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Full Length Research Paper

Molecular surveillance of antimalarial resistance in Chad

Issa Mahamat Souleymane[1,3], Adam Batrane, Kerah Hinzoumbé Clément[2], Mahamat Saleh Issakha Diar[1], Kodbesse Boulotigam[1], Mahamat Idriss Djaskano[1], N'garadoum Olivier[1,] Riguide Mbaïssanadjé[1], Mahamat Moussa Hassane Taisso[3], Honoré Djimrasengar[4], Fissou Henri[8], Djidi Ali Sougoudi[3,5], Mahamat Adano[6], Issa Ali Hagar[6], Hassoumi Manah[7], Kaltam Abderaman Yakhoub[5], Ngam-Asra[3], Ongram Kouleta II[8,] Djimadoum Djionadji[8,] Meram Atteib[8], Kaltam Abderaman Yakhoub[5], Oumaima Mahamat Djarma[3,5], Mahamat Ali BOLTI[3,5], and Brahim Boy Otchom[3]

[1,3]Chad National Malaria Control Program, N'Djamena, Chad (NMCP). [2] Projet d'appui à la lutte antipaludique au Tchad (PALAT/PNUD. [3]Faculty of Science and Human Health, University of N'Djamena, Chad. [4]World Health Organization, N'Djamena, Chad. [5]Renaissance National University Hospital / Infectious Diseases Department, N'Djamena, Chad [6]Ecole Nationale des agents sanitaires et Sociaux (ENASS). [7]Hôpital de l'amitié Tchad-Chine. [8]Ministry of public health.

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Abstract

Background: The emergence of *Plasmodium falciparum* resistance to antimalarial drugs remains a major public health concern in Africa. In Chad, molecular data on resistance markers are scarce. This study aimed to assess the prevalence of mutations associated with resistance to sulfadoxine-pyrimethamine (SP), chloroquine (CQ), amodiaquine (AQ), and artemisinin. **Methods:** Blood samples infected with *P. falciparum* were collected from 21 health districts across Chad. The samples were collected from patients with uncomplicated malaria, and parasite DNA was extracted from dried blood spots. Molecular markers of antimalarial resistance were investigated by nested PCR amplification of *pfdhfr*, *pfdhps*, *pfcrt*, *pfmdr1*, and the *pfk13* propeller domain, followed by sequencing and polymorphism analysis. **Results:** A total of 2053 P. falciparum isolates were successfully sequenced and analyzed. Mutations in *pfdhps* were detected at codons A437G (36.6%) and K540E (10.3%), while the triple *pfdhfr* mutant $I^{51}R^{59}N^{108}$ was highly prevalent (97.1%). Combined *pfdhfr/pfdhps* haplotypes showed 35.2% quadruple and 3.9% quintuple mutants. Moderate frequencies of *pfcrt* K76T (37.9%) and *pfmdr1* N86Y (20.6%) suggest partial persistence of CQ/AQ resistance. Only one non-synonymous k13 mutation (Asp-605) was observed at 0.5% frequency. **Conclusion:** In Chad, molecular markers indicate widespread resistance to sulfadoxine–pyrimethamine but preserved susceptibility to artemisinin, supporting the continued use of ACTs while underscoring the need for sustained resistance surveillance. These findings provide critical baseline data on antimalarial drug resistance in Chad, supporting current ACT-based treatment policies while emphasizing the need for continued molecular surveillance to inform national malaria control strategies.

Keywords: *Plasmodium falciparum*, molecular markers, antimalarial resistance, Chad, SP, CQ, AQ, ACTs.

Introduction

Malaria remains a major public health problem worldwide, with African countries accounting for the vast majority of cases and deaths, particularly among children under five (WHO, 2024). Despite substantial progress achieved through the deployment of artemisinin-based combination therapies (ACTs), the emergence and spread of resistance to antimalarial drugs continues to threaten efforts to control and eliminate malaria. Resistance to chloroquine and sulfadoxine-pyrimethamine (SP) is documented by molec-

Corresponding author. Email: issa.mahamatsoul@gmail.com

ular markers such as *pfcrt* (Plasmodium falciparum chloroquine resistance transporter), *pfmdr1*(Plasmodium falciparum multidrug resistance 1), *pfdhfr* (Plasmodium falciparum dihydrofolate reductase), and *pfdhps* (Plasmodium falciparum dihydropteroate synthase) mutations. In particular, the high prevalence of the triple *pfdhfr* ($I^{51}R^{59}N^{108}$) mutant and quadruple and quintuple *pfdhfr/pfdhps* haplotypes has been associated with reduced SP efficacy, even in settings where SP is currently limited to intermittent preventive treatment (Agaba et al., 2024; WHO, 2024). Artemisinin resistance, characterized clinically by delayed parasite clearance and molecularly by mutations in the *pfk13* gene, has been firmly established in Southeast Asia. More recently, increasing attention has been drawn to the detection of *pfk13* polymorphisms in Africa, raising concerns about the potential emergence of partial artemisinin resistance on the continent (Rosenthal et al., 2024; Zheng et al., 2024). Although validated resistance-associated *pfk13* mutations such as C580Y, R539T, Y493H, and I543T remain rare in Africa, sporadic reports from East and Central Africa underscore the need for sustained molecular surveillance (Agaba et al., 2024;). In Chad, malaria transmission remains intense, and ACTs are widely used as first-line treatment. However, data on the molecular landscape of antimalarial resistance, particularly regarding *pfk13* and partner drug resistance markers, remain limited. Continuous monitoring of molecular markers is therefore essential to detect early signals of resistance, guide national treatment policies, and support malaria control strategies in high-transmission settings (WHO, 2009). In this context, the present study aimed to evaluate the prevalence of molecular markers associated with resistance to artemisinin and related drugs in Plasmodium falciparum isolates collected in Chad, with a particular focus on *pfk13*, *pfcrt*, *pfmdr1*, *pfdhfr*, and *pfdhps* polymorphisms.

Methodology

A cross-sectional study was conducted in Chad to assess molecular markers of antimalarial drug resistance in *Plasmodium falciparum*. Blood samples were collected from patients with uncomplicated malaria, and parasite DNA was extracted from dried blood spots. Target genes associated with resistance to sulfadoxine–pyrimethamine (*pfdhfr* and *pfdhps*), chloroquine and partner drugs (*pfcrt* and *pfmdr1*), and artemisinin (*pfk13* propeller domain) were amplified by nested PCR and sequenced.

Period, study areas and sample collection

The samples were collected during the national malaria indicator survey conducted from October to November 2017. A total of 2,600 samples were collected in 19 regions (N'Djamena, Kanem, Lake Chad, Barh El Gazal, Wadi Fira, Ouaddai, Hadjer Lamis, Batha, Guera, Salamat, Sila, Mandoul, Moyen Chari, Logone Oriental, Logone Occidental, Tandjilé, Mayo-Kebbi Est, Mayo-Kebbi Ouest, and Chari Baguirmi). Blood samples were collected from patients of all ages. Patient blood was collected on Whatman® 3 MM filter paper. Molecular analyses were performed in the laboratories of the Malaria Research and Training Center (MRTC) in Mali **(**Figure I).

DNA extraction

DNA was extracted using the QIAGEN kit, according to the manufacturer's instructions. After cutting the confetti into a 1.5 mL microtube, 180 µL of ATL buffer was added and incubated at 85°C for 10 minutes. Twenty µl of proteinase K was added and incubated at 56°C for one hour. A volume of 200 µl of AL buffer was added and incubated at 70°C for 10 minutes, then 200 µl of ethanol was added and centrifuged at 8000 rpm for 1 minute. A volume of 500 µl of AW1 buffer wash solution was added, followed by 500 µl of AW1 buffer, then centrifuged at 8000 rpm for 1 minute. Next, 500 µl of AW2 buffer was added, then centrifuged at 14,000 rpm for 3 minutes. Finally, 150 µL of AE buffer was added, incubated at room temperature for 1 minute, then centrifuged at 8000 rpm for 1 minute. The extracted DNA was stored at -20°C.

DNA amplification

For the *pfdhfr* gene, during the first cycle, 5 µL of DNA extract was added to 20 µL of a mixture composed of: 11.875 µL of PCR water, 5 µL of buffer (2.5 µl mM) (ref. SIG-P2317-1.5ML), 5 µL of dNTP (200 µM) (ref. INV-10297018), 2.5 µL of primer (1 µM) FR519-A: GCGCGCTAATAACTACACATTTA, 0.25 µL of primer (1 µM) FR519-B: CCCGGGCTCTTATATTTCAATTT and 0.25 µL of Taq polymerase (5 U/µL). The amplification program was as follows: 95°C for 2 min, 92°C for 30 s, 45°C for 30 s, 65°C for 45 s, 72°C for 15 min. For the second cycle, the mixture consisted of: 15,875 µl of PCR water, 5 µl of buffer (1X/2.5 mM), 2.5 µl of dNTPs (200 µM), 0.25 µl of primers (1 µM) FR51-D: CTAGGAAATAAAGGAGTATTACCATGGAAATGGA, 0.25 µL primers (0.25 µL) FR59-D: ATTTTTCATATTTTGATTCATTCACATATGTTGTAACT GTAC and 0.125 µL Taq polymerase. The amplification program is the same. For the *pfdhps* gene, during an initial cycle, 5 µL of extracted DNA was added to 20 µL of a mixture composed of 11.875 µL of PCR water, 5 µL of buffer (3.5 mM), 2.5 µL of dNTP (200 mM), 0.25 µL of specific primers (1 µlM) for codon 437 (PS500-A: GGGCCCAAACAAATTCTATAGTG, PS500-B: GGCCGGTGGATACTCATCATATA), 0.25 µL of specific primers (1 µLM) 540 (PS500-A: GGGCCCAAACAAATTCTATAGTG, PS500-B: GGCCGGTGGATACTCATCATATA) and 0.125 µL of Taq

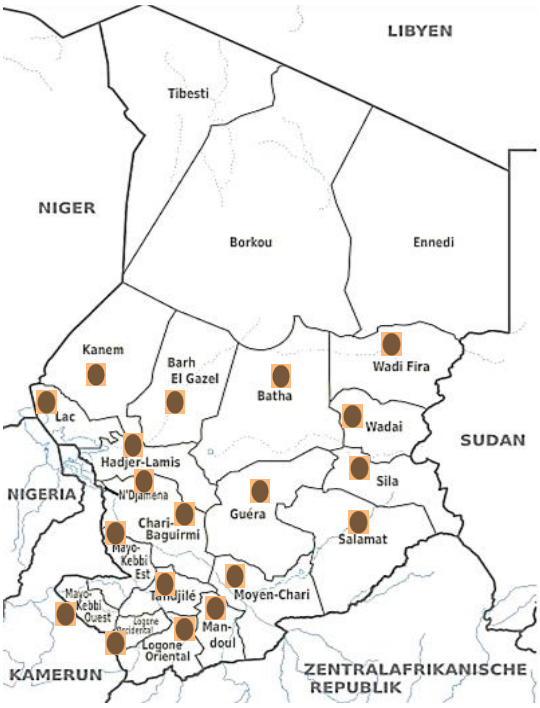

Figure 1: sample collection sites.

polymerase 5U/µL. Same amplification program as for *pfdhfr*. The second cycle, mixture, and amplification program are identical to those of the first cycle. These 437 codon-specific primers (PS400-D1: TGTTCAAAGAATGTTTGAAATGA, PS400-D2: CCATTCTTTTTGAAATAATTGTAAT) and 540 (PS500-D1: GCGCGCGTTCTAATGCATAAAAGAGG, PS500-D2: CGGGTAAGAGTTTAATAGATTGATCAGCTTTCTTC) were used. For the *pfpfcrt* and pfmdr1 genes, the amplification program is the same as for the *pfdhfr* and *pfdhps* genes. For the first cycle, the primers used were *pfcrt* (P1: CCGTTAATAATAAATACACGCAG and P2: CGGATGTTACAAAACTATAGTTACC) and *pfmdr1* (MDR-1: ATGGGGTAAAGAGAGAAAGA, MDR-2: AACGCAAGTAATACATAAAGTCA). For the second cycle, the primers used were *pfcrt* (D1: TGTGCTCATGTGTTTAAACTT, D2: CAAAACTATAGTTACCAATTTTG) and *pfmdr1* (MDR-3: TGGTAACCTCAGTATCAAAGAA, MDR-4: ATAAACCTAAAAAGGAACTGG).

Statistical analysis

Following data entry in Microsoft Access, statistical analyses were performed using STATA 13.0 (StataCorp, USA). Molecular sequence analyses were conducted using EMBOSS 6.6.0 and MAFFT 7.407 under Bio-Linux, with sequence alignments visualized and manually curated using BioEdit version 7.2.5

Results

Wild-type alleles predominated across *pfdhps*, *pfcrt*, and *pfmdr1*, although moderate frequencies of resistance-associated mutations, particularly *pfdhps* 437G and *pfcrt* 76T, were observed in the study population (Table I).

The *pfdhfr* triple mutant haplotype predominated in the study population, while the *pfdhfr/pfdhps* quadruple mutant was present at moderate frequency; the quintuple mutant haplotype was rare (Table II).

Sequencing of the *k13* propeller domain revealed four point mutations occurring at low frequencies, including three synonymous substitutions and one non-synonymous mutation at codon 605 (Glu→Asp), detected in 0.5% of isolates. The low frequency and predominance of synonymous mutations in the *k13* propeller domain, together with the absence of WHO-validated resistance-associated mutations, suggest preserved susceptibility to artemisinin in the study population, although continued molecular surveillance remains warranted (Table III).

Table II: Allele frequencies of resistance genes.

Gene	Mutation	Allele	n/N (%)
Pfdhps	A437G	Gly	751/2053 (36,6)
		Ala	1302/2053 (63,4)
Pfdhps	*K540E*	Glu	208/2014 (10,3)
		Lys	1806/2014 (89,7)
Pfcrt	*K76T*	Thr	744/1961 (37,9)
		Lys	1217/1961 (62,1)
pfmdr1	*N86Y*	Tyr	120/1819 (20,6)
		Asn	1699/1819 (79,4)

Table II: Resistance haplotypes and genotypes.

Haplotype	Genotype	n/N (%)
Double mutant (*pfcrt/pfmdr1*)	^{76}T/^{86}Y	120/1819 (6.6)
Triple mutant (*pfdhfr*)	I^{51}R^{59}N^{108}	1968/2026 (97.1)
Quadruple mutant (*pfdhfr/pfdhps*)	I^{51}R^{59}N^{108}/G^{437}	699/1978 (35.3)
Quintuple mutant (*pfdhfr/pfdhps*)	I^{51}R^{59}N^{108}/G^{437}E^{540}	74/1898 (3.9)

Table III: mutations observed on the *K13 propeller* gene.

Gene	Codon position	Nucleotide change	Amino acid change	Mutation type	n/N (%)
k13 propeller	567	GAG → GAA	Glu → Glu	Synonymous	2/201 (0.1)
	589	GTC → GTG	Val → Val	Synonymous	2/201 (0.1)
	605	GAA → GAC	Glu → Asp	Non-synonymous	1/201 (0.5)
	616	GCC → GCG	Ala → Ala	Synonymous	1/201 (0.5)

Discussion

Molecular markers of antimalarial drug resistance have rarely been investigated in Chad.

The present study provides an updated overview of molecular markers associated with antimalarial drug resistance in *Plasmodium falciparum* isolates collected in Chad. Overall, the results indicate a high prevalence of resistance markers to sulfadoxine–pyrimethamine (SP), persistent but declining chloroquine-related mutations, and an apparent absence of validated artemisinin resistance markers, supporting the continued efficacy of ACTs in this setting. This study provides significant findings on the genes *pfdhfr*, *pfdhps*, *pfcrt*, *pfmdr1*, and *k13-propeller*, which are respectively associated with resistance to sulfadoxine-pyrimethamine (SP), chloroquine (CQ), amodiaquine (AQ), and artemisinin (Nuwa et al. 2025; Mombo-Ngoma et al. 2025; Figueroa-Romero et al. 2022 ; Kerahb et al. 2006). Regarding the *pfdhps* gene, the frequency of the mutant allele Gly-437 and the wild-type

allele Ala-437 were 36.6% and 63.4%, respectively. The A437G mutation was present at a moderate frequency, suggesting a noticeable but not dominant circulation of sulfadoxine-resistant parasites. Similarly, the frequency of the K540E mutation was 10.3% for the mutant allele (Glu-540) and 89.7% for the wild-type allele (Lys-540), indicating that this mutation remains rare. In agreement with previous data from Pala (Issa MS et al. 2018), where the K540E mutation was not detected and the frequency of A437G was low (16.7%), our findings confirm that *pfdhps* mutations are still limited in Chad. These mutations are known to play a key role in SP resistance, particularly when combined with mutations in the *pfdhfr* gene (Kümpornsin et al. 2014 ; Sharma et al. 2015). Previous studies have shown that a high frequency of the double mutant *pfdhps* (A437G + K540E) confers a strong resistance to SP (Sowunmi et al. 2017; Plowe C V et al. 2017). In our study, the frequencies of mutant alleles remain lower than those of the wild types, suggesting that the double *pfdhps* mutation (A437G + K540E) à well-established marker of high-level SP resistance has not yet spread widely within the parasite in Chad. In contrast, several neighboring and East African countries have reported a continuous increase in SP resistance (Venkatesan et al. 2013; Muleta et al. 2017). Therefore, although the current levels of *pfdhps* mutations in Chad are moderate, their presence could signal the early stages of a potential decline in SP efficacy or an increased risk of resistance expansion. Consistent with other reports, mutations at codons 437 and 540 of the *pfdhps* gene are considered critical determinants of SP resistance (Venkatesan et al. 2013 ; Bungei et al. 2025; Matondo et 2014). Furthermore, for the *pfcrt* K76T gene, the frequency of the mutant allele Thr-76 was 37.9%, compared to 62.1% for the wild-type allele Lys-76. The K76T mutation, a key molecular marker of chloroquine resistance, remains present at a moderate level, suggesting a partial persistence of resistance despite the official withdrawal of the drug. In this study, the frequencies of mutant alleles *pfcrt* K76T and *pfmdr1* N86Y were 37.9% and 20.6%, respectively, while the combined double mutant (K76T/N86Y) represented only 6.6% of the isolates. The *pfcrt* K76T mutation is recognized as the principal molecular marker of chloroquine resistance, whereas *pfmdr1* N86Y is associated with cross-resistance to chloroquine and amodiaquine. The moderate frequency of the K76T mutant observed in Chad suggests that chloroquine resistance persists to some extent, even after its withdrawal from national malaria treatment policy.

Similarly, the low prevalence of the N86Y mutant reflects a tendency toward reversion to the chloroquine-sensitive genotype, a phenomenon previously observed in several African countries following the suspension of chloroquine use (Frosch et al. 2014; Fidock et al. 2000). The low frequency of the *pfcrt*/*pfmdr1* double mutant (6.6%) observed in this study further supports the hypothesis of a regression in drug resistance and suggests a progressive restoration of parasite sensitivity to chloroquine and amodiaquine. This trend mirrors findings from Senegal, Tanzania, and Malawi, where the re-emergence of the wild-type K76 allele was associated with the return of chloroquine clinical efficacy after its withdrawal (Achan et al. 2011; Kublinet al. 2003). The rarity of the *Pfcrt*/*Pfmdr1* double mutant (6.6%), which combines the K76T and N86Y markers responsible for chloroquine and amodiaquine resistance, suggests that resistance to these drugs is declining, likely due to their replacement by artemisinin-based combination therapies (ACTs) as first-line treatment. This trend could open the possibility of reconsidering chloroquine and amodiaquine as potential options for the treatment of uncomplicated malaria in Chad, provided that continued molecular and clinical monitoring confirms the sustained return of sensitivity. The triple mutant pfdhfr ($I^{51}R^{59}N^{108}$) was found at a high frequency of 97.1% in this study. In contrast, Issa et al. (2015) reported a much lower prevalence of this triple mutant genotype in Pala (3.33%). This substantial increase indicates a widespread pyrimethamine resistance, likely reflecting the strong selective pressure exerted by the continued use of sulfadoxine-pyrimethamine (SP), particularly through intermittent preventive treatment in pregnancy (IPTp-SP). Elsewhere, the presence of the *pfdhfr* triple mutant has been consistently associated with high-level pyrimethamine resistance, one of the SP components. Several authors have identified the triple and quintuple mutants as the major determinants of SP resistance (Adeel et al. 2016; Muleta et al. 2017; Madanitsa et al. 2016). In this study, the quadruple mutant *pfdhfr*/*pfdhps* $I^{51}R^{59}N^{108}/G^{437}$) was detected at a frequency of 35.2%, indicating an established but not generalized resistance to SP. The addition of the *pfdhps* A437G mutation to the *pfdhfr* triple mutant is known to enhance SP resistance (Issa MS et al. 2018). The quintuple mutant *pfdhfr*/*pfdhps* $I^{51}R^{59}N^{108}/G^{437}E^{540}$) represented only 3.9% of isolates. These combined haplotypes are recognized as the most predictive molecular markers of high-level SP resistance. Their low frequency in Chad suggests that high-level SP resistance remains uncommon, though close monitoring is warranted. In other words, the combined *pfdhfr*/*pfdhps* haplotypes (quadruple and quintuple) indicate a gradual progression of SP resistance. The moderate prevalence of the quadruple mutant reflects an established but not widespread resistance, whereas the rarity of the quintuple mutant suggests that SP efficacy remains largely preserved in Chad. Studies conducted in East Africa (Kenya, Tanzania, Uganda) have reported quintuple mutant frequencies exceeding 60%, which correlate with high clinical failure rates (Naidoo et al. 2013; who, 2023). In contrast, in West and Central Africa, these values remain below 10% (Coulibaly et al. 2001; Boussougou-Sambe et al. 2018). Thus, the situation observed in Chad

reflects an intermediate resistance profile, marking a gradual but still controlled evolution of SP resistance. These findings support the continued use of SP in preventive strategies (IPTp-SP and SMC) while emphasizing the need for ongoing molecular surveillance to detect early expansion of the quintuple mutant, often a precursor to complete clinical resistance.

Analysis of the *k13-propeller* gene revealed a single non-synonymous mutation at position 605, resulting in an amino acid substitution, whereas the three other observed mutations were synonymous. The *Pfkelch13* gene encodes a protein involved in the parasite's ubiquitin–proteasome pathway, and specific mutations within its propeller domain are recognized as major molecular markers of partial artemisinin resistance (Ariey et al. 2013; Straimer et al. 2014). The most strongly associated substitutions (C580Y, R539T, Y493H, I543T, and F446) have been primarily identified in Southeast Asia, while in Africa, confirmed mutations such as R561H, A675V, and C469Y remain rare (WHO, 2013). The mutation detected at position 605 does not belong to the list of WHO validated mutations known to confer clinical resistance to artemisinin. It is therefore likely to represent a neutral or emerging polymorphism, with no demonstrated impact on parasite susceptibility to artemisinin derivatives. Several African studies (Uwimanaet al. 2020; Balikagala et al.2021) have shown that most variations observed in the *k13* gene correspond to natural polymorphisms, without correlation to delayed parasite clearance. Nevertheless, the detection of a unique non-synonymous mutation warrants continued vigilance, as some emerging mutations initially observed at low frequencies, such as R561H in Rwanda were later linked to reduced in vitro sensitivity and slower parasite clearance. In this context, it is recommended to confirm the precise nature of the amino acid substitution (A605V, E605K), perform Ring-stage Survival Assays (RSA) on isolates carrying this variant, and pursue longitudinal molecular surveillance of the k13 gene in different regions of the country. In the absence of other functionally significant mutations, the present findings suggest that *P.falciparum* parasites in Chad remain sensitive to artemisinin derivatives, consistent with observations from most Central African countries. However, the establishment of continuous molecular monitoring of the *k13-propeller* gene remains crucial to detect, at an early stage, any emergence or spread of variants associated with reduced efficacy of artemisinin-based combination therapies (ACTs).

In Chad, the rarity of *k13* propeller mutations and the absence of WHO-validated artemisinin resistance markers indicate preserved ACT efficacy, in line with observations from Central Africa, while supporting the need for continued molecular surveillance in a high-transmission setting.

Conclusion

This study highlights moderate but persistent resistance to sulfadoxine-pyrimethamine (SP), characterized by a high prevalence of the *pfdhfr* triple mutant ($I^{51}R^{59}N^{108}$) and moderate frequency of *pfdhps* A437G, while K540E remains rare. CQ and AQ resistance appear to be declining, as indicated by reduced *pfcrt* and *pfmdr1* mutation frequencies. No validated *k13-propeller* mutations were observed, confirming the continued efficacy of ACTs. Molecular resistance in *P. falciparum* in Chad remains limited but dynamic. Strengthening national molecular surveillance and local laboratory capacity is essential to prevent future resistance expansion.

Limitations

This study is limited by its cross-sectional design, the absence of therapeutic efficacy data, and restricted geographic coverage. In addition, only selected molecular markers were analyzed, and low-frequency variants may have been underestimated.

Perspectives

Future research should focus on longitudinal and nationwide molecular surveillance integrated with therapeutic efficacy studies to detect emerging antimalarial resistance and guide malaria control strategies in Chad.

Findings for public health policy

These findings provide critical baseline data on antimalarial drug resistance in Chad, supporting current ACT-based treatment policies while emphasizing the need for continued molecular surveillance to inform national malaria control strategies.

Abbreviations

SP : Sulfadoxine-Pyrimethamine ; CQ : Chloroquine ; AQ : Amodiaquine ; SNPs : Single Nucleotide Polymorphisms : RSA : Ring-stage Survival Assays ; Ala (A): Alanine ; Arg (R): Arginine; Asn (N): Asparagine; Asp (D): Aspartate ou acide aspartique ; Cys (©): Cysteine; GLu (E): Glutamate ou acide glutamique; Glu (G): Glycine; Lys (K): Lysine; Val (V) : Valine; Tyr (Y): Tyrosine; Phe (F): Phénylalanine; Thr (T: Thréonine; pfcrt: Plasmodium falciparum chloroquine résistance transporter; pfmdr 1: Plasmodium falciparum multidrug résistance1; pfdhfr: Plasmodium falciparum dihydrofolate reductase; pfdhps: Plasmodium falciparum dihydropteroate synthetase; K13 propeller: Kelch13 propeller; pf: Plasmodium falciparum; ACT : Artemisinin-based combination therapies; NMCP **:** National Malaria Control Program ; MSPP: Ministry of Public Health and

Prevention; GF: Global Fund; MRTC: Malaria Research and Training Center; DNA: Deoxyribonucleic acid ; PCR: Polymerase chain reaction; WHO: World Health Organization.

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Authors' contributions

IMS, ABM, KHC: article writing; MMHT, MSID, KB, MID, HD: protocol design and writing; NO, MA, IAH, HM, RM: supervisors; MAB, DAS, OMD, KAY: protocol correction and validation.

Competing interests

The authors declare no conflict of interest.

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