

Efficacy of Artemether-lumefantrine and Dihydroartemisinin- piperazine for the Treatment of Uncomplicated Plasmodium falciparum Malaria in The Gambia, 2022

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Abstract

Background

Artemisinin-based combination therapies (ACTs) remain the cornerstone of treatment for uncomplicated *Plasmodium falciparum* malaria. However, the emergence and spread of artemisinin partial resistance highlights the need for continuous monitoring of ACT's therapeutic efficacy in endemic areas. In The Gambia, artemether–lumefantrine (AL) and dihydroartemisinin–piperaquine (DHA–PPQ) are used as first- and second-line antimalarial treatments, respectively.

Methods

A prospective therapeutic efficacy study was conducted between September 2022 and January 2023 across three sentinel sites in The Gambia, following the WHO protocol. Patients aged ≥ 6 months with uncomplicated *P. falciparum* malaria were treated with AL (28-day follow-up) or DHA–PPQ (42-day follow-up). The primary endpoint was PCR-corrected adequate clinical and parasitological response (ACPR). Molecular markers of resistance were analysed by targeted amplicon sequencing, and *ex vivo* susceptibility to dihydroartemisinin, lumefantrine, and piperazine was assessed using a SYBR Green I fluorescence assay.

Results

Of 199 enrolled participants (146 in AL; 53 in DHA–PPQ arm), 126 (86.3%) and 39 (73.6%) completed their follow-up, respectively. PCR-corrected ACPR was 98.3% (95% CI 96.0–100%) for AL and 100% for DHA–PPQ. Two recrudescence infections were identified in the AL group, one clinical and one parasitological failure, respectively, at day 22 and day 7. Parasite clearance was fast with no parasitaemia detected on day 3 in either treatment arm. None of the validated *Pfkelch13* resistance mutations was detected. High frequencies of legacy resistance markers were observed, including the *Pfcr1* CVIET haplotype (60% in monoclonal infections) and *Pfdhfr* triple mutants (89.3%). *Ex vivo* assays demonstrated high susceptibility to dihydroartemisinin (median IC_{50} : 2.7 nM; IQR (1.14–2.33)), while lumefantrine and piperazine showed broader IC_{50} distributions (median IC_{50} : 53.53 nM; IQR (17.11–170.1) and 76.21 nM; IQR (33.76–98.63), respectively).

Conclusion

AL and DHA–PPQ remain highly efficacious for the treatment of uncomplicated *P. falciparum* malaria in The Gambia, supporting their continued use as first- and second-line therapies. The absence of delayed parasite clearance and *Pfkelch13* mutations is reassuring. However, the persistence of legacy resistance markers and the high variability in partner-drug susceptibility underscore the importance of continued and integrated monitoring with molecular and phenotypic surveillance.

Introduction

Over the past two decades, large-scale implementation of control interventions has significantly reduced the global malaria burden. Between 2000 and 2024, an estimated 2.3 billion malaria cases and 14 million malaria deaths were averted worldwide; most averted cases (75.8%) and deaths (93.3%) occurred in the World Health Organization (WHO) African Region (1). Despite these gains, malaria remains a major public health problem, particularly in sub-Saharan Africa, where progress has stalled since 2015 (2). In 2024, an estimated 282 million cases and 610,000 deaths occurred globally, representing an increase of about 9 million cases compared to 2023 (1). Currently, one of the biggest challenges in malaria control is the growing threat of anti-malaria drug resistance.

Artemisinin-based combination therapies (ACTs) are the standard treatment for uncomplicated *P. falciparum* malaria (3). ACTs combine a fast-acting artemisinin derivative that clears most parasites in the first three days, with a long-acting partner drug clearing residual parasitaemia (3). Currently, the WHO recommends six different ACTs, namely artemether-lumefantrine (AL); artesunate-amodiaquine (AS-AQ); artesunate-mefloquine (ASMQ); artesunate+sulfadoxine-pyrimethamine (AS + SP); dihydroartemisinin-piperaquine (DHA-PPQ) and artesunate-pyronaridine (AS-PY) (3).

ACTs are highly effective antimalarials; however, the emergence and spread of the artemisinin-resistant *Plasmodium falciparum* poses a major threat to malaria control and elimination. Artemisinin resistance, first documented in the Greater Mekong Subregion in 2008, has recently emerged in East Africa, including in Rwanda, Uganda, Tanzania and Eritrea (3–5). Artemisinin partial resistance, primarily associated with mutations in the *P. falciparum* Kelch 13 (K13) gene, is defined as delayed parasite clearance after treatment with a drug containing an artemisinin derivative (5, 6). This partial resistance increases the risk of failure of the partner drug due to prolonged exposure of malaria parasites to the partner drug alone (7). Therefore, monitoring drug efficacy and molecular markers of resistance is essential for early detection and for informing national treatment policies. The WHO recommends routine monitoring of first-line antimalarial therapies in sentinel sites at least every two years (5, 8).

In The Gambia, AL was adopted as the first-line treatment for uncomplicated malaria in 2005 and rolled out nationwide in 2008. In 2013, DHA-PPQ was introduced as second-line therapy. In line with the WHO recommendation, the Ministry of Health, through the National Malaria Control Programme (NMCP), has conducted biennial therapeutic efficacy studies (TES) to monitor AL and DHA-PPQ efficacy in selected sentinel sites. The last TES, in 2018, reported high cure rates, *i.e.*, 94.3% for AL and 100% for DHA-PPQ (*unpublished data, NMCP*), supporting their continued use.

In 2022, the TES was jointly conducted by the NMCP and the Medical Research Council Unit The Gambia at the London School of Hygiene & Tropical Medicine (MRCG at LSHTM). We report here the treatment outcomes, frequencies of resistance markers to legacy antimalarials and artemisinin partner drugs, as well as *ex-vivo* susceptibility to dihydroartemisinin, lumefantrine, and piperaquine.

Methods

Study design

The TES consisted of a one-arm, prospective evaluation of the clinical and parasitological responses to directly observed treatment with either AL (first-line treatment) or DHA-PPQ (second-line treatment) in patients with uncomplicated *Plasmodium falciparum* malaria.

Study sites

The study was implemented between September 2022 and January 2023 in three different health facilities across The Gambia (Fig. 1): Brikama Health Centre (West Coast Region), Kuntaur Health Centre (Central River Region), and Gambissara Health Centre (Upper River Region). These sentinel sites were chosen to reflect the geographical diversity and heterogeneity of malaria transmission across The Gambia.

Brikama is a peri-urban settlement located approximately 35 km from Banjul, the capital city of The Gambia. In 2021, the malaria incidence rate in Brikama was 32 cases per 1,000 population. Gambissara is a rural town in the south-eastern part of The Gambia, near the Senegalese border, where the malaria incidence rate in 2021 was estimated at 23.65 cases per 1,000 population. In comparison, the incidence rate was estimated at 11.73 cases per 1,000 population in Kuntaur, a smaller rural town situated on the north bank of the Central River Region of the Gambia (NMCP, *unpublished data*).

Participant eligibility and enrolment

Patients aged ≥ 6 months presenting to study health facilities with suspected clinical malaria (fever or history of fever in the past 24h or any other symptoms suggestive of malaria) were screened for eligibility. Patients were enrolled if they met all inclusion criteria and none of the exclusion criteria. Inclusion criteria were age ≥ 6 months, *P. falciparum* mono-infection with parasite density between 500 and 200,000/ μL , and fever (axillary temperature $\geq 37.5^{\circ}\text{C}$) or history of fever in the past 24 hours and written informed consent. Exclusion criteria included malaria danger signs, haemoglobin < 8 g/dL, weight < 5 kg, inability to follow the study procedures, or not a permanent resident in the study area.

Treatment and follow-up

Enrolled patients were monitored daily for the first three days and subsequently followed weekly from day 7 through day 28 for AL treatment and through day 42 for DHA-PPQ treatment. AL was administered twice daily at 0, 8, 24, 36, 48, and 60 hours, while DHA-PPQ was given once daily for three consecutive days, both according to WHO weight-based dosing guidelines. Participants receiving AL were given biscuits at the time of the first daily dose to ensure administration with a fatty meal. During each first daily dosing, patients were observed for 30 minutes post-administration; if they vomited within 30 minutes, the full dose was re-administered, and they were re-observed for an additional 30 minutes. In case the patient vomited again, s/he was withdrawn and referred for hospital care, where parenteral artesunate was given according to the national treatment policy.

The second daily AL dose was usually not observed (late evening), but the study staff would confirm its correct administration with the patient during the next scheduled visit. AL and DHA-PPQ were supplied

by the WHO Geneva Office.

Demographic data, including age, sex, weight and height, were recorded at screening. A medical history was obtained, and physical examination was performed, including assessment of vital signs, malaria signs and symptoms, and adverse events at each scheduled and unscheduled visit. Finger-prick blood samples were collected at each visit (3 blood slides and 2 dry blood spots (DBS) on filter paper (Watman 3MM) for parasitological and molecular evaluations. At Day 0, 14 and 28, an additional drop of blood was collected for direct haemoglobin level measurement (HemoCue Hb 301, SE262 71 Angelholm, Sweden). Moreover, 2ml of venous blood sample was collected from participants who met the enrolment criteria at Day 0, for the *ex vivo* antimalarial susceptibility assay.

Treatment outcome was classified as early treatment failure (ETF), late clinical failure (LCF), late parasitological failure (LPF) or adequate clinical and parasitological response (ACPR) following the standard WHO guidelines (8).

Laboratory analyses

Microscopy

Three blood slides per patient were obtained at enrolment: two thick blood films and one thin blood film. The first thick blood film was stained rapidly (10% Giemsa for 15 min) for initial screening. If the patient was enrolled, the second thick blood film was stained with 3% Giemsa for 45 min. The thin film was used for parasite speciation to exclude mixed infections. Parasite density was estimated by counting with a hand tally counter, the number of asexual parasites by 200 white blood cells (WBCs), assuming 8,000 WBCs per μl of blood. A blood film was considered negative when the examination of 500 WBCs showed no asexual parasites. Giemsa-stained thick and thin blood smears for parasite identification and quantification were read independently by two microscopists. Discordant results (difference in *Plasmodium* species, or in parasite density count > 25%) were reviewed independently by a third microscopist, and parasite density was estimated by averaging the two closest counts. An external quality control by a WHO-accredited senior microscopist was performed on more than half of all slides collected (56.9%, 865/1,519).

Parasite genotyping

Parasite genomic DNA was extracted from paired blood samples (D0 and day of recurrence) collected on filter paper. For each sample, three 6mm spots were punched from a DBS into a 96 well rounded bottom plate. Punched samples were then digested with proteinase K and buffer ATL (1:10) at 56°C for 1 hour, and DNA extracted using the Qiacube extraction robot. All extracted DNA were eluted in 80 μl elution buffer (Qiagen) and subsequently analysed by polymerase chain reaction (PCR) genotyping, targeting *msp1*, *msp2* and *Poly-a* microsatellite. The 3D7 parental line and five knockout parasites lines (3D7, K1, MAD20, R033 and FC27) were used as positive controls (9).

The post-PCR analysis was performed by capillary electrophoresis. For each allelic marker, samples were diluted 1:10 using nuclease-free water (Qiagen) and nested PCR (nPCR) product. Samples for each allelic family were then mixed as follows: Poly α-FAM (1 µL), PfPK2-HEX (1 µL), and TA81-PET (1 µL), combined with 27 µL of nuclease-free water. Subsequently, 1 µL of the mixed sample dilution was added to 9 µL of master mix containing Hi-Di formamide (8.5 µL) and 600 LIZ size standard (0.5 µL) (Thermo Fisher Scientific). The samples were analysed using an ABI SeqStudio Genetic Analyzer (Applied Biosystems).

The WHO 2021 genotyping method (“3/3”) was used to distinguish recrudescence and new infection (10). The 3/3 method classified an infection as a recrudescence if one or more alleles were present at all three genotyped markers on both day 0 and the day of the recurrent malaria infection. Marker scoring was performed using the following criteria: (i) within each dye channel, a minor peak was considered valid if its height was at least one-third that of the dominant peak; and (ii) relative fluorescence unit (RFU) height across dye channels was used to confirm true allele calls. Alleles were considered different between initial and recurrent samples if fragment sizes differed by > 10 bp for *msp1* and *msp2*, and by > 5 bp for microsatellite markers.

Antimalarial resistance genotyping by sequencing

DNA from the DBS of each *P. falciparum* positive sample was amplified using a selective whole genome amplification (sWGA) protocol targeting the parasite genome for enrichment as previously reported (11). Amplicons for specific parasite genomic targets were generated using either the CleanPlex Paragon genomics panel (Paragon Genomics Inc, USA) or the SPOTMalaria *P. falciparum* Amplicon Toolkit Protocol (12). The SPOTMalaria Amplicon toolkit generates 136 amplicons in three pools and the CleanPlex protocol generates 278 amplicons in two pools, with both protocols targeting known mutations in genes associated with antimalarial resistance (*Pfcr*t - codons 72–76 resulting in the wildtype haplotype CVMNK and mutant haplotype CVIET; *Pfmdr1* – wildtype haplotype NYD for mutations at codons 86, 184 and 1246; *Pfdhfr* – wildtype haplotype NCSI and mutant haplotype IRNI at codons 50, 59, 108 and 164; and *Pfdhps* – wildtype haplotype SAKAA at codons 436, 437, 540, 581 and 613) (Supplementary Table 1). Library pools were bead-cleaned (CleanMag® Magnetic Beads or AMPure XP beads), quality checked using TapeStation and Qubit Fluorimeter or qPCR and the purified pool was sequenced on MiSeq (Illumina, San Diego, USA).

Ex-vivo antimalarial susceptibility assay

At a minimum of 0.5% parasitaemia, serum was removed from the venous blood samples and the packed cells suspended in incomplete RPMI medium at 50% haematocrit followed by layering on lymphoprep and centrifuged for 20 mins at 1,500 rpm with no brake, for phase separation. The peripheral blood mononuclear cell (PBMCs) and buffy coat layer containing predominantly white blood cells was removed to deplete the human DNA. Infected red blood cell pellets were washed thrice in incomplete media and adjusted to 2% hematocrit and a density range of 0.1% – 0.5% using uninfected group O red blood cells, with assays conducted in duplicate.

Drugs were initially prepared in 96-well tissue plates at concentrations ten times higher than the desired starting concentration. Serial dilutions were performed in complete growth media, using either two-fold or three-fold dilutions across nine dilution points, following established WHO and standardized *ex-vivo* susceptibility assays protocols (9). Control wells comprised a no-drug column, uninfected red blood cells at 2% haematocrit, and media-only wells for background fluorescence. Each sample well received 10µl of the appropriate drug dilution, and 90µl of the prepared parasite culture. The mixture was then incubated for 72 hours, after which the drug assay plates were removed from standard culture conditions. To assess parasite growth, a 2X SYBR lysis buffer containing SYBR Green stain was prepared. The lysis buffer consisted of 10 mM Tris (pH 7.5), 3 mM EDTA, 0.1% saponin solution, and 2% Triton X-100 solution. The 10,000X stock SYBR Green was diluted in this buffer to achieve a final concentration of 1:5,000. Subsequently, 100µl of this solution were added to each well of the drug-parasite plate, which was then incubated for 30 minutes at 37°C in dark conditions. A Fluorskan Ascent microplate reader (Thermo Scientific) was used to measure fluorescence from the stained parasite DNA in the RBCs, which correlates with parasite growth. Fluorescence measurement was done using a 485-nm excitation filter and a 535-nm emission filter (13).

Sample size and data analysis

The sample size calculation assumed a 5% PCR-adjusted treatment failure rate (based on prior NMCP data), 5% precision, a 95% confidence level, and 20% loss to follow-up. This resulted in a minimum of 88 patients per site. Data were collected using REDCap (version 12.0.20 LTS), then cleaned and analysed in *R software* (version 4.2.2). Data were also entered into the WHO-designed Excel spreadsheet for analysis.

Treatment efficacy was evaluated using a *per-protocol* (PP) analysis and a Kaplan-Meier (K-M) survival analysis to estimate the cumulative risk of treatment failure by day 28 for AL and by day 42 for DHA-PPQ. Adequate clinical and parasitological response (ACPR) was estimated both before and after PCR correction, stratified by site and by drug.

In the PP analysis, only patients who completed follow-up - either until treatment failure or the scheduled end of follow-up - were included, while withdrawals and lost-to-follow-up cases were excluded. In the K-M analysis, all enrolled patients were included and censored at their last recorded visit. For PCR-corrected analyses, patients with undetermined genotyping results were considered as recrudescence.

Changes in mean haemoglobin concentration between baseline and follow-up visits (Days 14 and 28) were assessed separately within each treatment group using paired t-tests. Mean differences with corresponding 95% confidence intervals were reported.

The analysis of drug assay readings for assessing half the maximal inhibitory concentration (IC₅₀) was performed using GraphPad Prism software version 10.2.0. Log transformation of the concentrations represented by fluorescence readouts was used to perform a nonlinear regression test for the resulting dose-response curves analysis.

Custom bioinformatics pipelines developed at the MRCG's Data Science platform were used for amplicon sequence data analyses, using R software environment (Version 4.2.2) available on Github (https://github.com/mrcg-mpb/grcMPB_Package). Single-nucleotide polymorphism (SNP) variant calling of the genotyping by sequencing samples was done using standard GATK pipelines, while microhaplotypes (short DNA segments containing two or more closely linked SNPs) were detected using the Mad4Hatter bioinformatics pipeline (<https://github.com/EPPIcenter/mad4hatter>).

Ethical Considerations

Written informed consent was obtained from all participants or their legal guardians for children below 18 years old, and additional written assent was obtained for children aged 12–17 years. The study was approved by the Gambia Government/MRC Joint Ethics Committee and the London School of Hygiene & Tropical Medicine Ethics Committee (Ref number 28009).

Results

Baseline characteristics of the study participants

Between 28 September 2022 and 15 January 2023, 748 patients with suspected clinical malaria were screened, 321 were assessed for eligibility, and 199 were enrolled. Of these, 53 patients enrolled at Kuntaur Health Centre received a 3-day DHA-PPQ treatment, while 146 patients, including 93 at Brikama- and 53 at Gambissara Health Centres, received AL for 3 days (Fig. 2). Baseline characteristics of study participants are summarized in Table 1. Overall, there were slightly more males than females (57.8%, 115/199), with the largest difference in Kuntaur (67.9%, F102/199) were children aged 5–15 years, with only 2.5% (5/199) under 5 years. At enrolment, the mean axillary temperature was 37.9°C (SD 1.2), mean parasite density was 9,935 (95%CI:(8,247–11,968) parasites/μL, and mean haemoglobin concentration was 12.6 g/dL (SD 1.8) (Table 1).

Table 1

Baseline characteristics of study participants by site and overall. *Geometric mean, 95%CI: 95% Confidence interval, IQR: Interquartile range, SD: Standard deviation

Characteristic	Brikama site (N = 93)	Gambissara site (N = 53)	Kuntaur site (N = 53)	Total (N = 199)
Age, median (IQR)	15 (10, 20)	14 (9, 19)	15 (11, 20)	14 (10, 20)
Age groups, n (%)				
<5y	1 (1.1%)	3 (5.7%)	1 (1.9%)	5 (2.5%)
5-15y	52 (55.9%)	26 (49.0%)	24 (45.3%)	102 (51.3%)
>15y	40 (43.0%)	24 (45.3%)	28 (52.8%)	92 (46.2%)
Gender, n (%)				
Male	49 (45.6%)	30 (56.6%)	36 (67.9%)	115 (57.8%)
Female	44 (44.4%)	23 (43.4%)	17 (32.1%)	84 (42.2%)
Axillary temperature, mean (SD)	37.8 (1.2)	38.3 (1.0)	37.6 (1.2)	37.9 (1.2)
Haemoglobin, mean (SD)	12.6 (1.8)	12.7 (2.0)	12.5 (1.7)	12.6 (1.8)
Parasite density/ μ *, (95%CI)	7,620, (5,981- 9,708)	10,273 (6,995 – 15,087)	15,227 (10,444 – 22,200)	9,935 (8,247 – 11,968)

Primary treatment outcome

A total of 146 participants were enrolled in the AL group in Brikama and Gambissara, of whom 126 (86.3%) completed the 28-day follow-up. In the DHA-PPQ group in Kuntaur, 39 of 53 participants (73.6%) completed the 42-day follow-up (Fig. 2).

In the *per-protocol* analysis, the ACPR for DHA-PPQ was 100% as no treatment failure was observed. Among the AL-treated participants, two late clinical failures (LCFs) occurred in Gambissara, one at day 21 and the other at day 22, along with seven late parasitological failures (LPFs: 5 in Brikama, 2 in Gambissara), resulting in an overall PCR-uncorrected ACPR of 92.9% (95% CI 86.9–96.7%) (Table 2). Genotyping of the nine recurrent infections showed that four of them were new infections, two recrudescence infections, and three were indeterminate. The two recrudescence were identified in Gambissara: one LPF at day 7 and one LCF at day 22, yielding an overall PCR-corrected ACPR of 98.3% (95% CI 96.0–100%). The Kaplan-Meier survival analysis for AL showed a PCR-uncorrected cumulative treatment success rate of 93.0% (95% CI 79.0–97.0%) by day 28, and 98.4% (95% CI 96.0–100%) after

PCR correction. For DHA-PQ, the PCR-corrected treatment success rate remained unchanged at 100% by day 42.

Table 2
Artemether-lumefantrine treatment outcome (*per-protocol* analysis) per site and overall

Total			Brikama		Gambissara	
PCR - uncorrected (day 28)						
Total analysed (N)	126		80		46	
Outcome	n (%)	95% CI	n (%)	95% CI	n (%)	95% CI
ETF	0 (0.0)	0	0	0	0	0
LCF	2 (1.6)	0.2–5.6	0	0	2 (4.3)	0.5–14.8
LPF	7 (5.6)	2.3–11.1	5 (6.3)	2.1–14.0	2 (4.3)	0.5–14.8
ACPR	117 (92.9)	86.9–96.7	75 (93.8)	86.0–97.9	42 (91.3)	79.2–97.6
PCR- corrected (day 28)						
Total analysed (N)	119		75		44	
Outcome	n (%)	95% CI	n (%)	95% CI	n (%)	95% CI
ETF	0	0	0	0	0	0
LCF (day 22)	1 (0.8)	0.0–4.6	0	0	1 (2.3)	0.1–12
LPF(day 7)	1 (0.8)	0.0–4.6	0	0	1 (2.3)	0.1–12
ACPR	117 (98.3)	96.0–100.0	75 (100)	100%	42 (95.5)	89.5–100
95%CI: 95% Confidence interval, ETF-early treatment failure, LCF-late clinical failure, LPF-late parasitological failure, ACPR- Adequate Clinical and Parasitological response						

Secondary treatment outcomes

Fever and Parasite clearance

Parasite clearance was fast, with all patients in both the AL and DHA-PPQ group achieving complete clearance by day 3. Similarly, fever resolved promptly with all febrile patients becoming afebrile by day 2.

Haemoglobin recovery

Mean haemoglobin (Hb) levels declined significantly from baseline to Day 14 in both treatment groups. In the AL group, the mean Hb decreased by 0.77 g/dL (95% CI: -1.05 to -0.48; $p < 0.001$), while in the DHA-PPQ group, the mean decrease was 0.99 g/dL (95% CI: -1.56 to -0.42; $p = 0.002$) (Table 3). Between Day 14 and Day 28, Hb levels increased significantly in both groups, with mean increases of 0.44 g/dL (95% CI: 0.17 to 0.71; $p < 0.001$) in the AL group and 0.75 g/dL (95% CI: 0.31 to 1.19; $p < 0.001$) in the DHA-PPQ group. Overall, from baseline to Day 28, a modest but statistically significant reduction in mean Hb was observed in the AL group (mean change: -0.37 g/dL, 95% CI: -0.67 to -0.07; $p = 0.024$), whereas the reduction in the DHA-PPQ group was not statistically significant (mean change: -0.31 g/dL, 95% CI: -0.80 to 0.17; $p = 0.226$).

Table 3
Haemoglobin recovery over 28 days post treatment measured as mean differences between Day0, 14 and 28, in participants treated with Artemether-lumefantrine (AL) and Dihydroartemisinin-piperaquine (DHA-PPQ)

Timepoints	N	Mean Difference (g/dL)	95% CI	p-value
Artemether-lumefantrine (AL)				
Day 14 – Day 0	124	-0.77	-1.05, -0.48	< 0.001
Day 28 – Day 0	119	-0.37	-0.67, -0.07	0.024
Day 28 – Day 14	121	+ 0.44	0.17, 0.71	< 0.001
Dihydroartemisinin-piperaquine (DHA-PPQ)				
Day 14 – Day 0	40	-0.99	-1.56, -0.42	0.002
Day 28 – Day 0	40	-0.31	-0.80, 0.17	0.226
Day 28 – Day 14	37	+ 0.75	0.31, 1.19	< 0.001

Molecular markers of drug resistance

A total of 143 Day0 samples were analysed across all sites for markers of antimalarial resistance, with 98% successful genotypes across all loci. An overall average of 3.5% of the samples had mixed haplotypes (more than one allele call at a codon) at the different drug resistance loci (*Pfcr*t = 4.7%; *Pfmdr*1 = 3.5%; *Pf dhfr* = 2.3%; *Pf dhps* = 3.3%). Kuntaur, located in the Central River Region had the highest frequency of mixed haplotypes across all drug resistance targets (Fig. 3a). The antimalarial resistance mutation profiles were similar across the three sites, with low frequencies of wildtype haplotypes. Gambissara had the highest frequency of *Pfmdr*1 partial resistance compared to the other two sites, and Brikama had the highest frequency of *Pf dhps* partial resistance (Fig. 3a). Overall, the drug-resistant haplotype CVIET of the *Pfcr*t gene associated with full chloroquine resistance was detected in 60% (84/140) of the infections, and in 7.9% (11/140) as mixed alleles, resulting in an overall haplotype frequency of 67.9% (95/140). For the *Pfmdr*1 gene, resistance mutation at codons 86 and 1246 were

detected in 7.9% (11/140) and 1.4% (2/140) of the infections respectively, while the frequency of mutations at codon 184 was 66.4% (93/140) (Fig. 3b). High frequencies of *Pfdhfr* mutants (96.6% overall) were observed at codons N51I (136/140), C59R (133/140), and S108N (137/140), whereas no mutation was identified at codon I164L (Fig. 3b). For the *Pfdhps* gene, the mutation at codon 437 was the most frequent, *i.e.*, with overall frequency of 83.6% (117/140). The mutation at codon 436 was 31.4% (44/140); mutation at codon 581 was 3.6% (5/140) and at codon 613 it was 12.9% (18/140); all of which contribute to the high frequency of sulphadoxine partial resistance observed. All parasites sequenced had wildtype genotypes for the *kelch13* gene associated with artemisinin resistance. The two recrudescence isolates carried the *Pfmdr1* NFD resistance haplotype. One had the wild-type *Pfcrt* CVMNK background whereas the other harboured the *Pfcrt* CVIET chloroquine-resistant haplotype.

Drug susceptibility of clinical isolates

IC₅₀ values were determined for 23 *P. falciparum* samples that met the minimum threshold of 0.1% parasitemia, against chloroquine (CQ), amodiaquine (AMD), mefloquine (MEF), lumefantrine (LUM), dihydroartemisinin (DHA), and piperazine (PIP). Summary IC₅₀ values for each drug are presented in Table 4. All isolates showed high susceptibility to dihydroartemisinin, translating to a low median IC₅₀ estimate value of 1.29 nM and IQR [1.14; 2.33] (Fig. 4). Of the 23 isolates, nine (39.1%) were resistant to chloroquine, with IC₅₀ values above 100 nM, and 11 isolates (47.8%) were above the median IC₅₀ value for CQ. The IC₅₀ estimates for other drugs spanned a broad range of values, the widest being for lumefantrine followed by piperazine, with median IC₅₀ estimates of 53.53 nM and 76.21 nM, respectively. The *ex vivo* analysis of the two recrudescence infections identified following AL treatment showed that one was chloroquine resistant (IC₅₀ = 327.9 nM), and the other was chloroquine susceptible (IC₅₀ = 8.0 nM), but with a high piperazine IC₅₀ estimate (462.6 nM) of more than five times the median piperazine IC₅₀ for all the isolates tested. The isolate with the *Pfcrt* CVIET showed relatively high IC₅₀ for all drugs including LUM (75.7 nM) except for DHA (1.076 nM), whilst the isolate with the *Pfcrt* CVMNK background had elevated IC₅₀s for all drugs including DHA (4.23 nM).

Table 4
IC₅₀ values of antimalarial drugs tested against clinical isolates (N = 23)

Drug	Median IC ₅₀ (nM)	IQR
Chloroquine (CQ)	69.0	13.11–140.50
Mefloquine (MEF)	48.18	24.75–61.09
Amodiaquine (AMD)	9.28	4.71–15.26
Dihydroartemisinin (DHA)	1.29	1.14–2.33
Lumefantrine (LUM)	53.53	17.11–170.1
Piperaquine (PIP)	76.21	33.76–98.63
IC ₅₀ : Half-maximal Inhibitory Concentration, IQR: interquartile range.		

Discussion

AL and DHA-PPQ remain highly efficacious for the treatment of uncomplicated *Plasmodium falciparum* malaria in The Gambia. The PCR-corrected ACPR for both treatments was higher than the WHO threshold of 90% for continued use as first- and second-line treatments (8). Two late treatment failures in the AL group (one LPF and one LCF) were confirmed as recrudescence infections, yielding a recrudescence rate of 1.7% by day 28. These results are consistent with previous therapeutic efficacy studies conducted in The Gambia (94.3% for AL and 100% for DHA-PPQ in 2018, *unpublished data*) and other West African countries (14–16). A meta-analysis of TES conducted between 2010 and 2019 in Sub-Saharan Africa reported PCR-corrected cure rates of 98.0% for AL and 99.4% for DHA-PPQ, highlighting the sustained high efficacy of these ACTs (16).

Rapid fever and parasite clearance were observed in all participants, with no fever or parasites detected by day 3, indicating preserved efficacy of the artemisinin component in both regimens. Delayed parasite clearance on day 3 is a validated surrogate marker for artemisinin partial resistance, as documented in Southeast Asia and recently in Rwanda, where the Pfk13 R561H mutation was associated with parasitaemia after day 3 in over 15% of treated individuals (5). In contrast, the absence of infection at day 3 in all three sites provides reassuring evidence that ACTs remain fully effective in The Gambia. Molecular analyses further confirmed the absence of validated or candidate Pfk13 resistance mutations, consistent with prior studies in the West Africa region (17, 18). Nevertheless, ongoing therapeutic efficacy monitoring and molecular surveillance remain essential to detect any early shifts in drug response.

Haemoglobin level declined significantly during the first two weeks after treatment, reflecting malaria-associated haemolysis and clearance of parasitized erythrocytes, but partially recovered by day 28, with both AL and DHA-PPQ showing a significant Hb increase between days 14 and 28. This pattern is

consistent with typical post-treatment haematological dynamics in endemic settings and supports the short-term safety of both ACTs regarding anaemia (19)

The high rate of recurrent infection classified as indeterminate is attributable to the low parasite densities observed in recurrent infection. At low parasite densities, genotyping assays may fail to amplify sufficient parasite DNA across all markers, limiting their ability to reliably distinguish recrudescence from reinfection (20). This challenge was further exacerbated by the new 3/3 algorithm, which was unable to generate complete results across all three markers for three of these low-parasitemia samples.

The IC₅₀ estimates of the laboratory strains (chloroquine sensitive Pf3D7 and chloroquine resistant PfDd2 strains) for the antimalarial drugs tested were comparable to those reported in other studies, confirming the reliability of the drug susceptibility assay results (21, 22). The *ex vivo* susceptibility testing reported a wide range of IC₅₀ estimates for lumefantrine, indicating high variability among the 23 isolates and increasing tolerance to the drug, which had been already reported in The Gambia (23, 24). On the other hand, the high susceptibility of our isolates to dihydroartemisinin is in line with the high efficacy of ACTs observed *in vivo*, and is similar to findings from a previous study where the viability of Gambian parasites was observed using a ring survival assay (RSA), following exposure to dihydroartemisinin (23).

The high frequency of *Pfcr* Chloroquine resistance haplotype was surprising though consistent with the reports in the MalariaGEN Pf7 and Pf8 data releases (25, 26). With the withdrawal of chloroquine drug pressure, *P. falciparum* populations have been shown to revert to wild-type *Pfcr* gene in neighbouring countries such as Ghana, Mali, Burkina Faso and in East Africa where chloroquine-sensitive genotypes currently dominate (27, 28). This persistence of chloroquine resistance markers in the absence of chloroquine drug pressure in The Gambia is not fully understood but could be driven by seasonal malaria chemoprevention (SMC) that combines sulfadoxine-pyrimethamine with amodiaquine, or by the use of artesunate-amodiaquine, one of the first line antimalaria treatment in neighbouring Senegal. It is however possible that chloroquine resistant isolates in the region of “far-west Africa” may have gained fitness compensating variants as shown for the S258L variant of *Pfaat1* gene, although these are yet to be fully investigated (29). Drug-resistant haplotypes and alleles for the *Pfmdr1* gene were found at low frequency, except for the Y184F mutation which is associated with reduced susceptibility to lumefantrine (29). However, only 2 isolates with the *Pfmdr1* NFD haplotype resulted in late treatment failure, which limited further genotype-phenotype relationship assessment. However, the chloroquine-sensitive recrudescence isolate showed elevated DHA and LUM IC₅₀ values, which is consistent with the reduced lumefantrine susceptibility and DHA sensitivity as previously reported in Gambian isolates (24, 30). The prevalence of *Pfdhfr* triple mutants was extremely high (96.6%), similar to other West Africa countries (31), probably due to the continuous pressure from SP used for SMC and intermittent preventive treatment in pregnant women.

Study limitations

This study has several limitations. The target sample size was not achieved in Kuntaur and Gambissara, largely due to lower-than-expected malaria incidence and higher refusal rates. In addition, the lost to follow-ups was much higher in Kuntaur (21%) compared to the other two sites (around 10%) mainly due to the larger distances of most villages to Kuntaur Health Centre. These limitations reflect the operational challenges inherent to prospective studies requiring frequent follow-up visits in rural area with remote communities. Another limitation to the study is the overall low parasitaemia observed, which limited the number of samples assayed ex-vivo for drug susceptibility phenotypes.

Conclusion

AL and DHA-PPQ remain highly effective for the treatment of uncomplicated *Plasmodium falciparum* malaria in The Gambia, supporting their continued use as first- and second-line therapies. In the context of emerging artemisinin resistance in East Africa, sustained therapeutic efficacy surveillance is essential for the early detection of reduced drug sensitivity. Strengthening routine pharmacovigilance and periodic TES will be critical to ensure timely updates to national treatment guidelines and to mitigate the risk of resistance spreading across the continent.

Abbreviations

ACT
Artemisinin-based combination therapy
ACPR
Adequate clinical and parasitological response
AL
Artemether-lumefantrine
AMD
Amodiaquine
CQ
Chloroquine
DHA -PPQ
Dihydroartemisinin-piperaquine
ETF
Early treatment failure
IC₅₀
Half maximal inhibitory concentration
IQR
Interquartile range
K-M
Kaplan-Meier
LCF
Late clinical failure

LPF
Late parasitological failure
LTF
Loss to follow-up
LUM
Lumefantrine
MEF
Mefloquine
NMCP
National Malaria Control Programme
PCR
Polymerase chain reaction
PP
Per-protocol
PIP
Piperaquine
RBC
Red blood cell
SMC
Seasonal Malaria Chemoprevention
SP
Sulfadoxine-pyrimethamine
TES
Therapeutic efficacy study
WHO
World Health Organization
WBC
White blood cell

Declarations

Authors contributions

ED, AE and BG developed the protocol and the case report forms. ED and AB coordinated the field implementation. ED, AE, BG, and AS monitored and supervised data collection. BB, HN, NFD, OR, AS and MON performed laboratory analyses, including molecular genotyping and ex vivo drug susceptibility assays. SC performed the external quality control of the microscopy reading. BNAN contributed to the genomic data analysis. ED drafted the first version of the manuscript. All authors contributed to data interpretation, critically reviewed the manuscript, and approved the final version.

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Competing interests

The authors declare that they have no competing interests.

Ethics approval and consent to participate

The study was approved by the Gambia Government/MRC Joint Ethics Committee and the London School of Hygiene & Tropical Medicine Ethics Committee (Ref number 28009). Written informed consent was obtained from all participants or their legal guardians for children below 18 years old, and additional written assent was obtained for children aged 12-17 years.

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Data Availability

The datasets generated and analysed during the current study are available from the corresponding author on reasonable request. Any data sharing will be conducted in accordance with institutional data sharing policies and ethical approvals

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Figures

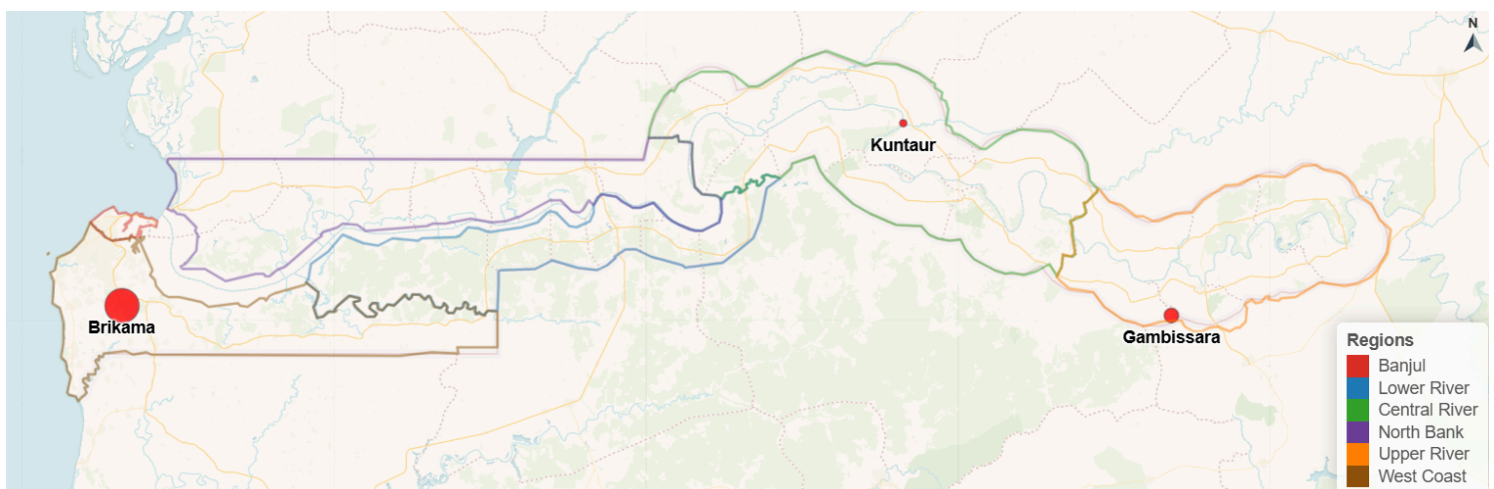


Figure 1

Map of The Gambia showing the six different administrative regions and the three study sites (red dots)

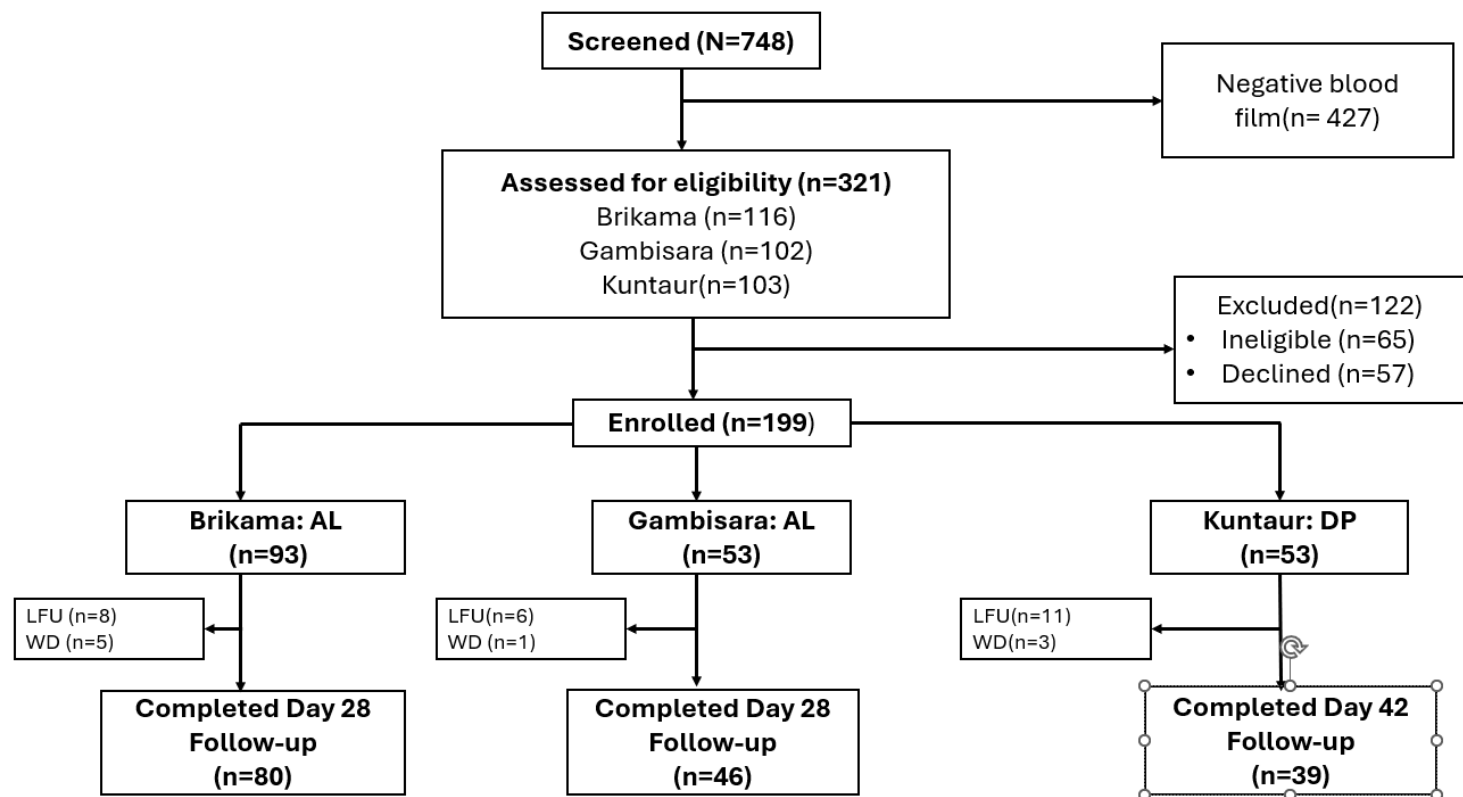


Figure 2

Study profile

AL: Artemether-lumefantrine; DHA-PPQ: Dihydroartemisinin-piperaquine; LTFU: Lost to Follow-Up, WD: Withdrawn

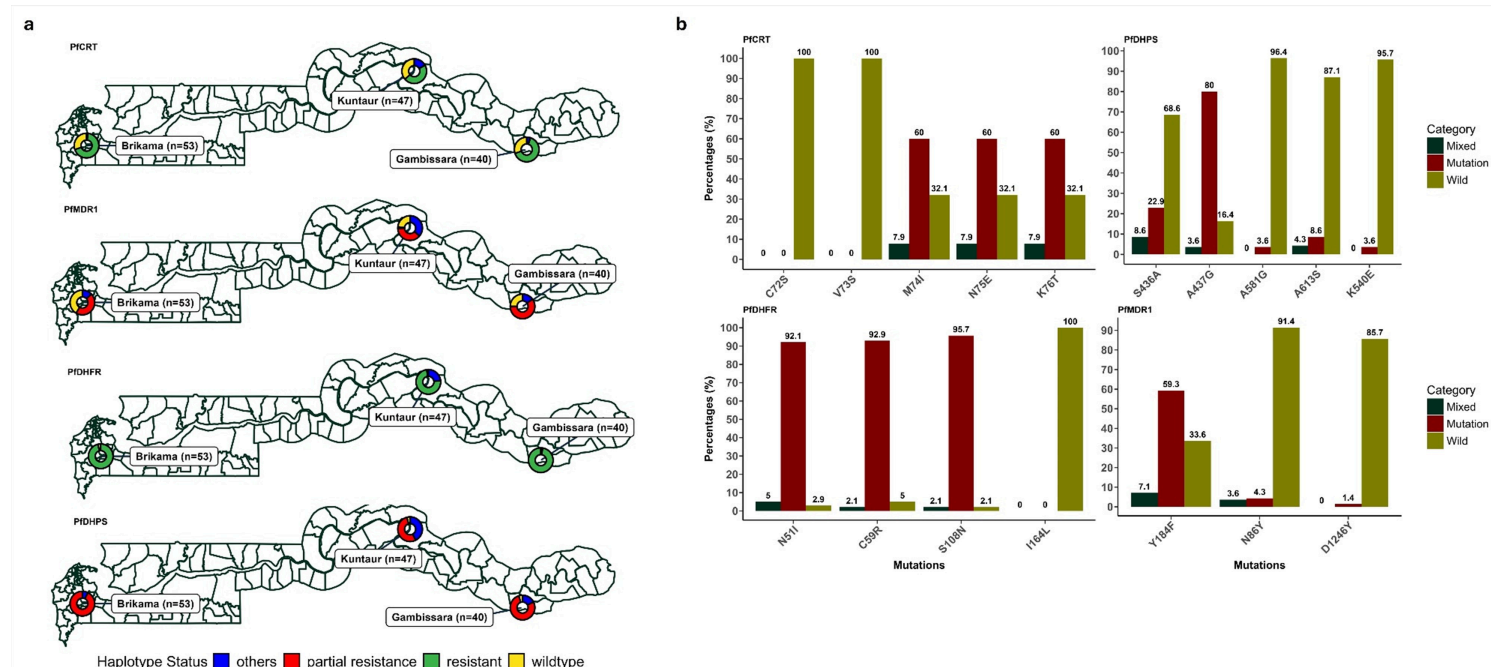


Figure 3

Parasite prevalence of antimalarial resistance to quinolines (PfCRT and PfMDR1) and antifolates (PfDHFR and PfDHPS). (a) Spatial distribution of drug resistance profiles in the three sites; ‘partial resistance’ refers to mutations in more than one genotyped codon and ‘others’ refer to undetermined resistance call due to mixed haplotypes; (b) bar charts of specific mutation frequencies for each codon of associated antimalarial resistance gene.

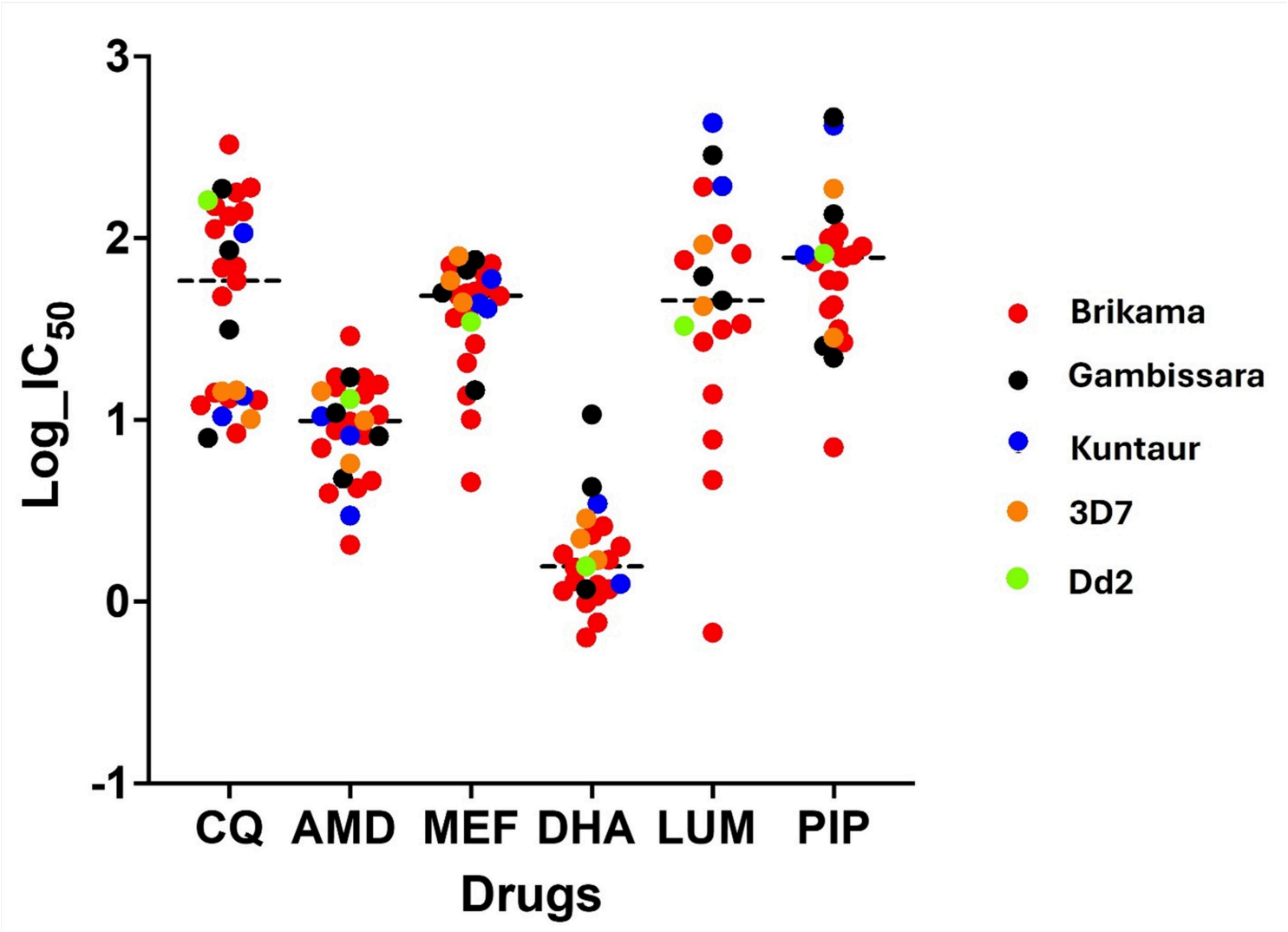


Figure 4

Ex vivo susceptibility of Gambian *P. falciparum* isolates to antimalarial drugs (IC₅₀ values). Each point represents a single isolate. Median IC₅₀ values are shown by the black horizontal dashed lines. Dihydroartemisinin (DHA); lumefantrine (LUM); mefloquine (MEF); piperazine (PIP); chloroquine (CQ). Laboratory strains – 3D7 (drug-sensitive) and Dd2 (drug-resistant) were added as controls.

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