Original Research Article

Non-synonymous mutations Associated with *Plasmodium falciparum*Artemisinin Resistant Gene (*Pfkelch13*) in Malaria cases, Jos Nigeria

ABSTRACT

Aim: The aim of this study was to explore the *Plasmodium falciparum* Artemisinin Resistant Gene (*Pfkelch13*) in malaria cases in Jos and profilethe nature of the mutations. The *Plasmodium falciparumkelch13* gene is a potential molecular marker for tracking artemisinin-resistant malaria parasites.

Study Design: The Study Design was Experimental

Place and Duration of Study: The study was conducted within Jos North, Jos, Plateau State, Nigeria between October 2019 and January 2021.

Methodology: Thirty-six clinically screened 2 plus (++) and above positive malarial whole blood samples were collected from Bingham University Teaching Hospital, Jos, Plateau State, Nigeria in EDTA bottles after being granted ethical approval. The DNA extraction was carried out using Zymo Research extraction kits according to the manufacturer's instructions. Detection of the *Plasmodium genus*, *Plasmodium falciparum* and *Pfkelch13* gene in the samples was done using PCR technique and gel electrophoresis. PCR amplicons were sequenced and bioinformatics software was used to analyze the sequences for mutations.

Results: From the results, only 41.67% of the positive samples collected were confirmed positive for the *Plasmodium genus*. Out of these, 93.33% were positive for *Plasmodium falciparum*. The *PfKelch13* (*Pfk13*) gene was detected in 78.57% of the *Plasmodium falciparum*. Non-synonymous mutations (S695C, C696M, C696H, H697S, H697V, F698I, 699L and 699S were observed at the amino acids level but didn't affect the structural conformation of the proteins.

Conclusion: Our findings suggest that the classical method of detecting malaria is not reliable compared to the PCR technique. Non-synonymous mutations on the *PfKelch13* gene (S695C, C696M, H697S, L699, C696H, H697V, F698I and S699) which could have the potential to cause resistance to artemisinin drugs that may not have been reported in the Jos human population were observed in this study.

Keywords: Plasmodium falciparum,Mutations,Pfkelch13 gene, Malaria

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1. INTRODUCTION

Malaria is a vector-borne infectious disease [1] caused by parasites of the genus Plasmodium [2]. It is transmitted by the bite of an infected anopheles female mosquito [3]. It is also a critical public health challenge, historically being responsible for the deaths of millions, particularly young children and expectant mothers [4–6]. Approximately 92% of the global

malaria burden occurred in sub-Saharan Africa [7]. There are five different species of Plasmodium that cause malaria in humans, *Plasmodium falciparum* is responsible for most of the mortality [2,8,9]. Since the 1940s, there have been continuous attempts to halt the spread of malaria and this has succeeded in Europe, North America, parts of Asia and Latin America, however, not in Sub-Saharan Africa where about 80% of the annual malaria patients are found [10].

Over time, great efforts devoted to the discovery and development of antimalarial drugs gave rise to several effective drugs. However, there are setbacks which include drug resistance issues [11]. Over the last century, all the antimalarial drugs deployed worldwide have inevitably suffered resistance by *P. falciparum* parasites, particularly in Southeast Asia [12]. Therefore, drug resistance and treatment failures are envisaged to be among the challenges to achieving the elimination of malaria in Nigeria [13].

Artemisinin resistance is defined, clinically, as delayed parasite clearance and a half-life for parasite clearance greater or equal to 5 hours [12,14]. Among all molecular markers, the most important breakthrough is the identification of an artemisinin-resistant genetic marker of the *PfKelch13* propeller domain (*Pfk13*-propeller) by Ariey and teammates in 2014 with the reference sequence found in PF3D7_1343700 [15]. The *Pfk13*-propeller mutation is currently proposed as a useful molecular marker for large-scale surveillance of artemisinin resistance [15]. Mbengue and teammates in 2016 demonstrated the presence of the C580Y mutation on the *Pfk13* gene led to decreased polyubiquitination of the *P. falciparum* phosphatidylinositol-3-kinase (*PfPI3K*) and increased production of the phosphatidylinositol-3-phosphate [PI3P] [12]. *PfKelch13* and the Ring-stage Survival Assay (RSA) have been used as tools to track resistance to artemisinin and its derivatives in endemic areas. *Pfk13*-propeller mutations associated with artemisinin resistance were mainly found in Southeast Asia [16,17]. The mutations on the *PfKelch13* gene regulate the dynamics of the export of *PfEMP1* [the major parasite virulence determinant] to the host red cell. [18]. The M476I, C580Y, R539T, Y493H, I543T and P574L in the propeller domain of the *PfKelch13* gene (PF3D7_1343700) were associated with in-vitro resistance to artemisinin. [19]. The *PfKelch13* resistance markers that have been reported as non-synonymous include N458Y, R539T, E556D, P574L, R575K, C580Y, S621F, Y493H, R539T and I543T [20]. However, the major mutations are C580Y, Y493H, R539T, I543T and N458Y which were observed in all isolates with the slow clearance phenotypic trait [20]. The principal mutations observed in African isolates were C580Y seen in two samples from Cameroon and Y493H seen in one sample from Ghana [20].
Therefore, this study was designed to explore the*Plasmodium falciparumkelch13*gene sequences in malaria cases in Jos, Nigeria. This is because it is a potential molecular marker for tracking artemisinin-resistant malaria parasites. Mutation in this gene is currently proposed as a useful molecular marker for large-scale surveillance of artemisinin resistance.

2. MATERIAL AND METHODS

2.1 Ethical approval

Ethical clearance with reference no PSSH/ADM/ETH.CO/2018/005 was obtained from the Health Research Ethics Committee of the Plateau state ministry of health, Jos.

2.2 Experimental Subject Sample Collections

Thirty-six clinically screened 2 Plus (++) and above positive malarial samples were collected from a Hospital in Jos Nigeria in EDTA bottles and stored at $4^{o}C$ before DNA extraction.

2.3 Parasite DNA extraction

Before the extraction began, 500µl of beta-mercaptoethanol was added to 100ml Genomic Lysis Buffer to make a final dilution of 0.5% [v/v]. Four hundred microliters of Genomic lysis Buffer were added to 100 µl of whole blood in the ratio of 4:1 and vortexed for 2min then incubated for 5min at room temperature. The mixture was transferred to a Zymo-SpinTM IICR column in a collection tube and centrifuged at 10,000 x g for 1 minute. The flow through as well as the collection tubes were discarded. The Zymo-SpinTM IICR column was transferred to a new collection tube and 200µl of DNA Pre-Wash Buffer was added to the spin column then centrifuged at 10,000 x g for 1 minute and the flow through was discarded. Five hundred microliters of g-DNA Wash Buffer were added to the spin column and centrifuged at 10,000 x g for 1 minute and the flow through was discarded. The spin column was transferred to a clean microcentrifuge tube and 70µl DNA elution buffer was added to the Spin column, incubated for 5minute at room temperature then centrifuged at 14,000 x g for 30 seconds. 30µl of the eluded DNA was transferred to another clean microcentrifuge tube and stored at 4oC for immediate use and the remaining 40µl of the eluded DNA was stored as a backup at -20oC for future use. These six steps were repeated for 36 blood samples.

2.4 Detection of *Plasmodium genus*

In the amplification of the *Plasmodium genus* targeting the 18S rRNA gene, the forward and reverse primers (rPLU5-5′-CCTGTTGTTGCCTTAAACT TC-3′) and (rPLU6 -5′TTAAAATTGTTGCAGTTAA AACG-3′) respectively were used. A total of 25 µL volume amplification reaction mixtures contained 12.5µl of One Tag Quick-load 2X Master Mix, 1µl each of 10µM forward and reverse primers, 5.5 µl of nuclease-free water, and 5 µl of DNA template [21]. The cycling conditions were 95oC (10 min); 35 cycles of: denaturation at 94 °C (1 min), annealing at 60°C (2 min), and extension at 72 °C (2 min); final

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extension at 72oC (10 min), held for an indefinite period at 4oC. The amplified products were visualized on 1.5% agarose gel with the expected product size of 1100bp [22].

2.5 Detection of *Plasmodium falciparum*

Five microliters (5µl) of Plasmodium species' amplicons were used for the amplification of *Plasmodium falciparum* using the forward and reverse primer rFAL-1 (5′TTAAACTGGTTTGGGAAAACCAAATATATT-3′) and rFAL-2 (5′-ACACAATGAACTCAATCATGACTACCCGT C-3') receptively. A total of 25µL volume amplification reaction mixtures which contained 12.5µl of One Tag Quick-load 2X Master Mix, 1µl each of 10µM forward and reverse primers, 6.5 of nuclease-free water, and 4µl of amplicon were used [21]. The cycling conditions were 95°C (10 min); 35 cycles of denaturation at 94 °C (1 min), annealing at 55°C (2 min), and extension at 72 °C (2 min); final extension at 72oC (10 min), held for indefinite period at 4oC and the amplified products were visualized on 1.5% agarose gel with the expected product size of 205 base pairs (bp) [22].

2.6 Detection of *PfKelch13* Gene

Two rounds of PCR were used to amplify the *PfKelch13* gene. The forward and reverse primers for the first round of PCR targeting the *PfKelch13* Gene are 5′-CGGAGTGACCAAATCTGGGA-3′ and 5′-GGGAATCTGGTGGTAACAGC-3′ respectively and the expected product size is 2095bp. A total of 25µL volume amplification reaction mixtures which contained 12.5µl of One Tag Quick-load 2X Master Mix, 1µl each of 20µM forward and reverse primers, 6.5 of nuclease-free water, and 4µl of template genomic DNA were used. The thermocycling conditions were set as follows: 95 °C for 2min, 30 cycles of 95 °C for 30 s, 56°C for 90s, 72 °C for 90s, and 72 °C for 10min final extensions then held indefinitely at 4°C.

For the second round of PCR amplification, forward and reverse primers were 5′GCCAAGCTGCCATTCATTTG-3′ and 5′-GCCTTGTTGAAAGAAGCAGA-3′ respectively, and the product size was expected to be 848bp. A total of 25µL volume amplification reaction mixtures which contained 12.5µl of One Tag Quick-load 2X Master Mix, 1µl each of 20µM forward and reverse primers, 6.5 of nuclease-free water, and 4µl of amplicons obtained from the first round of PCR. The thermocycling conditions were set as follows: 95 °C for 2min, 30 cycles of 95 °C for 30s, 60°C for 90s, 72°C for 90s, and 72°C for 10min final extension then held indefinitely at 4°C. The second PCR amplification was detected by 1.5% agarose gel electrophoresis plus 2 µL of ethidium bromide. These nested PCR procedures were adopted with some modifications from [23,24].

2.7 Sequencing and Bioinformatics Analyses

Three amplicons of the *PfKelch13* gene from samples 26, 28 and 29 were sequenced using the Sangar sequencing method at InqabaBiotec Industries, Pretoria, SA. The sequenced data were edited [base-called] using Bioedit software (version 7.2.5). The reverse complements of the sequences were done using the same version of Bioedit in order to obtain the Plus/Plus strands from the Plus/Minus strands of the edited sequences for further analysis. ORF Finder was used to translate the nucleotide sequence to the amino acids sequence. Emboss dotmatcher was used for the dot plots while muscle alignments were performed for the nucleotides and protein sequences using Mega 11 software. The 3D protein structures were predicted using the Swiss Model. The proteins' structural alignments were done using USCF Chimera.

3. RESULTS

3.1 *Plasmodium genus*, *Plasmodium falciparum* and *Pfk13* Gene Detected

Table 1 displays the summary of gel electrophoresis for *Plasmodium genus*, *Plasmodium falciparum* and *Pfk13* gene. The results show that, out of the 36 samples the presence of the *Plasmodium genus*was detected positive in 15 (41.67%) through the PCR technique. However, 58.33% of the total samples collected was found to be negative and thus reported as false positive obtained through the microscopic technique. Also, from the 15 positive samples of the *Plasmodium genus*, about 93.33% were detected positive for *Plasmodium falciparum.* A further analysis of this positive falciparum gave about 78.57% carried the *PfKelch13* gene.

Table 1. The Total Number of *Plasmodium* Genus, *Plasmodium falciparum* and *K13* Gene Detected

Parameters	*Plasmodium* Genus	*Plasmodium falciparum*	*PfKelch13* (*K13*) Gene
Total Sample N (%)	36(100)	15(100)	14(100)
Positives n (%)	15(41.67)	14(93.33)	11(78.57)
Negative n (%)	21(58.33)	1(6.67)	3(21.43)

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Note: N, total number; n, number; %, percentage.

The agarose gel electropherogram of PCR products produced by the amplification of the *Pfk13* gene is presented in Figure 1. From the gel's picture, samples 6,7,8,9,11,21,22,25,26,28, & and 29 weredetected positive for the *PFK13* gene with an expected band size of 848bp. The other samples were negative for this gene.

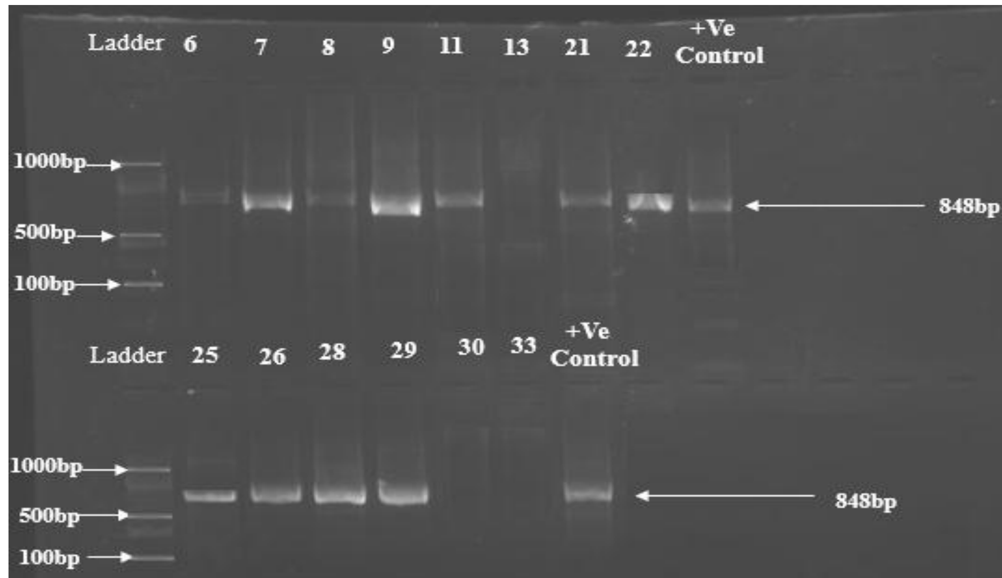

Figure 1 Agarose Gel Electropherogram of PCR Products of *PfKelch13* Gene.
A 100bp ladder, and Positive control (+ve control) with an 848bp known size were used as reference.

3.2 Nucleotides Sequence Alignments

The muscle alignments of the nucleotide sequences of samples 26, 28, and 29 in mega 11 with the wild type (XM_001350122.1 of PF3D7_1343700) are shown in Table 2 below. Both insertions and substitutions were observed. We identified insertions of G and C in sample 26 at positions 2085 and 2124 respectively. We also found the substitutions of A with G, and C with A at 2125 and 2133 positions respectively. At position 2133, we observed the substitution of C with G in sample 28. The insertions of C, C, and A at 2086, 2087, and 2124 positions respectively in sample 29 were also observed together with the substitution of C with G at position 2133.

Table 2. Mutations Observed in the Nucleotides Sequence of Chain A of *PfKelch13* Gene Fragments

Sample ID	Type of mutations	Mutations	Mutated positions
26	Insertion	G	2085
	Insertion	C	2124
	Substitution	A→G	2125
	Substitution	C→A	2133
28	Substitution	C→G	2133
29	Insertions	C, C	2086, 2087
	Insertion	A	2124
	Substitution	C→G	2133

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Note: ID, Identification; A, Adenine; C, Cytosine; G, Guanine. Reference sequence (XM_001350122.1 of PF3D7_1343700)

3.3 The Dotmatcher Plot

In order to visually depict the similarities and/or variations in the amino acid sequences of samples 26, 28, and 29 compared to the wild type (XM_001350122.1 of PF3D7_1343700), we conducted dotmatcher plots as shown in Figure 2. The plots of samples 26, 28, and 29 are represented as A, B, and C respectively. We observed that the sequences are highly similar to the reference sequence of the *PfKelch13* gene in both the global and local alignment views of the dotmatcher plots. The diagonal line in each plot shows the global alignment of the sequence, whereas the short-dashed lines represent local alignments of the sample sequences with the wild type. These plots demonstrate that the samples and wild type have high sequence similarity.

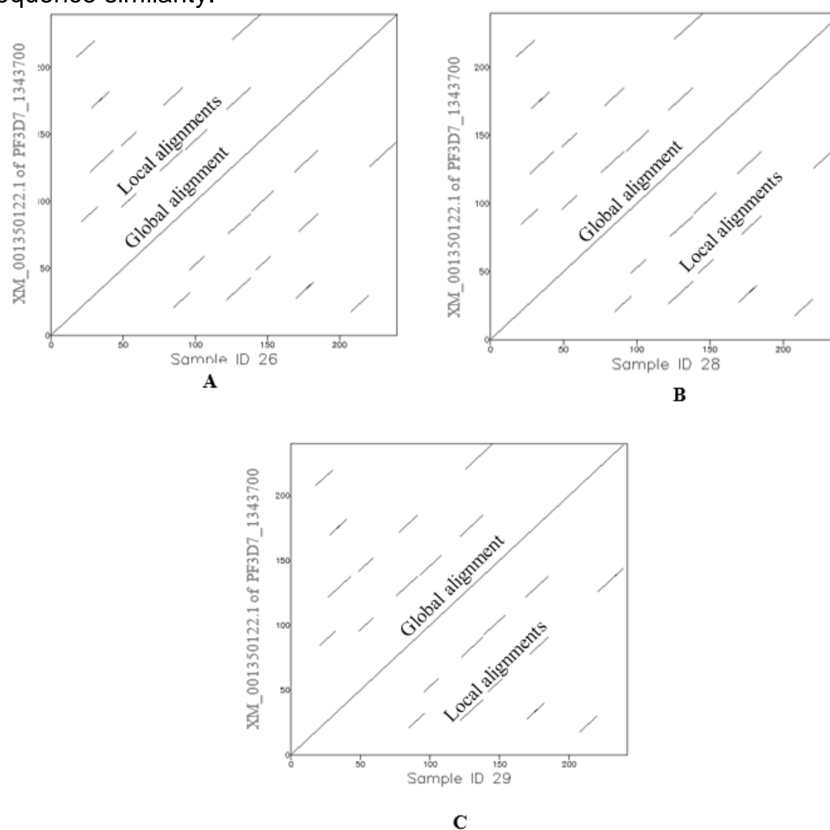

Figure 2 Dotmatcher Plot Illustrating Diversity Analysis of *PfKelch13* Amino Acid Sequences
Dotmatcher plot comparing amino acid sequences of samples 26, 28, and 29 against the 'chain A' reference sequence (XM_001350122.1 of PF3D7_1343700). Dashes indicate similarities between samples and the reference sequence.

3.4 Amino Acids Sequence Alignments

The muscle alignments of the three amino acids sequences *PfKelch13* gene sequences with the reference sequence (XP_001350158. 1) as shown in Figure 3, shows the following mutations: S695C, C696M, H697S, and L699 in sample 26, and C696H, H697V, F698I and S699 in sample 29 but no mutation was observed in sample 28. This means that in sample 26, there is substitution of serine with cysteine at position 695, cysteine with methionine at position 696, histidine with serine at position 697 and an insertion of leucine at position 699 respectively. In sample 28, there is a substitution of cysteine with histidine at position 696, histidine with valine at position 697, phenylalanine with isoleucine at position 698 and an insertion of serine at position 699 of the amino acids residues respectively.

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                ....|....| ....|....| ....|....| ....|....| ....|....|
                        460        470        480        490        500
XP_0013501     FDGVEYLNSM ELLDISQQCW RMCTPMSTKK AYFGSAVLNN FLYVFGGNNY
Sample 26      ---------M ELLDISQQCW RMCTPMSTKK AYFGSAVLNN FLYVFGGNNY
Sample 28      ---------M ELLDISQQCW RMCTPMSTKK AYFGSAVLNN FLYVFGGNNY
Sample 29      ---------M ELLDISQQCW RMCTPMSTKK AYFGSAVLNN FLYVFGGNNY

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        510        520        530        540        550
XP_0013501     DYKALFETEV YDRLRDVWYV SSNLNIPRRN NCGVTSNGRI YCIGGYDGSS
Sample 26      DYKALFETEV YDRLRDVWYV SSNLNIPRRN NCGVTSNGRI YCIGGYDGSS
Sample 28      DYKALFETEV YDRLRDVWYV SSNLNIPRRN NCGVTSNGRI YCIGGYDGSS
Sample 29      DYKALFETEV YDRLRDVWYV SSNLNIPRRN NCGVTSNGRI YCIGGYDGSS

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        560        570        580        590        600
XP_0013501     IIPNVEAYDH RMKAWVEVAP LNTPRSSAMC VAFDNKIYVI GGTNGERLNS
Sample 26      IIPNVEAYDH RMKAWVEVAP LNTPRSSAMC VAFDNKIYVI GGTNGERLNS
Sample 28      IIPNVEAYDH RMKAWVEVAP LNTPRSSAMC VAFDNKIYVI GGTNGERLNS
Sample 29      IIPNVEAYDH RMKAWVEVAP LNTPRSSAMC VAFDNKIYVI GGTNGERLNS

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        610        620        630        640        650
XP_0013501     IEVYEEKMNK WEQFPYALLE ARSSGAAFNY LNQIYVVGGI DNEHNILDSV
Sample 26      IEVYEEKMNK WEQFPYALLE ARSSGAAFNY LNQIYVVGGI DNEHNILDSV
Sample 28      IEVYEEKMNK WEQFPYALLE ARSSGAAFNY LNQIYVVGGI DNEHNILDSV
Sample 29      IEVYEEKMNK WEQFPYALLE ARSSGAAFNY LNQIYVVGGI DNEHNILDSV

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        660        670        680        690        700
XP_0013501     EQYQPFNKRW QFLNGVPEKK MNFGAATLSD SYIITGGENG EVLNSCHF-F
Sample 26      EQYQPFNKRW QFLNGVPEKK MNFGAATLSD SYIITGGENG EVLNCMSFLF
Sample 28      EQYQPFNKRW QFLNGVPEKK MNFGAATLSD SYIITGGENG EVLNSCHF-F
Sample 29      EQYQPFNKRW QFLNGVPEKK MNFGAATLSD SYIITGGENG EVLNSHVISF
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Figure **3 Amino Acid Sequence Alignments of *PfKelch13* Gene with 3D Reference (XP_001350158.1 of PF3D7_1343700)**

Muscle alignments of amino acid sequences from samples 26, 28, and 29 of the *PfKelch13* gene with the *PfKelch13* genes of *Plasmodium falciparum* 3D reference (XP_001350158.1 of PF3D7_1343700). Green marks signify amino acid substitutions, while yellow indicates amino acid insertions.

3.5 The Predicted Protein Structure *PfKelch13* Gene

Figure 4 shows the predicted structures of the *PfKelch13* gene for samples 26, 28, and 29, presented as A, B, and C respectively. Structures A, B and C represent the chain A portion of the *PfKelch13* gene, each containing 6 blades. The structure of A, B, and C were predicted with sequence identities of 97.1%, 100%, and 98.33% respectively. These A, B, and C structures have percentage coverages of 96%, 100%, and 98% respectively. Also, the Global Model Quality Estimate (GMQE) of the three respective structures are 92%, 95%, and 91%, indicating good quality models.

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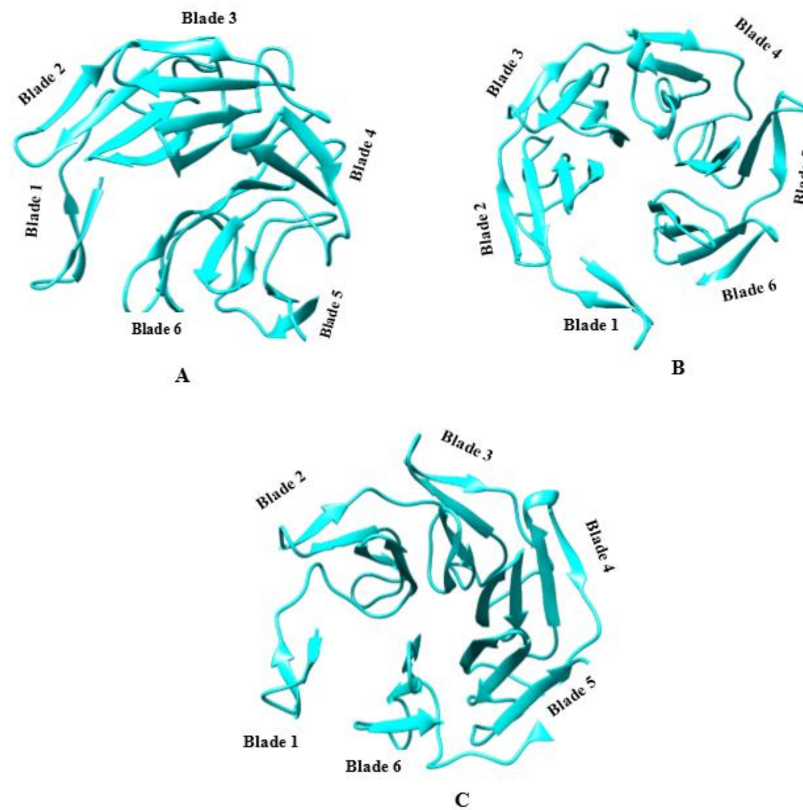

Figure 4 Predicted 3D Protein Structures of *PfKelch13* genes of Samples 26, 28, and 29
Predicted 3-dimensional protein structures of samples 26, 28, and 29, represented as A, B, and C respectively. Predictions were based on the reference PDB protein structure of 4ZGC.1.A.

3.6 The Structural Alignments of The Predicted Proteins

The alignments of the 3D structures of each of the predicted *Plasmodium falciparum*'s Keltch13 gene with the wildtype **(**4zgc.1. A**)** as shown in Figure 5 did not show any major changes in the 3D structures of the aligned proteins. The mutations observed on the amino acid residues of samples 26 and 29 occurred on the sixth blade each. The sixth blade **IS** the exact position where we expected to see the impact of the mutations on the Predicted structures.

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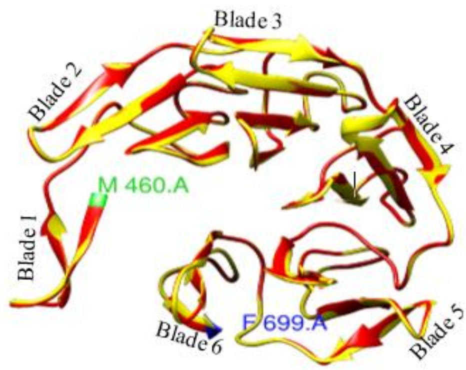

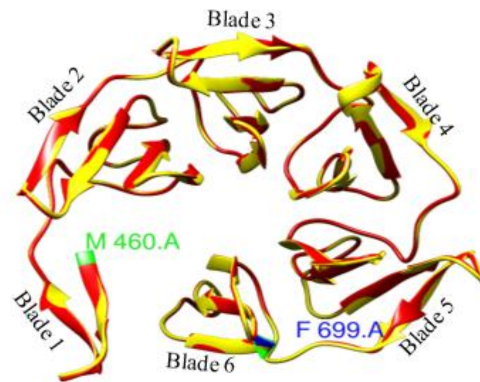

A

B

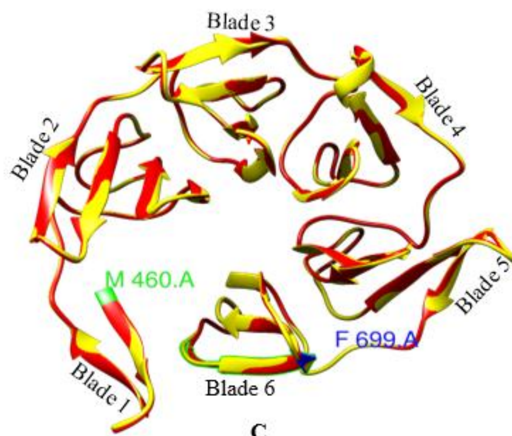

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Figure 5 The 3D Structural Alignments of *PfKelch13* Proteins with the Wild Type (4ZGC.1.A)

Structural alignments of samples 26, 28, and 29 (represented as A, B, and C) with the wild type (4ZGC.1.A). Predicted structures are in yellow, while the reference (wild type) structures are in red. Note: M denotes Methionine; F denotes Phenylalanine.

4. Discussion

Malaria control and treatments are hindered by widespread antimalarial drug resistance, especially in *Plasmodium falciparum* [8,25]. Our findings suggest that there is a high percentage of false positive test results of malaria parasites being reported. This might have resulted in patients being diagnosed and treated with the wrong antimalarial drugs which could be one of the major contributory factors of this artemisinin resistance [26,27]. This finding also confirmed the PCR technique has a higher level of accuracy/precision in malaria testing than the traditional method [microscopy] [28,29]. It was observed from this study that, there is a high level of *Plasmodium falciparum* detected in the population. Over 93% of the malaria parasite's positive sample is *Plasmodium falciparum*. Therefore, out of the five species of Plasmodium parasites that cause malaria in humans namely, P. falciparum, *P. vivax*, *P. malariaeP. ovale*, and *P. knowlesi*, [2,8], *Plasmodium falciparum* is the most prevalent and lethal in the studied area [30,7,8].

Scientific reports have proven that *PfKelch13* is the primary marker for artemisinin resistance [31], and mutations in the *PfKelch13* gene are currently proposed as a useful molecular marker for the surveillance of artemisinin resistance [15,31]. Therefore, we propose that the mutations observed in the nucleotides and amino acid sequences are associated with artemisinin resistance in samples 26 and 29. This could have implications for the regulation and dynamics of the export of *Plasmodium falciparum* erythrocyte membrane protein1(PfEMP1) as the major virulence determinant [18].

Some studies have documented a potential association between specific mutations (M476I, C580Y, R539T, Y493H, I543T, P574L, F451L, N664I, V487E, V692G, Q661H, and Y493H) identified in the propeller domain of the *PfKelch13* gene and the development of in-vitro resistance to artemisinin, an antimalarial medication drug [19, 20, 32]. Notably, the C580Y mutation was detected in two samples obtained from Cameroon, while the Y493H mutation was identified in one sample from Ghana [20]. However, in our study, we could not identify any of the aforementioned mutations.

4. Conclusion

During our investigation, we detected non-synonymous mutations within the amino acid sequences in samples 26 and 29 as (S695C, C696M, H697S, and L699) and (C696H, H697V, and S699) respectively. These mutations, however, did not induce any significant alterations in the three-dimensional structure of the aligned protein, as illustrated in Figure 5. Therefore, we conclude that the observed mutations in samples 26 and 29 could potentially signify novel variations that may have not been previously reported or documented, particularly within Plateau State, Nigeria.

CONSENT

Not applicable

ETHICAL APPROVAL

Ethical clearance was obtained from the Health Research Ethics Committee of Bingham University Teaching Hospital Jos, Plateau state, Nigeria with reference no NHREC/21/05/2005/00677. We ensured that all experimental procedures were carried out in compliance with the relevant laws guiding the use of human subjects.

REFERENCES

1. Siwal N, Singh US, Dash M, et al. Malaria diagnosis by PCR revealed differential distribution of mono and mixed species infections by *Plasmodium falciparum* and *P. vivax* in India. PLoS One. 2018;13(3):e0193046. doi:10.1371/journal.pone.0193046

2. Hadni H, Elhallaoui M. Molecular docking and QSAR studies for modeling the antimalarial activity of hybrids 4-anilinoquinoline-triazines derivatives with the wild-type and mutant receptor pf-DHFR. Heliyon. 2019;5(8):e02357. doi:10.1016/j.heliyon.2019.e02357

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3. Nyaoke BA, Mureithi MW, Beynon C. Factors associated with treatment type of nonmalarial febrile illnesses in under-fives at Kenyatta National Hospital in Nairobi, Kenya. PLoS One. 2019;14(6):e0217980. doi:10.1371/journal.pone.0217980

4. Burrows JN, Hooft Van Huijsduijnen R, Möhrle JJ, Oeuvray C, Wells TN. Designing the next generation of medicines for malaria control and eradication. Malar J. 2013;12:187. doi:10.1186/1475-2875-12-187

5. Bloland PB. Drug resistance in malaria. Drug Resist Bact Fungi, Malaria, Cancer. 2017:429-447. doi:10.1007/978-3-319-48683-3_19

6. Maier AG, Matuschewski K, Zhang M, Rug M. *Plasmodium falciparum*. Trends Parasitol. 2019;35(6). doi:10.1016/j.pt.2018.11.010

7. Dieng, Gonzalez, Pestana, et al. Contrasting Asymptomatic and Drug Resistance Gene Prevalence of *Plasmodium falciparum* in Ghana: Implications on Seasonal Malaria Chemoprevention. Genes (Basel). 2019;10(7):538. doi:10.3390/genes10070538

8. Shahzadi S, Akhtar T, Hanif A, Sahar S, Niaz S, Bilal H. Molecular detection of malaria in south Punjab with a higher proportion of mixed infections. Iran J Parasitol. 2014;9(1):37-43.

9. Saba S, Akhtar T, Hanif A, Sahar S, Niaz S, Bilal H. Molecular detection of malaria in south Punjab with a higher proportion of mixed infections. Iran J Parasitol. 2014;9(1):37-43.

10. Ikram NKBK, Simonsen HT. A Review of Biotechnological Artemisinin Production in Plants. 2017;8(November):1966. doi:10.3389/fpls.2017.01966

11. Hadni H, Elhallaoui M. Molecular docking and QSAR studies for modeling the antimalarial activity of hybrids 4-anilinoquinoline-triazines derivatives with the wild-type and mutant receptor pf-DHFR. Heliyon. 2019;5(8):e02357. doi:10.1016/j.heliyon.2019.e02357

12. Duru V, Witkowski B, Ménard D. Review article *Plasmodium falciparum* resistance to artemisinin derivatives and piperaquine: A major challenge for malaria elimination in Cambodia. Am J Trop Med Hyg. 2016;95(6):1228-1238. doi:10.4269/ajtmh.16-0234

13. Yakubu B, Longdet IY, Horsefield T, Davou DT, Obishakin E. High-Complexity *Plasmodium falciparum* Infections, North Central Nigeria, 2015-2018. Emerg Infect Dis. 2019;25(7):1330-1338. doi:10.3201/eid2507.181614

14. Noisang C, Prosser C, Meyer W, et al. Molecular detection of drug-resistant malaria in Southern Thailand. Malar J. 2019;18(1):275. doi:10.1186/s12936-019-2903-y

15. Phompradit P, Chaijaroenkul W, Muhamad P, Na-Bangchang*K13* propeller domain mutations and pfmdr1 amplification in isolates of *Plasmodium falciparum* collected from the Thai-Myanmar border area in 2006-2010. Folia Parasitol (Praha). 2019;66. doi:10.14411/fp.2019.006

16. Foguim FT, Robert MG, Gueye MW, et al. Low polymorphisms in pfact, pfugt, and pfcarl genes in African *Plasmodium falciparum* isolates and absence of association with susceptibility to common antimalarial drugs. Malar J. 2019;18(1):293. doi:10.1186/s12936-019-2919-3

17. Zhang T, Xu X, Jiang J, Yu C, Tian C, Li W. Surveillance of antimalarial resistance molecular markers in imported *Plasmodium falciparum* malaria cases in Anhui, China, 2012-2016. Am J Trop Med Hyg. 2018;98(4):1132-1136. doi:10.4269/ajtmh.17-0864

18. Severini C, Menegon M. Resistance to antimalarial drugs: An endless world war against Plasmodium that we risk losing. J Glob Antimicrob Resist. 2015;3(2):58-63. doi:10.1016/j.jgar.2015.02.002

19. Suresh N, Haldar K. Mechanisms of artemisinin resistance in *Plasmodium falciparum* malaria. CurrOpinPharmacol. 2018;42. doi:10.1016/j.coph.2018.06.003

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20. FoguimTsombeng F, Gendrot M, Robert MG, Madamet M, Pradines B. Are *K13* and plasmepsin II genes, involved in *Plasmodium falciparum* resistance to artemisinin derivatives and piperaquine in Southeast Asia, reliable to monitor resistance surveillance in Africa? Malar J. 2019;18(1):285. doi:10.1186/s12936-019-2916-6

21. Matrevi SA, Opoku-Agyeman P, Quashie NB, et al. *Plasmodium falciparum*kelch propeller polymorphisms in clinical isolates from Ghana: 2007-2016. Antimicrob Agents Chemother. August 2019. doi:10.1128/AAC.00802-19

22. Lloyd YM, Esemu LF, Antallan J, et al. PCR-based detection of *Plasmodium falciparum* in saliva using mitochondrial cox3 and varATS primers. Trop Med Health. 2018;46(1):1-6. doi:10.1186/s41182-018-0100-2

23. Saba S, Akhtar T, Hanif A, Sahar S, Niaz S, Bilal H. Molecular detection of malaria in south Punjab with a higher proportion of mixed infections. Iran J Parasitol. 2014;9(1):37-43.

24. Dong Y, Wang J, Sun A, et al. Genetic association between the *PfPFK13* gene mutation and artemisinin resistance phenotype in *Plasmodium falciparum* isolates from Yunnan Province, China. Malar J. 2018;17(1):351. doi:10.1186/s12936-018-2500-5

25. Huang B, Deng C, Yang T, et al. Polymorphisms of the artemisinin resistant marker (*PFK13*) in *Plasmodium falciparum* parasite populations of Grande Comore Island 10 years after artemisinin combination therapy. Parasit Vectors. 2015;8(1):634. doi:10.1186/s13071-015-1253-z

26. Frasse PM, Odom John AR. Haloacid Dehalogenase Proteins: Novel Mediators of Metabolic Plasticity in *Plasmodium falciparum*. Microbiol Insights. 2019;12:1178636119848435. doi:10.1177/1178636119848435

27. D'Alessandro U, Buttiéns H. History and importance of antimalarial drug resistance. Trop Med Int Health. 2001;6(11):845-848. doi:10.1046/j.1365-3156.2001.00819.x

28. Omondi B. Factors contributing to antimalarial drug resistance in Rachuonyo district, Kenya. 2016;7(1). doi:10.4314/eajph.v7i1.64670

29. Berzosa P, Lucio A De, Barja MR, et al. Comparison of three diagnostic methods (microscopy, RDT, and PCR) for the detection of malaria parasites in representative samples from Equatorial Guinea. Malar J. 2018;17(1):335. doi:10.1186/s12936-018-2481-4

30. Kang JM, Cho PY, Moe M, et al. Comparison of the diagnostic performance of microscopic examination with nested polymerase chain reaction for optimum malaria diagnosis in Upper Myanmar. Malar J. 2017;16(1):119. doi:10.1186/s12936-017-1765-4

31. Ouji M, Augereau JM, Paloque L, Benoit-Vical F. *Plasmodium falciparum* resistance to artemisinin-based combination therapies: A sword of Damocles in the path toward malaria elimination. Parasite. 2018;25:24. doi:10.1051/parasite/2018021

32. Ariey F, Witkowski B, Amaratunga C, et al. A molecular marker of artemisinin-resistant *Plasmodium falciparum* malaria. Nature. 2014;505(7481):50-55. doi:10.1038/nature12876

DEFINITIONS, ACRONYMS, ABBREVIATIONS

Pfkelch13*:Plasmodium falciparum* Artemisinin Resistant Gene;**PCR:** Polymerase Chain Reaction; **EDTA**: Ethylenediaminetetraacetic Acid; **RNA**: Ribonucleic Acid; **DNA**: Deoxyribonucleic Acid; **GMQE:** Global Model Quality Estimate

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