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Non-Synonymous Mutations Associated with *Plasmodium falciparum* Artemisinin Resistant Gene (*Pfkelch13*) in Malaria Cases, Jos Nigeria

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Authors' contributions

This work was carried out in collaboration among all authors. Authors IYL and JP were responsible for the study design. Authors JP, NJN, MOS, SKG, TF, MMM and DTD conducted the laboratory investigations. Author JP wrote the initial draft of the manuscript. Authors IYL, BY and RJK supervised the work and contributed to manuscript editing. All authors have reviewed, revised, and ultimately approved the final manuscript for publication.

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ABSTRACT

Aim: The aim of this study was to explore the *Plasmodium falciparum* Artemisinin Resistant Gene (*Pfkelch13*) in malaria cases in Jos and profile the nature of the mutations. The *Plasmodium falciparum kelch13* 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, Nigeria between October 2019 and January 2021.
Methodology: Thirty-six clinically screened 2 plus (++) and above positive malarial whole blood samples were collected from a Hospital in Jos, Plateau State, Nigeria in EDTA bottles after being granted ethical approval. The DNA extraction was done 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: Only 41.67% of the 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: The classical method of detecting malaria is not reliable compared to the PCR technique. Non-synonymous mutations on the *PfKelch13* gene have the potential to cause resistance to artemisinin drugs in the Jos human population.

Keywords: Malaria; plasmodium falciparum; mutations, pfkelch13 gene.

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 with *Plasmodium falciparum* being responsible for most of the mortality [2,8,9]. Since the 1940s, there have been continuous attempts to halt the spread of malaria. This has succeeded in Europe, North America, parts of Asia and Latin America, however, it is not so in Sub-Saharan Africa where about 80% of the annual malaria patients are found [10].

Over time, great efforts devoted to research and development into antimalarial drugs have given 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 *Plasmodium falciparum*, particularly in Southeast Asia [12]. Therefore, drug resistance and treatment failures are envisaged to be among the challenges slowing down the process of malaria elimination 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) 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]. It has been demonstrated that 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 the major

parasite virulence determinant (*PfEMP1)* 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 falciparum kelch13* 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. MATERIALS 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

In order to extract the DNA, 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 -20^{o}C 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. The primer set was initially validated and described [21]. 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 95^{o}C (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 extension at 72^{o}C (10 min), held for an indefinite period at 4^{o}C. 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 earlier validated [21]. 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 primers used were previously validated and described [21]. 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 Gel Electrophoresis

The polymerase chain reaction (PCR) products from the nested amplification were analyzed by agarose gel electrophoresis to separate and visualize the DNA fragments. A 1.5% agarose gel solution was prepared by dissolving 1.5 grams of agarose powder in 100 millilitres of Tris-acetate-EDTA (TAE) buffer. Two microliters of ethidium bromide was added to the agarose gel solution to serve as an intercalating agent that fluoresces under ultraviolet light when bound to DNA, in order to allow the separated DNA fragments to be visualized. The PCR products and 100bp ladder were loaded into the wells of the agarose gel after it was cast and submerged in TAE buffer. The gel was run at 90 volts for 35 minutes and visualized under UV light using the Gel Doc XR system from BioRad.

2.8 Sequencing and Bioinformatics Analyses

Three amplicons of the *PfKelch13* gene from samples 26, 28 and 29 were sequenced using the Sangar sequencing method at Inqaba Biotec 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 Plasmodium genus was detected positive in 15 (41.67%) through the PCR technique. However, 58.33% of the total samples collected were negative and thus reported as false positives 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.

The agarose gel electropherogram of PCR products produced by the amplification of the *Pfk13* gene is presented in Fig. 1. From the gel's picture, samples 6,7,8,9,11,21,22,25,26,28, & and 29 were detected positive for the *PFK13*

gene with an expected band size of 848bp. The other samples were negative for this gene.

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 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)

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

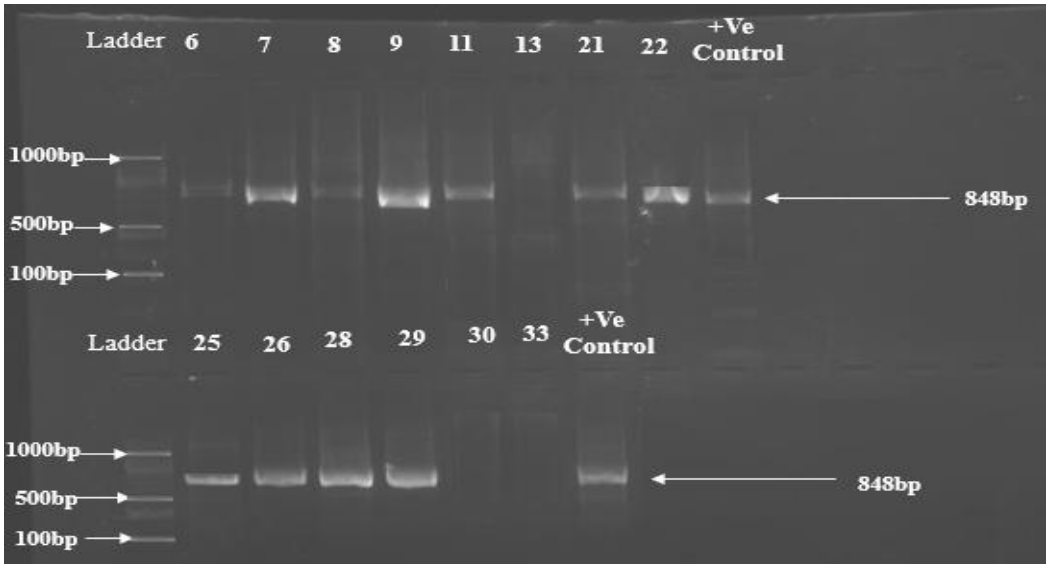

Fig. 1. Agarose gel electropherogram of PCR Products of *PfKelch13* Gene
A 100bp ladder, and Positive control (+ve control) with an 848bp known size were used as reference.

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

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 dot-matcher plots as shown in Fig. 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 dot-matcher 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.

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 Fig. 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.

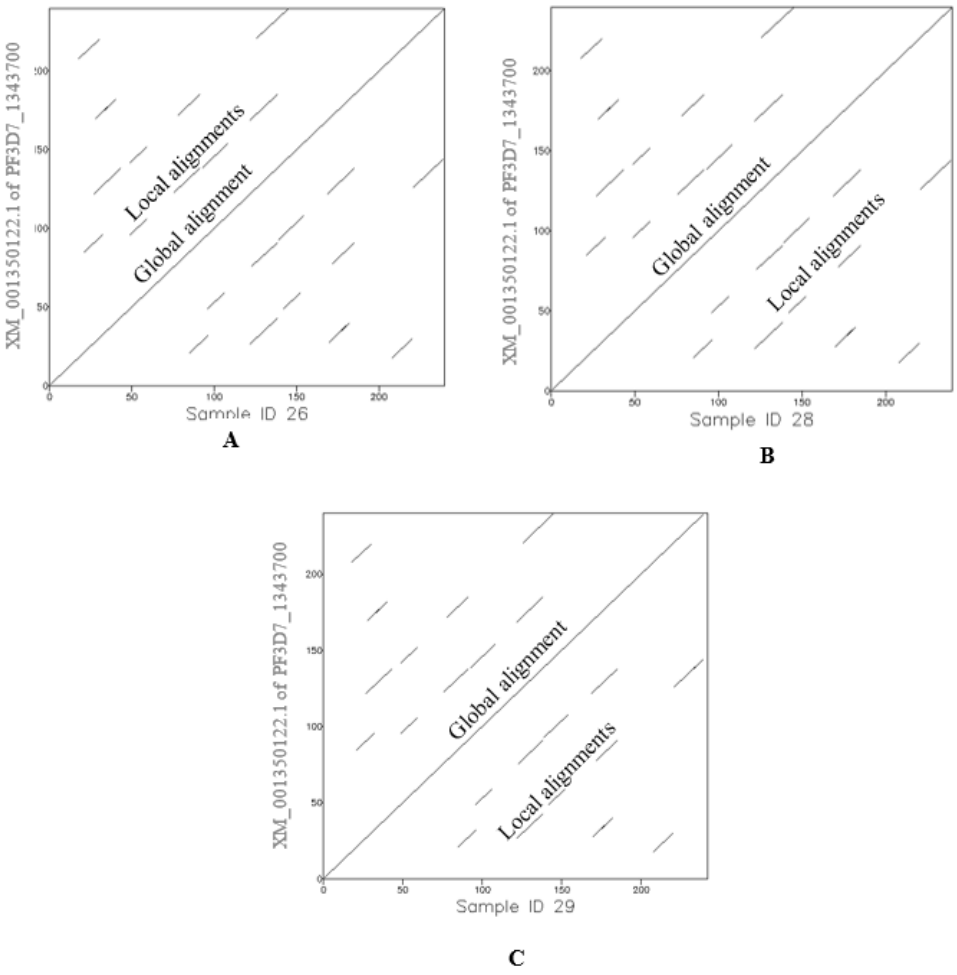

Fig. 2. Dot-matcher plot illustrating diversity analysis of *PfKelch13* amino acid sequences

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.

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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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Fig. 3. Amino acid sequence alignments of *PfKelch13* Gene with 3D reference (XP_001350158.1 of PF3D7_1343700) indicating amino acid substitutions (green) and amino acid insertions (yellow)

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

Fig. 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.

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 Fig. 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.

4. DISCUSSION

Malaria control and treatments are hindered by widespread antimalarial drug resistance, especially in *Plasmodium falciparum* [8]. 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. This could be one of the factors responsible for artemisinin resistance [25,26]. This finding also confirmed the PCR technique as having a higher level of accuracy/precision in malaria testing than the traditional method by microscopy [27-28]. 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 was *Plasmodium falciparum*. Therefore, out of the five species of Plasmodium parasites that cause malaria in humans namely, *P. falciparum, P. vivax, P. malariae P. ovale,* and *P. knowlesi*, [2,8], *Plasmodium falciparum* was the most prevalent and lethal in the studied area [7,8,29].

Scientific reports have proved that *PfKelch13* is the primary marker for artemisinin resistance [30], and mutations in the *PfKelch13* gene are currently proposed as a useful molecular marker for the surveillance of artemisinin resistance [15,30]. 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].

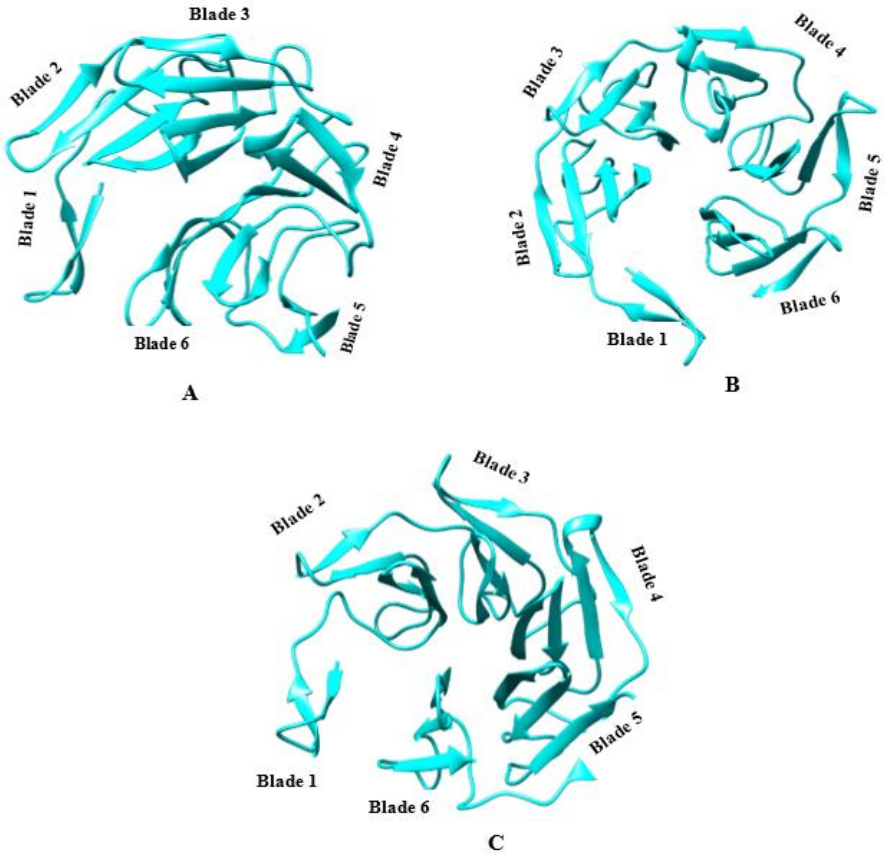

Fig. 4. Predicted 3D protein structures of *PfKelch13* genes of Samples 26, 28, and 29

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.

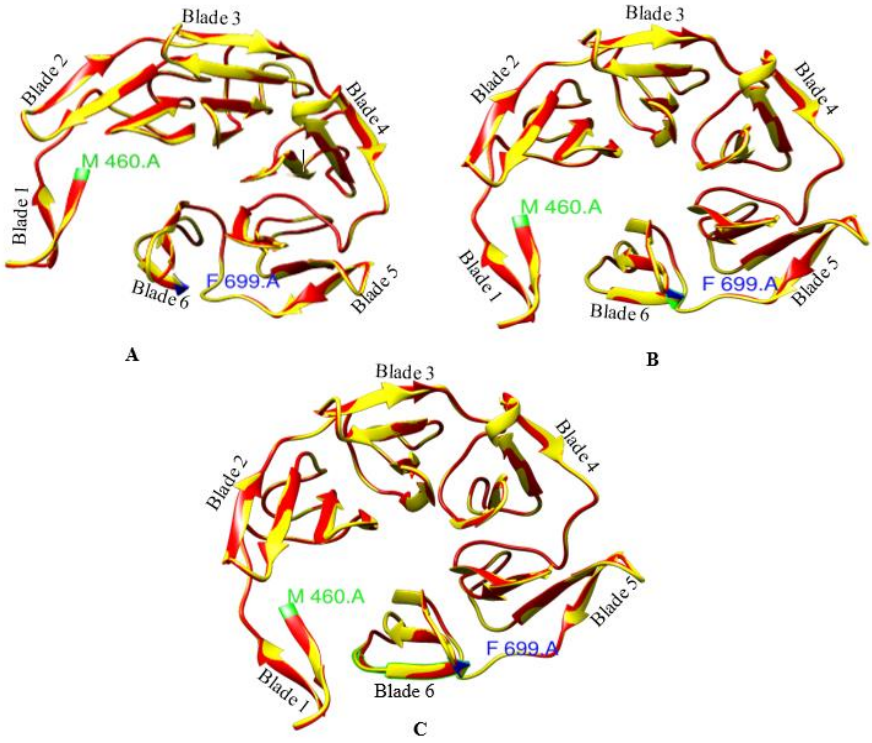

Fig. 5. The 3D structural alignments of *PfKelch13* Proteins with the wild type (4ZGC.1.A)

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.

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 [19,20,31]. 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.

5. 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 Fig. 5. Therefore, 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.

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.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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