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Original Article

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Molecular surveillance of artemisinin resistance-linked *PFK13* gene polymorphisms in Adamawa State, Nigeria

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Abstract

***Background & aims*:** The evolution and spread of *Plasmodium falciparum* malaria parasite capable of evading antimalarials, particularly artemisinin (ART), is a prime concern for malaria control. Mutations in the *P. falciparum Kelch 13* (*Pfk13*) gene confer resistance to artemisinin, necessitating molecular surveillance of *Pfk13* mutations. This study is aimed at investigating artemisinin resistance linked *Pfk13*-propeller polymorphisms in clinical isolates of *P. falciparum* from three Local Government Areas (Yola North, Numan and Mubi North), of Adamawa State, Nigeria.

***Materials & methods*:** A total of 240 symptomatic malaria patients were recruited for this study. Eighty febrile patients diagnosed with uncomplicated *P. falciparum* malaria attending two major selected healthcare facilities in each of the three Local Government Areas were used. *P. falciparum* parasite was identified by Rapid Diagnostic Tests (RDTs) and Microscopy. DNA extraction and nested PCR were performed on positive samples to amplify the *Pfk13* propeller domain. Sequencing and sequence analysis were conducted to check for mutations at validated codon positions.

***Results*:** Out of the 240 samples collected, RDT revealed 100% to be positive for *P. falciparum* while microscopy confirmed *P. falciparum* presence in 214 samples (89.17%). Extraction and amplification of *Pfk13* gene were successful in 163 samples (67.92%). Out of 163 successfully amplified samples, no validated mutations linked to artemisinin resistance were found, but the A578S mutation was detected in 15.09% of the analyzed samples.

***Conclusion*:** The absence of *Pfk13* gene mutations indicates the sensitivity of the parasites in this study location to artemisinin treatments, but the mutant A578S observed needs to be investigated to determine its functional relevance in the *Pfk*13 propeller-domain. However, continuous surveillance and research are crucial to maintain these successes and address any future challenges posed by drug resistance.

***Keywords*:** Artemisinin resistance, Malaria, Molecular surveillance, PFK13 gene

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Introduction

Antimalarial drugs have been central to malaria control efforts, particularly in sub-Saharan Africa, where *Plasmodium falciparum* malaria remains the deadliest form of the disease (1). Resistance to chloroquine necessitated the adoption of artemisinin-based combination therapies (ACTs), which remain the gold standard for malaria treatment globally (2). ACTs combine a fast-acting artemisinin derivative with a slower-acting partner drug to enhance efficacy and reduce the emergence of resistance. Artemisinin derivatives, such as artemether, artesunate, and dihydroartemisinin, reduce the parasite load by 10,000 times during the 48-hour erythrocytic cycle, with the remaining parasites eliminated by the partner drug (3).

Since their introduction and scale-up in 2005, artemisinin-based therapies have significantly reduced malaria morbidity and mortality in endemic regions, bringing global malaria elimination back onto the agenda (4). Derived from the leaves of *Artemisia annua*, artemisinin, and its derivatives act by inhibiting *Plasmodium falciparum* phosphatidylinositol-3-kinase (PfPI3K), providing rapid parasite clearance with minimal adverse effects (5). Six ACTs, including Artesunate-Amodiaquine (AS+AQ) and Artemether-Lumefantrine (AL), are recommended by the World Health Organization (WHO) for treating malaria globally (6). Despite these advancements, *P. falciparum* resistance to all major antimalarial drugs, including artemisinin, poses a significant threat to malaria control and eradication goals (7).

Artemisinin resistance (ART-R), characterized by delayed parasite clearance following treatment, first emerged in Southeast Asia between 2006 and 2008 and is linked to non-synonymous single nucleotide polymorphisms (NS-SNPs) in the *P. falciparum Kelch 13* (*Pfk13*) gene (8). Mutations such as C580Y, R539T, and Y493H are associated with ART-R and prolonged parasite survival (9). Although ART-R mutations are prevalent in Southeast Asia, recent studies have identified low-frequency mutations in Africa, such as R561H and P574L, with potential resistance implications (10,11).

In Nigeria, which bears approximately 25% of Africa's malaria cases, ACTs remain the cornerstone of malaria treatment (10, 12). However, molecular surveillance of resistance marker genes, such as *Pfk13*, is limited. While studies from Southeast, Southwest, and Northwestern Nigeria have provided some insights, data from Northeastern Nigeria, including Adamawa State, remain scarce. Understanding *Pfk13* polymorphisms is critical for detecting emerging resistance and informing malaria treatment policies in this region. This study investigates *pfk*13 gene polymorphisms in Adamawa State to identify potential artemisinin resistance and contribute to regional malaria control strategies.

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Materials & Methods

Study Area

This study was conducted in August 2023 in three Local Government Areas (LGAs) of Adamawa State, Nigeria: Yola North, Numan, and Mubi North. Yola North (9°14′N 12°29′E), the state capital, covers 13,840.83 km² with a population of 1,255,691. Numan (9°10′N 12°55′E) spans 2,193 km² with a population of 91,549, while Mubi North (10°16′N 13°16′E) covers 1,212 km² with a population of 151,515. These areas are characterized by tropical climates, seasonal rainfall, and high malaria endemicity. The LGAs were each selected to represent the three geopolitical zones of the state, high malaria prevalence, and accessibility to healthcare facilities for sampling collection (Figure 1).

Inclusion and Exclusion Criteria

Males and females of all age groups diagnosed with uncomplicated malaria were included in this study. However, pregnant women, individuals with complicated malaria, and those with a history of any recent treatment with antimalarial drugs were excluded from the study.

Sampling

The convenience sampling method was applied in this study. Participants were selected based on ease of access, availability at the time of research, and willingness to participate in the research. A total of 240 symptomatic malaria patients were recruited for this

study. Eighty febrile patients diagnosed with uncomplicated *P. falciparum* malaria attended two major selected healthcare facilities in each of the three Local Government Areas mentioned above. Following the informed consent of the participants, parent or guardian, they were tested with malaria rapid diagnostic test (RDT) kit and microscopy was also carried out. Dried blood spots (DBS) of positive samples were made on 3MM Whatman filter paper.

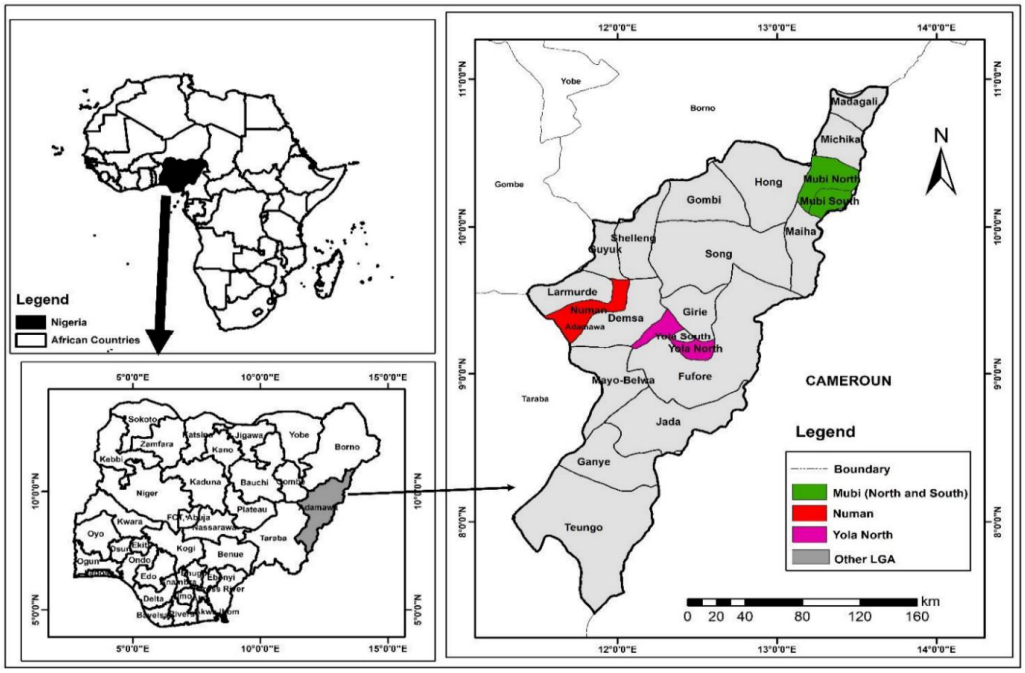

Fig. 1. Map of Adamawa state by the right, map of Nigeria showing Adamawa state by the bottom left and map of Africa showing Nigeria by the top left

Extraction of *P. falciparum* DNA from Positive Samples

P. falciparum DNA was extracted from the positive samples using the Quick-DNA Mini Prep (Zymo-Spin) extraction kit based on the manufacturer's instructions (Zymo Research).

PCR Amplification of the *Pfk13* Gene

DNA samples were first screened with *P. falciparum*-specific primers to confirm parasite presence. Positive samples were then amplified for the *K13* gene propeller domain using a nested polymerase chain reaction (PCR) protocol based on Ariey et al. (9), with modifications to thermocycling conditions. The first-round primers (5'-GGGAATCTGGTGGTAACAGC-3' and 5'-CGGAGTGACCAAATCTGGGA-3') amplified a 2095 bp fragment (1724435–1726531 bp on chromosome 13, GenBank CP017003.1). Second-round primers (5'-GCCTTGTTGAAAGAAGCAGA-3' and 5'-GCCAAGCTGCCATTCATTTG-3') targeted an 849 bp region (1724469–1725318 bp, coding region 444–691 in the propeller domain).

Both reactions used 2.6 µL template DNA (first-round product for the second reaction), 14 µL 2× Taq PCR mix (New England Biolabs, UK), 0.7 µL of each primer (20 µmol/L), and ddH2O to 25 µL. Thermocycling for the first round included 95°C for 2 min, 30 cycles of 95°C for 30 s, 56°C for 90 s, 72°C for 90 s, and a final extension at 72°C for 10 min. Second-

round conditions were similar, with annealing at 60°C. Amplicons were visualized by gel electrophoresis.

Gel Electrophoresis of the Nested PCR Products

The products of Nested PCR, 2095bp (long Fragment) and 849bp (Short Fragment) were detected by 1.5% agarose gel electrophoresis; the agarose was procured from Cleaver Sci. Ltd. (France). Agarose gel was treated with Ethidium bromide dye that was purchased from Applied Biological Materials Inc. (Canada) for Visualization of amplicons on the gel documentation system. DNA was eluted in nuclease-free water (10).

Sequencing

Successful DNA amplicons of the nested PCR were sent to Inqaba BioLabs. (Ibadan) for Sanger sequencing.

Data Analysis

Sequence electropherograms obtained were trimmed and edited with the help of Chromas. Sequence alignment and translation of nucleotide sequence to amino acid sequences were done using Geneious Prime (Version 2023.2) bioinformatics data analysis tool, and the results were expressed as percentages.

Results

Identification of *P. falciparum* and DNA Extraction

Out of the 240 samples collected, RDTs detected *P. falciparum* in 100% of samples, microscopy confirmed it in 89.17%, reflecting potential issues with technician expertise or parasitemia levels. Extraction and amplification of the *Pfk13* gene were successful in 163 samples representing 67.92% (Table 1).

Table 1. Identification of *P. falciparum* from samples collected across selected healthcare facilities in Yola North, Numan, and Mubi North of Adamawa State

Healthcare facility	Samples collected	RDT	Microscopy	*P.f* PCR-confirmed (positive)
Specialist Hospital (SH) Yola	40	40 (100%)	39 (97.5%)	26 (65%)
Major Aminu UHC (MA) Yola	40	40 (100%)	37 (92.5%)	21 (52.5%)
General Hospital Numan	40	40 (100%)	35 (87.5%)	31 (77.5%)
Sabon Pegi Clinic Numan	40	40 (100%)	33 (82.5%)	28 (70%)
General Hospital Mubi	40	40 (100%)	37 (92.5%)	28 (70%)
Lokuwa Clinic Mubi	40	40 (100%)	33 (82.5%)	29 (72.5%)
Total	240	240 (100%)	214 (89.17%)	163 (67.92%)

Detection of Single Nucleotide Polymorphisms

Analysis of sequences revealed that validated codon positions (F446I, N458Y, C469Y, M476I, Y493I, R539T, I543T, P553L, R561H, P574L, and C580Y) were uniformly wild type across all samples, suggesting no resistance-associated SNPs in the study population.

However, the A578S mutant, not associated with artemisinin resistance but prominent in African isolates, was observed in 8 samples, 3 from Yola North LGA and 5 from Mubi North LGA representing 15.09 % of the analyzed total samples (53). This is summarized in Table 2.

Table 2. Single nucleotide polymorphisms of *Pfk13* sequences for the six selected healthcare facilities across Adamawa State

Gene (codon)	Genotypes	Specialist Hospital (SH)Yola		Major Aminu UHC (MA)Yola		General Hospital Numan (GHN)		Sabon Pegi Clinic Numan (SPC)		General Hospital Mubi (GHM)		Lokuwa Clinic Mubi (LCM)	
		n	%	n	%	N	%	n	%	n	%	n	%
Pfk13 (F446I)	Wild	10	100	8	100	9	100	8	100	9	100	9	100
	Mutant	0	0	0	0	0	0	0	0	0	0	0	0
Pfk13 (N458Y)	Wild	10	100	8	100	9	100	8	100	9	100	9	100
	Mutant	0	0	0	0	0	0	0	0	0	0	0	0
Pfk13 (C469Y)	Wild	10	100	8	100	9	100	8	100	9	100	9	100
	Mutant	0	0	0	0	0	0	0	0	0	0	0	0
Pfk13 (M476I)	Wild	10	100	8	100	9	100	8	100	9	100	9	100
	Mutant	0	0	0	0	0	0	0	0	0	0	0	0
Pfk13 (Y493I)	Wild	10	100	8	100	9	100	8	100	9	100	9	100
	Mutant	0	0	0	0	0	0	0	0	0	0	0	0
Pfk13 (R539T)	Wild	10	100	8	100	9	100	8	100	9	100	9	100
	Mutant	0	0	0	0	0	0	0	0	0	0	0	0
Pfk13 (I543T)	Wild	10	100	8	100	9	100	8	100	9	100	9	100
	Mutant	0	0	0	0	0	0	0	0	0	0	0	0
Pfk13 (P553L)	Wild	10	100	8	100	9	100	8	100	9	100	9	100
	Mutant	0	0	0	0	0	0	0	0	0	0	0	0
Pfk13 (R561H)	Wild	10	100	8	100	9	100	8	100	9	100	9	100
	Mutant	0	0	0	0	0	0	0	0	0	0	0	0
Pfk13 (P574L)	Wild	10	100	8	100	9	100	8	100	9	100	9	100
	Mutant	0	0	0	0	0	0	0	0	0	0	0	0
Pfk13 (C580Y)	Wild	10	100	8	100	9	100	8	100	9	100	9	100
	Mutant	0	0	0	0	0	0	0	0	0	0	0	0
A578S	Wild	7	70	8	100	9	100	8	100	6	66.7	7	77.8
	Mutant	3	30	0	0	0	0	0	0	3	33.3	2	22.2

Discussion

Identification of *P. falciparum* and DNA Extraction

Pfk13 polymorphism is the variations in the *Kelch 13* (*k13*) gene of the *P. falciparum* parasite, which is associated with resistance to artemisinin, a critical component of malaria treatment. The *k13* gene encodes a protein that is crucial for the parasite's survival and proliferation. Mutations in this gene can lead to changes in the structure and function of the protein, thereby conferring resistance to artemisinin-based therapies (13).

The emergence of artemisinin-resistant strains of *P. falciparum* poses a significant challenge to malaria control and eradication efforts. *Pfk13* polymorphisms are a key marker for monitoring and understanding the

spread of artemisinin resistance. Studies have identified several specific mutations in the *k13* gene, such as C580Y, Y493H, and R539T, that are associated with delayed parasite clearance and treatment failure (14).

The results from the identification and DNA extraction of *P. falciparum* from clinical isolates provide significant insights into the detection methods of malaria in the study region. The RDT results indicated a 100% presence of *P. falciparum* in the samples collected from all the health facilities (Table 1). However, the discrepancy between RDT results and microscopic examination underscores the importance of confirmatory testing. Microscopy, while more labor-intensive, remains the gold standard for malaria diagnosis and can identify mixed infections and lower parasite densities that RDTs might miss (15).

The slightly lower detection rates by microscopy compared to RDTs may be attributed to several factors, including the skill level of laboratory technicians, the quality of the blood smears, and the inherent limitations of microscopic diagnosis in detecting low parasitemia. These findings align with studies that advocate for the use of both RDTs and microscopy to improve diagnostic accuracy in clinical settings (16).

The successful extraction of *P. falciparum* DNA with a high percentage of the samples (77.5%) from one of the health centers demonstrates the effectiveness of the extraction protocols used (Table 1). Agarose gel electrophoresis confirmed the integrity of the extracted DNA, which is crucial for subsequent molecular analyses such as PCR and sequencing. These results are in line with previous studies that have reported high success rates of DNA extraction from clinical samples using similar methodologies (14).

The effectiveness of RDTs in initial screening, combined with the confirmatory power of microscopy and molecular diagnostics, provides a robust framework for accurate diagnosis and effective treatment. Furthermore, the successful extraction of parasite DNA paves the way for genetic studies that can explore drug resistance patterns and contribute to the development of targeted interventions.

PCR amplification targeting the *Pfk13* gene propeller domain was successful in 67.92% of the samples collected after PCR identification of *P. falciparum* (Table 1). The efficiency of amplification, as indicated by the presence of PCR products approximately 849 base pairs in size, reflects the quality of the extracted DNA and the robustness of the PCR protocols used. The amplification success rates observed in this study are consistent with findings from other regions. A study by Ménard et al. (17) reported amplification success rates of around 75% for the *Pfk13* gene in samples from Southeast Asia, where the prevalence of artemisinin-resistant *P. falciparum* strains is high. Similarly, similar amplification success rates were noted by Kamau et al. (18) in African samples, indicating that the methods used in this study are reliable and effective for detecting *Pfk13* polymorphisms across different settings.

Detection of Single Nucleotide Polymorphisms

The current findings revealed that all examined codons (446, 458, 469, 476, 493, 539, 543, 553, 561, 574, and 580) in the *Pfk13* gene propeller domain retained their wild-type sequences. This finding indicates the absence of known artemisinin resistance-associated mutations in the isolates studied. The findings align with several studies conducted within Nigeria, which have reported the absence of *Pfk13* mutations associated with artemisinin resistance. For instance, a study conducted in Gombe State, which borders Adamawa State, found no mutations in the *Pfk13* gene at validated positions linked to artemisinin resistance (19). Similarly, studies conducted in Kano State (10) and Lagos State (20) also reported no mutations in the *Pfk13* gene at these validated positions.

Moreover, a retrospective, cross-sectional, community-based study carried out in three Nigerian states (Enugu, Plateau, and Kano) by Ajogbasile et al. (21) corroborates the findings of this study. Additionally, a study conducted in Southwestern Nigeria found no mutations in the *Pfk13* propeller domain, suggesting that artemisinin resistance has not yet emerged as a major problem in these areas (22).

In neighboring West African countries, similar trends have been observed. Studies in Ghana and Burkina Faso have reported the predominance of wild-type *Pfk13* sequences, with minimal occurrences of resistance-associated mutations (23, 24). These findings suggest a consistent pattern across West Africa, where ACTs remain effective.

Comparative data from East and Central Africa present a slightly different scenario. In countries like Uganda and Kenya, studies have detected a few *Pfk13* mutations, though these have not yet compromised the efficacy of ACTs (25, 26). In contrast, reports from the Democratic Republic of Congo indicate emerging resistance with mutations such as R561H and A675V appearing more frequently (11). These regional differences within Africa highlight the importance of localized surveillance and tailored public health strategies.

Outside Africa, particularly in Southeast Asia, the situation is more concerning. The high prevalence of *Pfk13* mutations, such as C580Y, R539T, and Y493H, has been well-documented in countries like Cambodia, Thailand, and Myanmar, where they are associated with delayed parasite clearance and increased treatment failures (27). These mutations have led to significant challenges in malaria control and necessitated changes in treatment policies.

However, apart from the validated *Pfk13* mutations not detected in this present study the most prominent SNP observed among sub-Saharan African countries like Ghana, Kenya, Gabon, DRC, Uganda, Cameroon, and Mali (A578S) which changes the amino acid from a non-polar Alanine (GCT) to a polar Serine (TCT) (18), though not associated with artemisinin resistance was observed in eight (8) out of the total samples analyzed in this study. Three SNPs were identified in the samples collected from Yola North, specifically from Specialist Hospital. In Mubi North, five SNPs were detected, with three from General Hospital Mubi and two from Lokuwa Clinic Mubi. However, this SNP was not found in any of the samples collected from Numan. The presence of the mutant allele (A578S) might be attributed to commercial activities in those regions where it was observed like the International Cattle Market in Mubi which harbors different people from different parts of Africa. This mutation can alter the shape of the *Pfk13* protein in the regions it has been observed. Although this allele (A578S) was not previously associated with clinical or *in vitro* resistance to artemisinin a study by Hawkes et al., (28) associated it with prolonged parasite clearance in Ugandan children who had severe malaria and were treated with intravenous artesunate (28). Also, its closeness to the C580Y-validated mutation makes it significant. Therefore, more attention must be paid to this mutation as it is emerging to be the most prominent mutation observed in the *Pfk13* gene in sub-Saharan Africa (21).

The prominent African allele (A578S) observed in this present study corresponds with the finding of Igbasi et al., (20) in Lagos State in which 1 (0.5%) sample out of 195 samples analyzed exhibit the allele and is also in line with the study conducted by Ajogbasile et al., (21) in the isolates obtained from Enugu State but all observed in low frequencies. But this result is contrary to the findings reported by Muhammad et al., (19) who reported zero prevalence of A578S in Gombe State bordering the State of this present study. Ndwiga et al., (29) in their review of the frequencies of *P. falciparum K13* artemisinin resistance mutations in Africa reported that A578S has been detected in fourteen (14) countries across Africa at frequencies of up to 11% (29). While A578S is not directly associated with artemisinin resistance, its increasing frequency in sub-Saharan Africa, including Adamawa, suggests potential evolutionary significance.

The absence of *Pfk13* mutations in the study area is reassuring, suggesting that artemisinin-based therapies continue to be effective in treating *P. falciparum* infections in Adamawa State. However, the findings also emphasize the necessity for continuous molecular surveillance to detect any emerging resistance early. Continuous molecular surveillance should be integrated into Nigeria's malaria control program to preempt resistance emergence. Regular monitoring will ensure timely intervention and prevent the spread of resistant

strains, safeguarding the efficacy of current treatment regimens.

Conclusion

In conclusion, this surveillance study, although restricted to a limited number of field samples, revealed the absence of *Pfk13* propeller-validated mutations associated with artemisinin resistance thereby confirming the sensitivity of the parasites in this study location to artemisinin treatments. Therefore, any artemisinin-therapy failure observed in this study area may not be attributed to artemisinin resistance. However, the presence of A578S mutation close to the validated C580Y needs to be further explored and thoroughly investigated to determine its functional relevance in the *pfk13* propeller domain because of its rising frequency in Africa. Future research should focus on *in vitro* studies of A578S to determine its functional impact on the *pfk13* protein and explore its role in parasite survival.

Acknowledgments

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Ethical statement

The ethical clearance to conduct this study was obtained from Adamawa state Ministry of Health with reference no: S/MoH/1131/1.

Data availability

The supporting data of this study is always obtainable on request from the corresponding author.

Conflict of interest

Authors declared that there is no conflict of interest.

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Author contributions

Author MSJ designed the study and wrote the first draft of the manuscript; authors YU and AAP performed the experiments; author AA conducted the statistical analysis and authors HAZ and AUW managed the analyses of the study and literature searches. All authors read and approved the final manuscript.

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