

High Prevalence of Artemisinin-Resistant *Plasmodium falciparum*, Southeastern Sudan

Appendix

***Plasmodium falciparum* malaria in Sudan.** Sudan is a major contributor to malaria in the WHO Eastern Mediterranean Region, accounting for 33% of estimated malaria cases and 44% of estimated deaths (1,2). *Plasmodium falciparum* is the predominant malaria parasite in Sudan, responsible for approximately 95% of malaria cases (3). Malaria transmission in the country is characterized as low to moderate, highly seasonal and occasionally epidemic (3).

From 2017 to 2019, Sudan experienced high malaria incidence, with regional variability and fluctuations driven by environmental and epidemiological factors. The situation has been exacerbated by armed conflict, political instability and economic disruption, leading to a collapse of the health system and an increased burden of malaria (1). In response to a more than 40% increase in malaria incidence (4), Sudan adopted the High Burden to High Impact (HBHI) strategy in 2022 (1,4). However, despite interventions, several biological threats continue to challenge malaria control efforts. These include *P. falciparum* resistance to antimalarial drugs (5–7) and insecticides (8–10), *pfhrp2/3* gene deletions affecting the performance of rapid diagnostic tests (11,12), and the recent invasion of *Anopheles stephensi*, a highly adaptable urban malaria vector (13).

Efforts to map malaria incidence using spatio-temporal methods have provided insights into its spread and intensity (3). Nevertheless, the emergence of drug-resistant malaria strains and vector adaptation remain major challenges.

***Plasmodium falciparum* antimalarial drug resistance in Sudan.** Sudan has faced challenges with antimalarial drug resistance, leading to changes in treatment policies.

Chloroquine resistance emerged in the late 1970s (14), prompting a switch to artemisinin-based combination therapies (ACTs) in 2004-2006 (15). Studies have shown high prevalence of mutations in drug resistance genes, particularly in *Pfcr*, *Pfmdr-1*, *dhfr*, and *dhps* (15,16). The *Pfcr*-76T and *Pfmdr1*-86Y mutations associated with chloroquine resistance have decreased, while *dhfr* and *dhps* mutations linked to sulfadoxine-pyrimethamine resistance have increased (15). No *Pfkelch13* mutations associated with artemisinin partial resistance (ART-R) were detected in central and eastern Sudan (15,17). However, novel *Pfkelch13* mutations not linked to ART-R (F375S, K378R, D389N, K430K, E433D, P443P, N594K) were observed in the Blue Nile State (17).

Antimalarial drug efficacy studies in Sudan between 2004 and 2018 have shown varying results for different treatments. Chloroquine and sulfadoxine-pyrimethamine monotherapies demonstrated high failure rates, necessitating a shift to artemisinin-based combination therapies (ACTs) (18). Artesunate plus SP (AS+SP) and artemether-lumefantrine (AL) became first-line and second-line treatments, respectively (14). While AL maintained high efficacy (96.8-100%), AS+SP showed declining effectiveness in some regions, particularly eastern Sudan (14). A meta-analysis revealed overall high treatment success (98%) for ACTs, with AL slightly outperforming AS+SP (5). However, a comparative study in eastern Sudan found AS+SP to have marginally higher efficacy than AL after PCR correction (19). As reported by Adam et al., in 2023 (6) high efficacy rates (97%) of AL was confirmed in Sudan, except for Sennar site in the 2017–2018 where a lower cure rate (92.1%) was detected which was still above the 90% threshold for treatment policy change (20).

Supplementary materials and Methods

Screening and recruitment of study participants. Eligible patients were at least one year of age, presenting with suspected uncomplicated *P. falciparum* infection with fever (axillary temperature $\geq 37.5^{\circ}\text{C}$) and/or history of fever in the past 24h.

Enrolled patients were assigned to receive a supervised standard 3-day course of artemether-lumefantrine AL (IPCA 20 mg artemether and 120 mg lumefantrine per tablet; 6 tablets for 5kg to <15kg; 12 tablets for 15 to <25kg; 18 tablets for 25 to <35kg; 24 tablets for $\geq 35\text{kg}$) in 2017 according to the manufacturer's dosing schedule.

Informed written consent was obtained from the adult patient or parent/caretaker of a child prior to recruitment. If a patient or parent/caretaker was illiterate, consent was obtained in the presence of a witness (witness' signature and the thumbprint of the participant's parent/caretaker). Children aged 12 years and above also signed informed assents in addition to their parents' consent. The study was approved by the relevant committees of the Ministry of Health in Gezira State, which serves as the central committee in Sudan (MU/2019).

Sample collection. Patients were monitored clinically and parasitologically at recruitment. Thick and thin smears were obtained by finger prick prior to the initiation of treatment and blood were spotted onto filter papers for molecular studies. Patients were advised to come to the health facilities for unscheduled visits if symptoms developed.

DNA extraction and molecular analysis. Parasite DNA was extracted from dried blood spots (DBS) collected pretreatment according to the Zainabadi et al. protocol (21).

Molecular signatures associated with antimalarial drug resistance. Parasite DNA extracted from blood samples collected on day 0 was analyzed for molecular markers associated with antimalarial drug resistance. We assessed point mutations in the *Pfkelch13* propeller domain (codons 430–720), which are linked to artemisinin partial resistance (ART-R). Additionally, we examined mutations in the following genes associated with resistance to different antimalarial drugs: *Pfprt* (codons 72–76, 93, 97, 145, 218, 343, 350, 353, and 356) – associated with 4-aminoquinoline resistance; *Pfmdr1* (codons 86, 184, 1034, 1042, and 1246) – associated with amino-alcohol resistance; *dhfr* (codons 16, 50, 51, 59, 108, and 164) – associated with pyrimethamine resistance; *dhps* (codons 431, 436, 437, 540, 581, and 613) – associated with sulfadoxine resistance. We also tested for deletions in *hrp2* and *hrp3*, which can impact the performance of HRP2-based malaria rapid diagnostic tests (RDTs) (22).

PCR Amplification and Sequencing Protocol. Amplicons from the targeted regions (presented in the table below) were generated using multiplex nested PCR assays with indexed primers containing specific barcodes. A total of 4 µL of PCR products from each sample was pooled (96 samples per pool) to optimize sample usage in downstream steps. Each pool underwent AMPure XP bead purification (Beckman Coulter Life Sciences, Villepinte, France) following the manufacturer's protocol to remove dNTPs, salts, primers, and primer dimers. The purified amplicons were then analyzed on a Fragment Analyzer (Agilent, Agilent Technologies,

Les Ulis, France) to assess DNA quality, and DNA concentrations were measured using fluorometric quantitation (Qubit, Thermo Fisher, Scientific, Illkirch-Graffenstaden, France). The pooled libraries were denatured with NaOH (0.1 N final concentration) and diluted with hybridization buffer before sequencing. Sequencing was performed using the MiSeq v2 reagent kit (300-cycle, Illumina Inc., Evry-Courcouronnes, France), following the manufacturer's recommendations (22).

Appendix Table 1. List of the targeted regions and number of amplicons generated by multiplexing nested PCR assays

Gene Name	Gene ID	Chr.	Gene location	SNPs/CNVs detection	No. of amplicons
<i>Pfkelch13</i>	PF3D7_1343700	13	1,724,817 - 1,726,997 (-)	SNP from codon 440 to 727	12
<i>chloroquine resistance transporter</i>	PF3D7_0709000	7	403,222 - 406,317 (+)	Codons 72-76, 93, 97, 145, 218, 343, 350, 353	5
<i>multidrug resistance protein 1</i>	PF3D7_0523000	5	957,890 - 962,149 (+)	Codons 86, 184, 1034, 1042, 1246	5
<i>bifunctional dihydrofolate reductase-thymidylate synthase</i>	PF3D7_0417200	4	748,088 - 749,914 (+)	Codons 16, 50, 51, 59, 108, 164	5
<i>hydroxymethyldihydropterin pyrophosphokinase-dihydropteroate synthase</i>	PF3D7_0810800	8	548,200 - 550,616 (+)	Codons 436, 437, 540, 581, 613	4
<i>histidine-rich protein 2</i>	PF3D7_0831800	8	1,373,212 - 1,376,988 (-)	Deletion	2
<i>histidine-rich protein 3</i>	PF3D7_1372200	13	2,840,236 - 2,842,840 (-)	Deletion	2

Bioinformatics Processing and Variant Calling. Raw sequencing reads were demultiplexed and quality-trimmed at a Phred score threshold of 30 to ensure high-quality data. Primer sequences were removed from the 5'-end of the reads to eliminate potential primer bias in downstream analyses. Base calling was performed by aligning reads against a custom database, which included the *Plasmodium falciparum* 3D7 reference genome (v45). Bioinformatic analyses were conducted using CLC Genomics Workbench 25 (Qiagen, Courtaboeuf, France) for sequence alignment, variant calling, and quality assessment. To ensure accuracy and reliability, laboratory reference *P. falciparum* strains with known alleles were included as controls. These strains comprised Dd2, 7G8, HB3, and 3601 (a Cambodian culture-adapted parasite), as detailed in the table below (22).

Appendix Table 2. List of the alleles of the parasite strains used as controls

Gene name	Dd2 allele	7G8 allele	HB3 allele	3601 strain
<i>Pfkelch13</i>	Wild-type	Wild-type	Wild-type	C580Y
<i>chloroquine resistance transporter</i>	CVIETTHFIMCGT	SVMNTTHFIMCGT	CVMNKTHFIMCGI	CVIETTHFIMCGT
<i>multidrug resistance protein 1</i>	YYSND	NFCDY	NFSDD	NFSND
<i>bifunctional dihydrofolate reductase-thymidylate synthase</i>	ACIRNI	ACICNI	ACNCNI	ACIRNI
<i>hydroxymethyldihydropterin pyrophosphokinase-dihydropteroate synthase</i>	IFGKAS	ISGKAA	ISAKAA	IAGEAA
<i>histidine-rich protein 2</i>	Deletion	NA	NA	NA
<i>histidine-rich protein 3</i>	NA	NA	Deletion	NA

Position of amino acid corresponds to codons 72, 73, 74, 76, 93, 97, 145, 218, 343, 350, 353 and 356 for pfprt, to codons 86, 184, 1034, 1042 and 1246 for pfmdr-1, to codons 16, 50, 51, 59, 108, 164 for dhfr and to codons 431, 436, 437, 540, 581, 613 for dhps.

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