-clusters (Group3D7, GroupDd2, GroupA, GroupB, and GroupC) determined by variants in *pfmdr1* and *pfdhps* (Fig. 1B). Groups A and B have the *pfprt* CVIET haplotype but vary in their *pfmdr1* NFD or NYD haplotypes. Group C also has the *pfprt* CVIET backbone but has members with both wild and mutant *pfmdr1* and *pfdhps*. Several other haplotype combinations represented by less than five infections were also in circulation.

3.3. Distribution of IC₅₀ responses against antimalarials in The Gambia

Gambian *P. falciparum* isolates were successfully tested for drug sensitivity, generating IC₅₀ estimates for six antimalarial drugs including AQ, CQ, DHA, LUM, MQ, and PPQ using the SYBR Green I fluorescence assay (Table 2). The median IC₅₀ estimate of the field isolates for CQ was 84.5 nM, below the predefined threshold for CQ resistance (>100 nM) [31]. The prevalence of isolates with CQ IC₅₀ >100 nM (resistance to CQ) was 44.7 %, while 49% had values above the median IC₅₀. There was a consistently low IC₅₀ to DHA, with a median IC₅₀ value of 1.24 nM. In addition, DHA IC₅₀ spanned the narrowest range across all isolates compared with other drugs (Fig. 2). For ACT partner drugs, the median IC₅₀ values were 54.99 nM, 38.06 nM, 80 nM, and 8.143 nM for LUM, MQ, PPQ, and AQ, respectively. The IC₅₀ ranges for these drugs (Fig. 2) were relatively narrow, with a difference of no more than threefold between the most susceptible and least susceptible isolate for each drug when outliers were excluded. The widest distribution of values was observed for LUM (Table 2). The strongest positive correlation between IC₅₀ values was between PPQ and LUM (*r* = 0.46, *P* = 0.0011; Supplementary Fig. S3A). The IC₅₀s of LUM displayed a significant negative correlation with those of AQ (*r* = −0.36, *P* = 0.01). CQ IC₅₀s positively correlated (*r* = 0.39, *P* = 0.0496) with those of AQ, which weakly correlated with MQ (*r* = 0.22). DHA IC₅₀s showed a significant negative correlation with those of CQ (*r* = −0.42, *P* = 0.037) but had a weak positive correlation with AQ (*r* = 0.1) and MEF (*r* = 0.21). For LUM, IC₅₀ estimates differed significantly between isolates from the urban coastal sites and the

Table 1
Prevalence of mutations and haplotypes in drug-resistance genes (*pfCRT*, *pfmdr1*, *pfdhfr*, and *pfDHPS*) among the 68 Gambian *Plasmodium falciparum* isolates tested with in vitro testing.

Gene	Alleles (frequency)	Haplotypes	Haplotype frequency
<i>pfCRT</i> (Codon 72–76)	M74I (0.62)	CVMNK (wild type)	0.33 (n = 21)
	N75E (0.62)	CVIET	0.62 (n = 39)
	K76T (0.62)		
	Mixed alleles (0.05)		
<i>pfMDR1</i> (Codons 86/184/1246)	N86Y (0.03)	NYD (wild type)	0.30 (n = 19)
	Y184F (0.67)	NFD	0.60 (n = 38)
	D1246Y (0)	YFD	0.02 (n = 1)
	Mixed alleles (0.03)		
<i>pfDHFR</i> (Codons 51/59/108/164)	N51I (0.97)	NCSI (wild type)	0.02 (n = 1)
	C59R (0.97)	IRNI	0.95 (n = 60)
	S108N (0.98)	NRNI	0.02 (n = 1)
	I164L (0.00)	ICNI	0.02 (n = 1)
	Mixed alleles (0.00)		
	S436A/F (0.17)	SAKAA (wild type)	0.02 (n = 1)
	A437G (0.86)	SGKAA	0.70 (n = 44)
<i>pfDHPS</i> (Codons 436/437/540/581/613)	K540E (0.05)	AAKAA	0.11 (n = 7)
	A581G (0.02)	AGKAA	0.03 (n = 2)
	A613S/T (0.05)	AGKAS	0.02 (n = 1)
	Mixed alleles (0.05)	SGEAA	0.05 (n = 3)
		AGKGS	0.02 (n = 1)
		SGKAS	0.02 (n = 1)

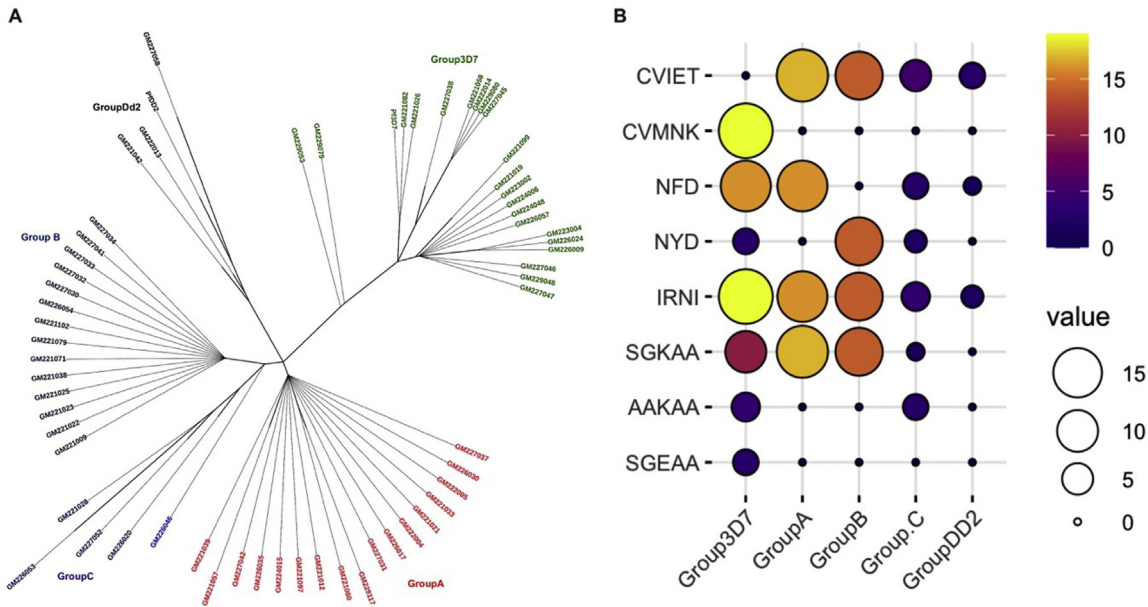


Fig. 1. Genetic cluster of 68 clinical isolates of *Plasmodium falciparum* collected from western and eastern Gambia in 2022 showing (A) Neighbour joining tree from drug-resistance haplotypes across *pfCRT*, *pfmdr1*, *pfdhfr*, and *pfDHPS* with clades labelled into representative subgroups and (B) balloon plot of the distribution of main drug-resistance haplotype frequencies in each clade.

rural eastern region of The Gambia ($P = 0.0327$; Supplementary Fig. S3B).

3.4. *PfCRT* and *pfmdr1* haplotypes affect CQ and MQ sensitivity in Gambian isolates

Isolates with the *pfCRT* resistance haplotype (CVIET) were significantly less sensitive to CQ ($P < 0.0001$), with median IC_{50} of 115.8 nM as against 12.97 nM for the susceptible CVMNK haplotype group. For two isolates with mixed haplotypes, the median CQ IC_{50} estimates was 64.56 nM (Fig. 3). In contrast, the CVIET haplotype group of isolates had a significantly lower IC_{50} estimates for DHA and MQ ($P < 0.01$). Considering *pfmdr1* haplotypes alone, isolates with the single mutant resistance NFD haplotype were less sensitive to MQ, with higher IC_{50} estimate values compared with those with the wildtype NYD haplotype. These *pfmdr1* haplotype

groups did not significantly differ in response to any of the other drugs, including CQ. However, the distribution of CQ IC_{50} s was bi-modal for *pfmdr1* haplotypes but less so for MQ (Supplementary Fig. S4).

3.5. Drug susceptibility to CQ, MQ, and DHA differ by multi-locus drug-resistance genetic backbone

The most susceptible isolates to CQ were those with a wildtype genetic backbone that clustered together with the CQ-sensitive *P. falciparum* 3D7 (Group3D7). These had a mean IC_{50} estimate of 18.9 nM. The mean IC_{50} estimates for the other groups were 104.3 nM, 93.95 nM, 165.7 nM, and 151.3 nM for GroupA, GroupB, GroupC, and GroupDd2, respectively. These were all significantly higher than those for the Group3D7 (P -values < 0.05), with an increasing trend towards GroupDd2 (Fig. 4). Isolates in Group3D7

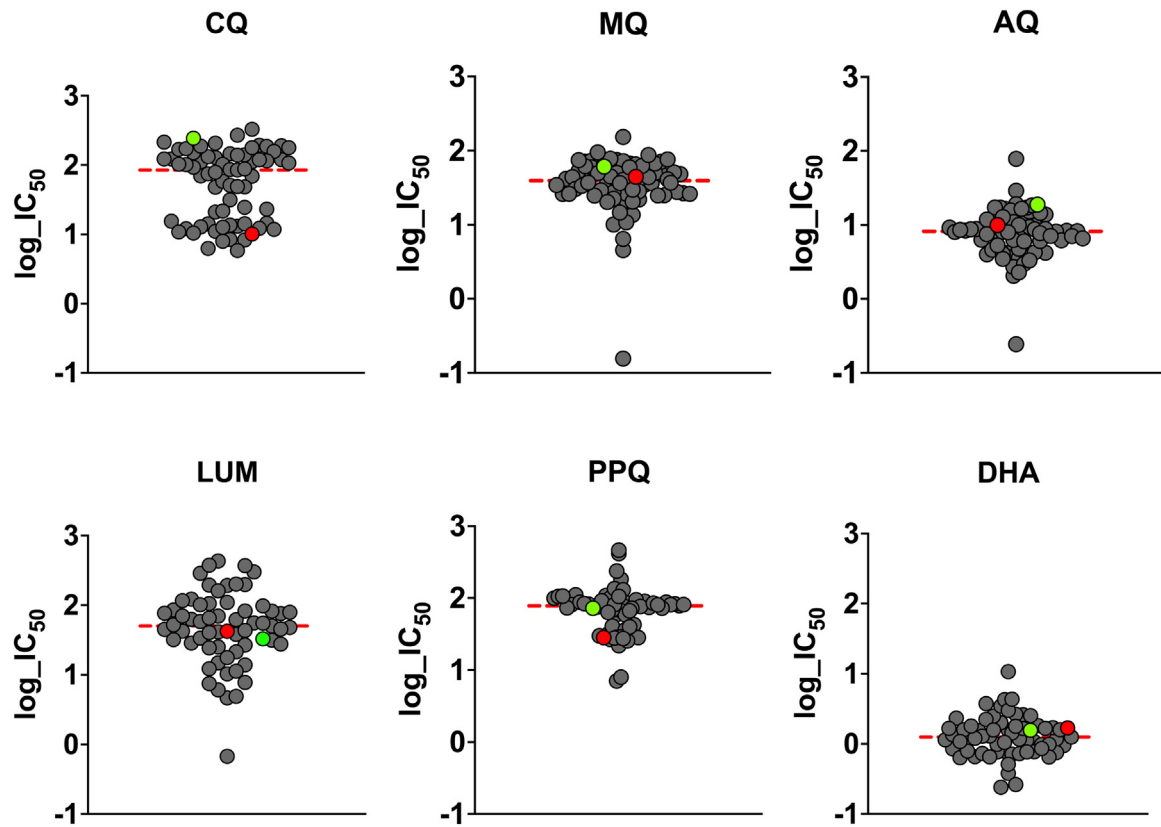


Fig. 2. Distribution of IC₅₀ estimates of *Plasmodium falciparum* in vitro susceptibility to chloroquine, mefloquine, amodiaquine, lumefantrine, piperazine, and dihydroartemisinin. Red dashed lines represent the median IC₅₀ for each drug, and each point represents the mean IC₅₀ from two technical replicates of each isolate ($N \leq 68$). Red points and green points represent PfDd2 and PfDd1, respectively.

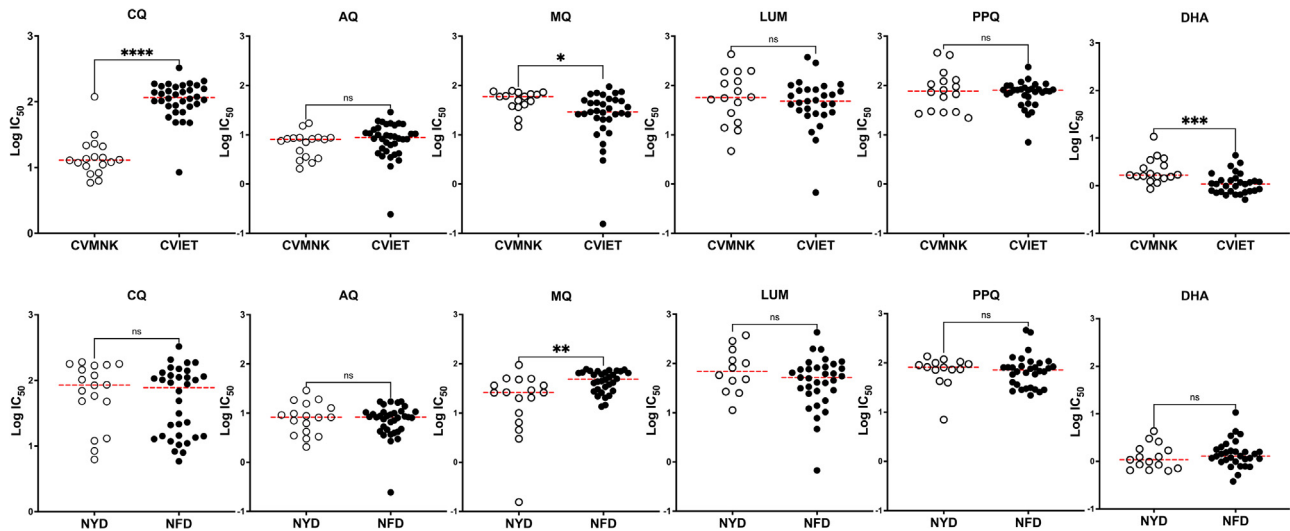


Fig. 3. Drug susceptibilities of *pfprt* (upper panel) and *pfmdr1* (lower panel) The wild type and resistant haplotype groups in Gambian *Plasmodium falciparum* isolates.

Table 2		
In vitro susceptibilities of natural Gambian field isolates to six antimalarial drugs.*		
Drug	Median IC ₅₀ (IQR)	Range
Amodiaquine ($n = 68$)	8.176 (4.7–10.6)	0.3–78.1
Chloroquine ($n = 67$)	84.52 (14.2–138.9)	5.9–327.9
Dihydroartemisinin ($n = 63$)	1.24 (0.8–1.8)	0.2–10.7
Lumefantrine ($n = 61$)	52.12 (26.0–99.4)	0.7–431.0
Mefloquine ($n = 68$)	37.51 (25.9–61.1)	0.2–152.8
Piperazine ($n = 59$)	78.32 (40.6–94.7)	7.1–462.6

* Median (IC₅₀) and IQR.

were mostly of the CVMNK-NFD *pfprt* and *pfmdr1* combined haplotype background (Supplementary Fig. S5). A small number of isolates in this group with the CVMNK-NYD joint haplotype were similarly sensitive to CQ. The mean IC₅₀ estimate for GroupA were non-significantly higher than those for GroupB because the *pfmdr1* NFD or NYD haplotype had no impact on responses to isolates that already had the CQ-resistant CVIET background. The IC₅₀ values against MQ were significantly higher for isolates in Group3D7, which were mostly wild type at *pfprt* but single mutant at *pfmdr1* 184F (CVMNK-NFD) compared with those in GroupB, which carry

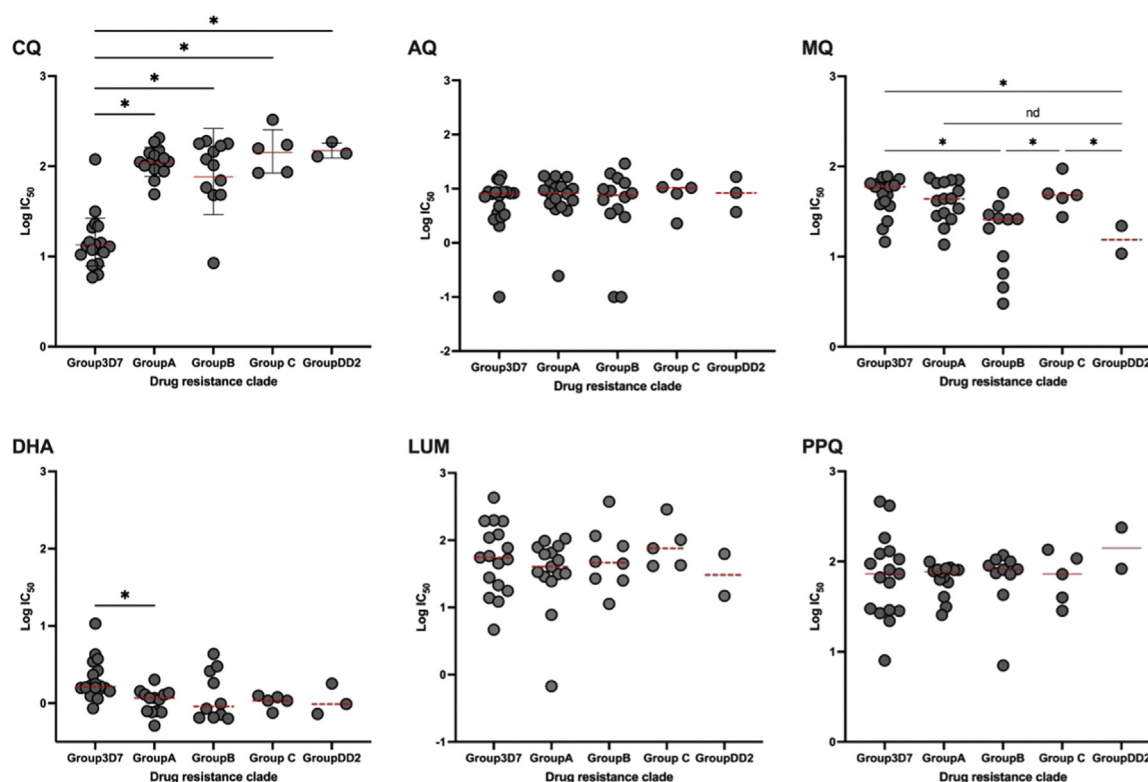


Fig. 4. In vitro responses to antimalarial drugs by isolates with different multi-locus drug-resistance genetic backgrounds. Isolates were classified by clades in the neighbour joining tree shown in Fig. 1.

the CVIET-NYD haplotype. GroupB isolates were significantly more sensitive to MQ than those in GroupC, while two isolates in GroupDd2 were the most sensitive to the drug. Lastly, Group3D7 isolates were less sensitive to DHA (q -value < 0.05) compared with GroupA that differs in having the CQ-resistant background. Response to AQ, LUM, and PPQ did not significantly differ by drug-resistance backbone.

4. Discussion

This study characterises the in vitro drug susceptibility and resistance markers of natural *Plasmodium falciparum* isolates from The Gambia, where low malaria prevalence coexists with high drug pressure from SMC and mass drug administration interventions. We determined two major multi-locus genetic backbones from five antimalarial-resistance genes that modulate drug sensitivities in Gambian *P. falciparum* isolates from the 2022 malaria season. Despite the continuous clinical efficacy of artemisinin derivatives and the absence of *pfk13* artemisinin-resistance mutations, the persistence of CQ resistance and strong influence on in vitro drug sensitivity to DHA and partner drugs calls for increased surveillance and further understanding of the polygenic mechanisms driving evolution to drug adaptation in *P. falciparum* in West Africa.

The Gambia withdrew CQ as a first line treatment for malaria more than a decade ago. However, two-thirds of *P. falciparum* infections carried the *pfprt* CVIET haplotype, with the K76T mutation that is strongly associated with CQ resistance consistent with high CQ IC₅₀ estimates, as previously reported in The Gambia and other parts of Africa [17,32]. Unlike seen in some parts of West Africa, such as Ghana, malaria parasites with CQ-resistance genetic markers persist in The Gambia [3,17,33]. While there are speculations of persistent CQ pressure from informal malaria treatment, the continuous fitness in the absence of significant CQ drug pressure may reflect compensatory genetic background for *pfprt* deleterious mu-

tations [17,34]. This fitness modulating genetic background could include *pfat1* S258L, which has been shown at similar frequencies as K76T, working in epitaxis with *pfprt* and co-selected by CQ [17,35]. Nonetheless, continued use of CQ for non-malarial conditions, including autoimmune diseases, may contribute to the maintenance of CQ-resistant parasites, as well as the selection of other drugs such as AQ in SMC and ACT partner drugs. For now, the most significant antimalarial drug pressure is from artemether and lumefantrine, the ACT combination recommended for first line treatment of uncomplicated malaria in The Gambia and most of West Africa [16,37]. *P. falciparum* isolates resistant to CQ are more susceptible to lumefantrine [38]. However, the mechanisms of lumefantrine resistance are not well known, despite some *pfmdr1* haplotypes, such as NFD, being strongly linked to lumefantrine selection in Africa and Asia [37,38]. The *pfmdr1* NFD haplotype and the Y184F allele frequencies were high (0.67) in the samples. Although lumefantrine showed the widest range of IC₅₀s, no clear haplotype or allele-specific correlation was observed, suggesting additional contributors in responses to ACT partner drugs that require further investigation [35]. This could include variants at other candidate markers; *pfh1* (Sodium-Hydrogen Exchanger 1), *pfatp6* (SERCA-type Ca²⁺-ATPase), *pfubp1* (ubiquitin-specific protease 1), and *pfcoronin*, possibly contributing to multidrug resistance [35,39]. The isolates were almost all resistant to pyrimethamine as the *pfdhfr* triple mutant IRNI was close to fixation (0.95). These also mostly carried the A437G in *pfdhps* (0.85), commonly as SGKAA (0.70) for sulphadoxine resistance, as seen for other West African countries [18,38]. These alongside the wild type *pfkelch13* variants represent the multi-locus drug-resistance genetic background of *P. falciparum* in West African malaria populations.

Two major multi-locus drug-resistance haplotypes across five clades were detected from variants across *pfprt*, *pfmdr1*, *pfdhfr*, and *pfdhps*. The two major genetic backgrounds were driven by the sensitive (CVMNK) or drug-resistant (CVIET) *pfprt* haplotypes, whereas the subgroups differed mainly in *pfmdr1* and *pfdhps* combinations. The two major clusters had significant differences in responses to drugs CQ, MQ, and DHA, supporting previous findings on the effect of *pfprt* and *pfmdr1* variants on efficacy of ACTs [40]. The variation in DHA responses surprisingly occurred, despite the absence of artemisinin-associated *pfkelch13* mutations in The Gambia and most of West Africa, contrary to their emergence in East Africa [41]. This suggests *pfkelch13*-independent molecular pathways to artemisinin tolerance as highlighted in other studies in West African isolates [42]. Nonetheless most isolates tested remained highly DHA-sensitive (median IC₅₀ 1.24 nM). This is particularly encouraging given the widespread use of ACTs as the first line treatment for malaria for almost two decades. Similar findings have been reported in other African countries [16,32]. Higher tolerance to DHA in isolates with CQ-sensitive backgrounds was unexpected. Artemisinin partial resistance has emerged in East Africa, alongside reversion to wild-type *pfprt*, whereas artemisinin-resistant strains in Southeast Asia of lower malaria prevalence typically carry mutant *pfprt* backgrounds [41,43]. The impact of *pfprt* haplotype on DHA response was independent of *pfmdr1* haplotype, an observation that warrants further investigation.

The *pfmdr1* resistance haplotype NFD was associated with higher IC₅₀ estimate for MQ, consistent with previous findings that link *pfmdr1* mutations to reduced sensitivity to ACTs [44]. Combined with *pfprt* variants, the CVIET-NFD combination was most prevalent (31.7%) and was associated with higher CQ and MQ IC₅₀ estimates. Already, there are several reports of reduced efficacy of ACTs in Burkina Faso [45], and the sporadic appearance of *pfkelch13* mutants in Ghana and regions of west Africa where CQ-resistance mutants are now at the lowest prevalence [33,46]. ACTs were introduced to combat resistance and emerging resistance threats in Africa have led to the push for triple ACTs. The variability in responses and presence of outliers underscore increasing tolerance and the need for vigilance as malaria interventions scale up [3,4]. With expanding ACT, SMC, and dihydroartemisinin-piperaquine (DHAPP) use under The Gambia's malaria elimination strategy, cumulative drug pressure may increase the risk of multidrug resistance.

The study was limited by the small sample size for isolates that went through drug susceptibility testing due to the labour and cost intensive nature of in vitro tests and the absence of ring survival and piperaquine survival assays for DHA and PPQ, respectively. An increased sample size in future work, including isolates from other West African settings for standardised IC₅₀ and drug-specific assays, can reveal additional response trends and may provide early warning of any emergence of reduced drug efficacy and better inform regional antimalarial policy.

5. Conclusions

In conclusion, we observed different resistance-genetic backgrounds in The Gambia that are largely susceptible to ACT drugs. The level of in vitro sensitivity is significantly dependent on the presence of legacy resistance to CQ, driven by the persistent *pfprt* CVIET haplotype. Elevated DHA tolerance in this subset of the isolates underscores the need to strengthen ACT-resistance monitoring in West Africa. This study refines our understanding of how combined haplotypes across resistance genes influence drug response dynamics in The Gambia and West Africa.

Funding: This work was funded by the European and Developing Countries Clinical Trials Partnership (EDCTP) under the Emerging

Genomic Selection and AntiMalarial Tolerance in Africa (EGSAT) consortium and by the Wellcome Trust through the Malaria Research Capacity Development Consortium (MARCAD-Plus) consortium. The funders had no role in the design and data collection, analyses, interpretation, or manuscript preparation.

Declaration of competing interests: None.

Ethical approval: This study was approved by The Gambia Government/Medical Research Council Unit The Gambia (MRCCG) Joint Ethics Committee and the Ministry of Health (The Gambia), for the West African Malaria In vitro Antimalarial Testing project (EGSAT-EDCTP-SFp). Written consent was obtained from all participants, and all procedures complied with MRCC guidelines.

Author Contributions: NFD and AAN. designed the experiment. NFD carried out the experiments, performed data analyses, and drafted the manuscript. FB, ASJ, NN, and BM contributed to methodology and phenotypic data generation. SC contributed to microscopic assessments. BNA and ECO contributed to sequencing data generation and bioinformatics analyses. CJS was involved in supervision of the project and reviewing. AAN supervised the project.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jgar.2026.04.003.

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