Open Access Research Journal of
Science and Technology
Journals home page: https://oarjst.com/
ISSN: 2782-9960 (Online)

OARJ OPEN ACCESS RESEARCH JOURNALS

(RESEARCH ARTICLE)

Kelch 13 gene polymorphisms of Plasmodium falciparum among human population in Nasarawa-West Senatorial District, Nasarawa State, Nigeria

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Open Access Research Journal of Science and Technology, 2024, 10(01), 128–140

Publication history: Received on 25 December 2023; revised on 04 February 2024; accepted on 07 February 2024

Article DOI: https://doi.org/10.53022/oarjst.2024.10.1.0032

Abstract

Kelch-13 is a gene produced by *Plasmodium falciparum*, one of the species of Malaria parasite. Malaria remains a major public health challenge especially in Sub-Saharan Africa, a major cause of mortality and morbidity, especially in children and pregnant women. WHO recommends Artemisinin-based combination therapies as the first-line drugs for the treatment of uncomplicated malaria but the emergence and spread of Artemisinin-resistance associated with mutations in K13 gene poses a threat to ACT efficacy. Detection of mutant K13 gene may provide the information on changes in parasite susceptibility to Artemisinin. This study was aimed at detecting K13 *g*ene polymorphisms among human population in Nasarawa-West Senatorial District, *Nigeria.* A total of 385 blood samples were collected from selected hospitals and screened for malaria by microscopy (the gold standard). Species specific screening of the *P. falciparum* was done using RDT. Dried blood spots made from RDT positive samples were investigated for the presence of K13 genes by nested PCR. Sequencing detected mutant K13 gene. Results: 103 samples were positive for *Plasmodium falciparum* by RDT, PCR confirmed 48 K13 genes with a band size of 848 bp. Nucleotide sequence alignment revealed six Single Nucleotides Polymorphisms. The nucleotide sequences were converted to protein sequences and results showed a point mutation in 1(5.0%) of the 20 *Pfkelch 13* gene sequenced. Conclusively, the need for continuous surveillance following the detection of mutant gene in the study population is recommended in order to have a wider picture of the parasite diversity for effective malaria control.

Keywords: Kelch 13; Malaria; Artemisinin; Nasarawa; Sequencing; RDT

1. Introduction

Kelch 13 is a gene produced by *Plasmodium falciparum*, one of the species of Malaria parasite. Malaria is a vector-borne deadly disease caused by *Plasmodium* species and remains a major public health challenge in Sub-Saharan Africa [1, 2]. It is estimated to cause 241 million clinical episodes and 627,000 deaths with an estimated 94% of deaths occurring in the WHO African Region [3, 4]. According to the 2020 World Malaria Report, Nigeria has the highest number of global malaria cases with 27% of global malaria cases in 2019 and accounted for the highest number of global malaria deaths which stands at 23% [2, 5, 6]. The control of Malaria disease relies heavily on: vector control, the administration of antimalarial drugs and recently, the use of malaria vaccines [3]. The use of these control measures has contributed to a significant reduction in the malaria burden in Africa by about 40% from 2000 to 2015 [7].

Despite substantial efforts to eliminate or control malaria, it remains the leading cause of morbidity and mortality in the world [1]. The use of antimalarial drugs in the control of malaria and the emergence and spread of Artemisinin-resistance parasites necessitated the recommendations by the World Health Organization (WHO) of the use of Artemisinin-based combination therapies (ACTs) as the first-line drugs for the treatment of uncomplicated malaria [8, 9, 10]

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Kelch 13 gene belongs to the Kelch superfamily of proteins that harbours multiple protein–protein interaction sites and mediates different cellular functions, including ubiquitin-regulated protein degradation and oxidative stress responses [8]. It is produced by all stages of *P. falciparum* (asexual trophozoites and schizonts) and sexual blood stages (gametocytes). It is responsible for the activation of the Artemisinin. Mutation at certain codons on K13 gene is associated with *in vitro* Artemisinin resistance and *in vivo* slow clearance of parasiteamia and therefore is used as a marker of Artemisinin resistance [8]. Antimalarial resistance is defined as the ability of a parasite strain to survive and/or multiply despite the administration and the absorption of a drug, given in doses equal to or higher than those usually recommended but within the tolerance of the subject [11]. The therapeutic efficacy of ACTs is therefore threatened by an unusual resistant phenotype that manifests as delayed clearance of *P. falciparum* blood forms following Artemisinin-based treatment [9]. To improve antimalarial resistance monitoring, molecular markers of Artemisinin resistance have been identified to occur within the *P. falciparum* Kelch 13 (*Pf*K13) - propeller gene.

This resistance of the malaria parasite to Artemisinin and its derivatives is due to mutation at certain codons on Kelch 13 gene [8]. This Mutation alters the amino acid sequence of the transporter genes and results in changing their physiochemical properties and functional characteristics [12]. Mechanisms of Artemisinin resistance by mutant K13 genes of *P. falciparum* could arise due to:

- Decreased availability of reduced heme for ART activation;
- An altered endoplasmic reticulum (ER) response or unfolded protein response (UPR) or
- Decoupling of the stress response and cell death pathways or rescue of a critical substrate [12].

In the highlight of the resistance, the cytostome (cell mouth) that the parasite uses to take up hemoglobin is the proposed location for the artemisinin-resistance marker Kelch 13 (K13). K13 mutants exhibit disrupted hemoglobin catabolism. Decreased hemoglobin-dependent activation likely underpins K13-mediated artemisinin resistance. The resultant decrease in hemoglobin-derived heme reduces artemisinin activation, which is sufficient to enable parasite survival in the early ring stage of infection. Mutations in other endocytic apparatus proteins may underpin alternative resistance mechanisms [12].

The risk of the emergence and spread of Artemisinin resistance in malaria parasite pose a greater challenge to the World health malaria control policies [13]. This study was therefore aimed at detection of Kelch-13 *g*ene Polymorphisms of *Plasmodium falciparum* among human population in Nasarawa West Senatorial District, Nasarawa State, Nigeria moreover that there is paucity of literature on the subject matter in the study area.

2. Material and methods

2.1. Study Area

This hospital-based prospective study was conducted in selected health facilities in Nasarawa-West Senatorial District, Nasarawa State, Nigeria. The senatorial district comprises of five (5) Local Government areas namely: Karu, Keffi, Kokona, Nasarawa and Toto. The selected health facilities include: General Hospital Garaku (GHG), General Hospital Nasarawa (GHN), General Hospital Keffi (GHK), Medical Center Mararaba Gurku (MCMG) and South Atlantic Petroleum Medical centre (SMC), Nasarawa State University, Keffi. The senatorial district has boundaries with Kaduna State in the North, Kogi State in the South, Akwanga and Nasarawa Eggon LGA in Nasarawa State in the East and the Federal Capital Territory, Abuja in the West. Nasarawa state is located in North Central Nigeria between latitudes 8˚51 and 8˚53[1] North of the equator and longitudes 7˚50' and 7˚51' East of the Greenwich meridian. It is located about 128 km away from Lafia, the Nasarawa State capital and about 52 km away from Abuja, the Federal Capital Territory of Nigeria [14, 15].

2.2. Study Population

The Senatorial District has a population of 723, 608 distributed into five local governments namely Karu (216, 230), Keffi (92, 550), Kokona (108, 558), Nasarawa (187, 220) and Toto (119, 051).

2.3. Sample Size Determination

The sample size was determined with a 95% Confidence Interval (CI) and precision level of 5% using the standard sample size calculation formula as described by [16].

$$n = \frac{Z^2(1-P)}{d^2}$$

Where:
n = Sample size if the target population is > 10,000
Z = Z statistic for a level of confidence (For 95% confidence level, Z = 1.96)
P = Expected prevalence or proportion (in proportion of one; if 50%, P = 0.5) and
d = Precision (in proportion of one; if 5%, d = 0.05).

Given that Z = 1.96, P = 0.5, d = 0.05 and substituting the values, sample size was calculated thus:

$$n = 1.96 \times 1.96 \times 0.5 (1 - 0.5) \div 0.05 \times 0.05$$

$$n = 3.8416 \times 0.5 \times 0.5 \div 0.0025$$

$$n = 0.9604 \div 0.0025$$

$$n = 384.2$$

n = ~ 385 samples were collected

2.4. Ethical Approval

The ethical approval was obtained from the Research and Ethical Committee of the Nasarawa State Ministry of Health, Nasarawa State, Nigeria.

2.5. Subject Selection (Eligibility criteria)

2.5.1. Inclusion criteria

Patients who had clinical symptoms of malaria determined by a headache, body aches and febrile condition of 37.5 °C and above and have not taken antimalarial drugs in the preceding week prior to visitation to the health facility were recruited in the study. Similarly, only consenting Patients residing within the senatorial district were enrolled in the study.

2.5.2. Exclusion criteria

Patients residing outside the senatorial district, without clinical symptoms of malaria determined by a headache, body aches and febrile condition of 37.5 °C and above, have taken antimalarial drug in the preceding week prior to visitation to the health facility and refused granting consent were exempted from the study.

2.6. Blood Collection

A total of 385 venous blood samples were collected by venipuncture from the study participants at the collection sites (77 samples per collection site) using standard procedure. The collected blood samples were let into labeled EDTA containers at the respective collection sites and conveyed to Microbiology Laboratory, Nasarawa State University, Keffi for analysis.

2.7. Laboratory Analysis

2.7.1. Microscopy for detection of malaria parasites

At the Microbiology laboratory at NSUK, thin and thick blood films made on well labeled clean grease free slides and air dried were stained for 10 min using a 1 in 10 dilution of freshly prepared Giemsa stain in buffer water of pH7.2. Parasite density of the stained dry films were read by two independent Microscopists, discrepancies were resolved by a third reader.

2.7.2. Detection of Plasmodium falciparum by Rapid Diagnostic Test

The microscopic Giemsa stained malaria parasite positive samples were confirmed for mono-infection of *Plasmodium falciparum* using Rapid diagnostic Test (RDT) specific for *P. falciparum* Histidine Rich Protein 2 (HRP2) (Standard Diagnostics, inc. Korea). Briefly, 5µl of whole blood from the test sample was added into the sample well of a labeled, warm SD Bioline malaria antigen *P. falciparum* test device placed on a flat surface. Two drops (100µl) of assay diluent were then added into the diluent well and timed for 15 minutes. Positive samples were indicated by show of 2 colour bands (one in the control line region and the other in the test line region). Results were considered negative when there

was colour band only in the control line but none in the test line. Similarly, results were also considered invalid when there was no colour band at all in either the test nor control line or if colour band appeared only in the test line but none in the control line.

The confirmed, mono-infected samples were then spotted on Whatman #3 filter paper (Whatman International Ltd., Maidstone, England) and labeled accordingly. The blood samples spotted on filter papers were air-dried as Dry Blood Spot (DBS) and individually inserted in a zip-lock bag to which a desiccant was added and kept at ambient temperature for molecular analysis at Nucleometrix Research laboratory, Yenagoa, Bayelsa State.

2.7.3. Molecular detection of Plasmodium falciparum genes

DNA Extraction

Genomic DNA was extracted from 100 µL of each mono-infected whole blood sample using a Quick-DNA Mini prep plus Kit (Zymo Research (ZR), USA) according to the manufacturer's protocol [17]. The extracted DNA was eluted in 50 µl of TE buffer (10 mM Tris–HCl, 0.1 M EDTA, pH 8.0) and stored at −20 °C until use.

The extracted DNA was quantified using a NanoDrop 1000 Spectrophotometer (Thermo Scientific). The quality of genomic DNA was detected using 1.0 % agarose gel electrophoresis using Safe view (Safe view ™Classic cat # G.108) staining as ethidium bromide substitute.

PCR amplification of *P. falciparum* K13 propeller genes

Plasmodium falciparum K13-propeller was amplified using a nested PCR amplification method following standard protocols as described by [17] with slight modifications. The primers and cycling conditions used are as presented in table 1. The total 30 µl amplification reaction mixtures contained: 8.8 µl of dH2O, 0.6 µl of each primer (0.6 pM), and 15 µl of *Taq* PCR Master Mix following the manufacturer's instructions (Inqaba, South Africa) prepared at a concentration of 0.4M. Primary amplification reactions were initiated with the addition of 5.0 µl of template genomic DNA prepared from the blood samples. For the nested PCR, 0.5 µl of primary PCR productions was used as template. The amplified PCR products were detected on 1.5 % agarose gel, and the sizes of the PCR products were measured visually based on a 100 bp DNA ladder (MW-1700-10 (Bio Labs).

Table 1 Primer sequences and cycling conditions used to amplify *Plasmodium falciparum* K13-propeller gene and Nested PCR of Kelch 13 genes

Genes	Primer sequence (5' - 3')	PCR Cycling conditions	Product size
Kelch 13 (P)	F: CGGAGTGACCAAATCTGGGA	94 °C 5 min/[94 °C 90 s, 55 °C 45 s, 72 °C , 90 s] × 40 cycles, 72 °C 10 min.	2096bp
	R: GGGAATCTGGTGGTAACAGC		
Kelch 13 (S)	F: GCCAAGCTGCCATTCATTTG	94 °C 5 min/[94 °C 90 s, 55 °C 45 s,72 °C 90 s] × 40 cycles, 72 °C 10 min	848bp
	R: GCCTTGTTGAAAGAAGCAGA		

P = Primary PCR reaction, S = Secondary PCR reaction, F = Forward primer, R = Reverse primer, bp = base pairs. Source: Huang *et al.,* 2015

Sequencing of *P. falciparum* K13 fragment

Twenty (20) samples of the nested PCR products of K13-propeller gene were directly sequenced in both directions using an ABI 3730XL sequencer (ThermoFisher). The obtained sequences were edited using the bio-informatics algorithm Trace edit. The reference *Pfkelch 13* nucleotide and protein sequence were downloaded from the National Center for Biotechnology Information (NCBI) data base using BLASTN. These sequences were aligned using ClustalX 2.0.

2.8. Statistical analysis

Taking microscopy as the gold standard, the performance of the Rapid Diagnostic Test used in selecting the species of malaria (*P. falciparum*) for amplification and sequencing was compared to it by computing the sensitivity, specificity, the negative predictive value, the positive predictive value and the accuracy of the test method (RDT) to ensure that

only *P. falciparum* was used in the PCR amplification and sequencing. The data obtained was analyzed using MedCalc version 22.009 statistical package [18]

3. Results

A total of 385 blood samples were collected during the period of this study and 115 samples were positive for malaria parasite by microscopy, (the WHO gold standard for malaria parasite diagnosis) representing 29.9% prevalence rate. Meanwhile, 103 samples (26.8%) were confirmed by RDT to be specifically positive for *P. falciparum*. The sensitivity, specificity and accuracy of the RDT was determined using standard protocol by [19]. The sensitivity of RDT was 89.6% (95% CI = 82.48% to 94.49%) while its specificity was 100.00% (95% CI = 98.64% to 100.00%). The Positive Predictive Value (PPV) was 100.00% (95% CI = 96.48% to 100.00%) while the Negative Predictive Value (NPV) was 95.8% (95% CI = 92.94% to 97.46%). The prevalence of malaria using microscopy in this study population was 29.9% (95% CI = 25.34 to 34.71%). The accuracy of this diagnostic tool was 96.88% (95% CI = 94.62 to 98.38%) meaning it was fit as a diagnostic tool for this research.

Following low parasitemia and/or poor DNA quality after DNA extraction and quantification, not all the 103 RDT positive samples were used for the amplification. Of the 103 RDT positive samples, only 90 samples (87.4%) were amplified by PCR from which only 48 samples (53.3%) were confirmed to be *P. falciparum.* The occurrence of malaria parasite by Microscopy, RDT and PCR diagnostic methods in representative samples in Nasarawa-West Senatorial District is presented in table 2 while an agar gel electrophoresis film of the amplified representative samples is shown in Figure 1.

Table 2 Occurrence of malaria parasite by Microscopy, RDT and PCR diagnostic methods in representative samples in Nasarawa-West senatorial zone.

Facility	**Microscopy (n=77)**		**RDT (n=77)**		**PCR (n=18)**	
	No +ve	**%**	**No +ve**	**%**	**No +ve**	**%**
GHK	20	25.9	18	23.4	11	61.1
GHN	26	33.8	23	29.9	9	50
GHG	25	32.5	21	27.3	11	61.1
MCMG	22	28.6	21	27.3	8	44.4
SMC	22	28.6	20	25.9	9	50
Total	115	-	103	-	48	-

GHK = General Hospital, Keff; GHN = General Hospital, Nasarawa; GHG = General Hospital, Garaku; MCMG = Medical Centre Mararaban Gurku; SMC = SAPETRO Medical Centre, NSUK; No = Number; +ve = Positive; % = Percent

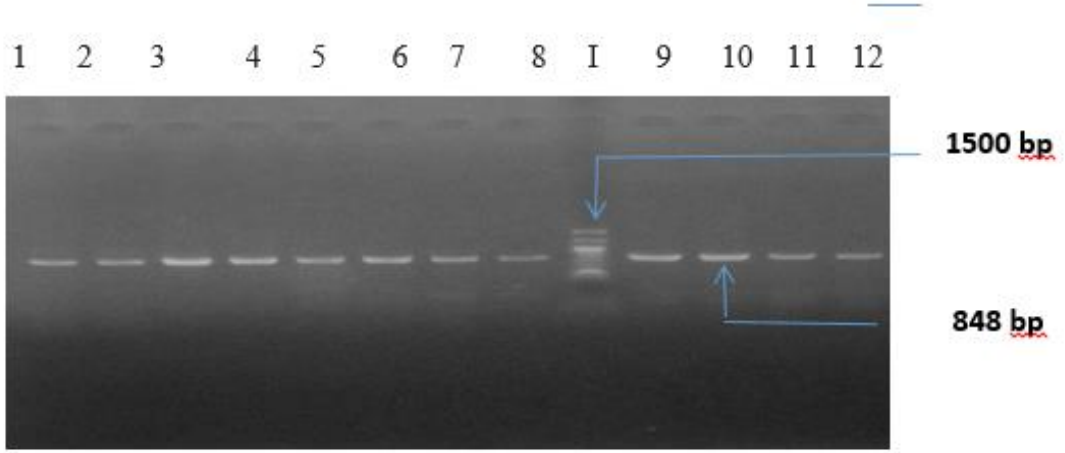

Figure 1 Agarose Gel Electrophoresis showing bands of representatives samples of amplified Kelch-13 genes of *P. falciparum.* Lanes 1-12 represent the positive Kelch-13 genes while lane I is the 100bp DNA ladder at 1500bp

Of the 48 PCR confirmed *P. falciparum* positive samples, only 20 samples were randomly selected and sequenced in other to detect/determine the Kelch 13 resistance (mutant) genes. The nucleotide sequence alignment revealed six (6) Single Nucleotides Polymorphisms (SNPs) among the sequences from all the sampled localities. This Nucleotide sequence alignment showed point mutations (indicated with red arrow) on some Kelch 13 gene sequences as shown on figure 2A - F. The six (6) SNPs detected include:

- A. Sample I5 at position 118, T was replaced with A
- B. Sample I7 at position 128, T is replaced with A
- C. At position 500 of sample I42, GC was replaced with TG
- D. At position 555 of sample I42, T was replaced with C
- E. Sample I7 at position 616 showed T replaced with A
- F. Sample I7 at position 638, A was replaced with C

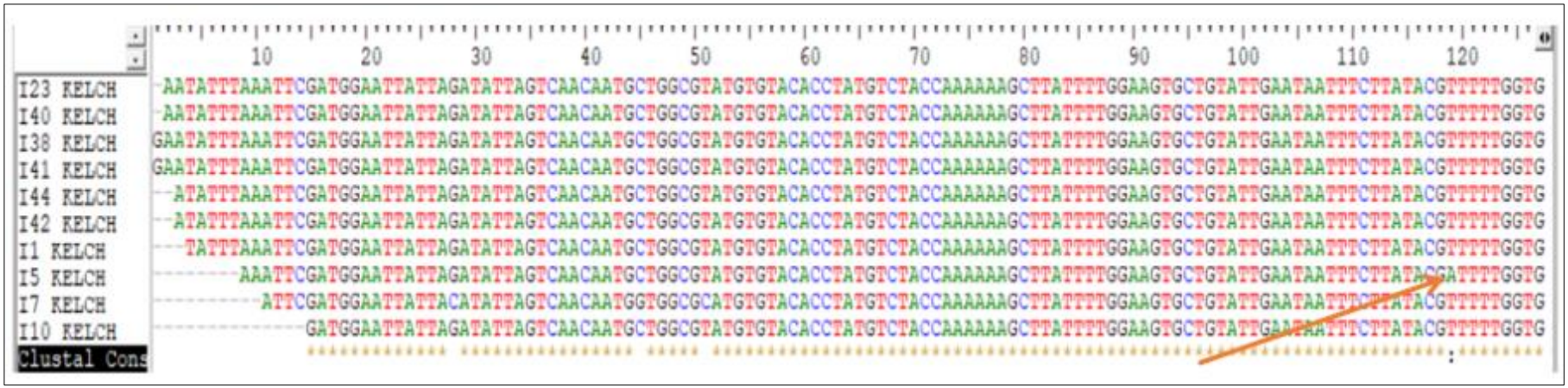

Figure 2A Nucleotide sequence alignment showing point mutation of sample I5 at position 118 where T was replaced with A

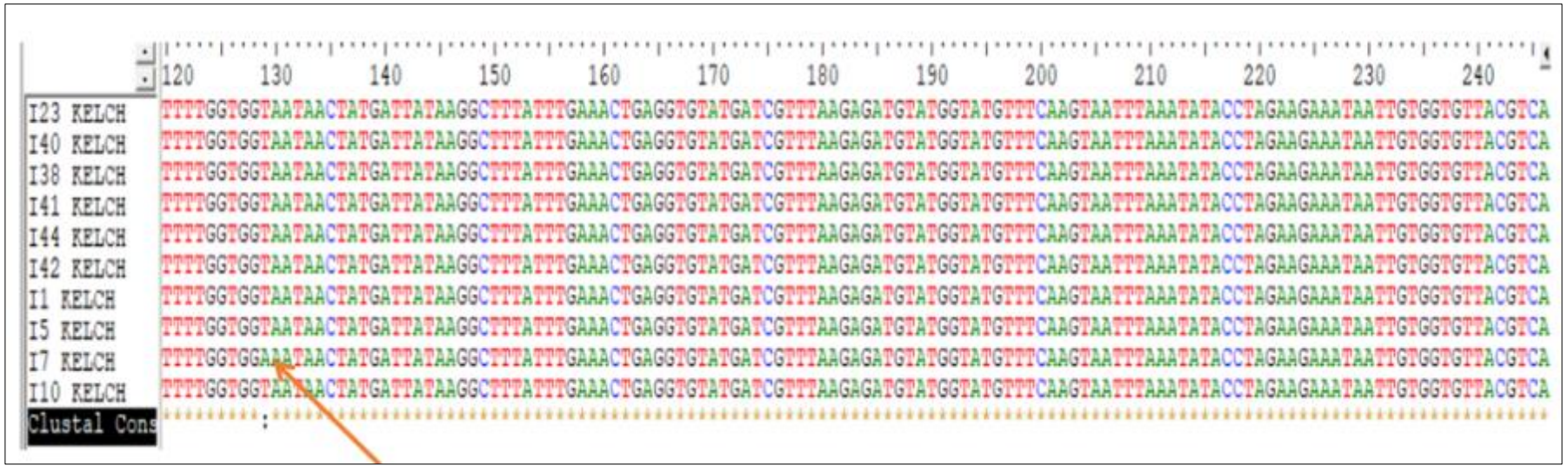

Figure 2B Nucleotide sequence alignment showing point mutation of sample I7 at position 128, T was replaced with A

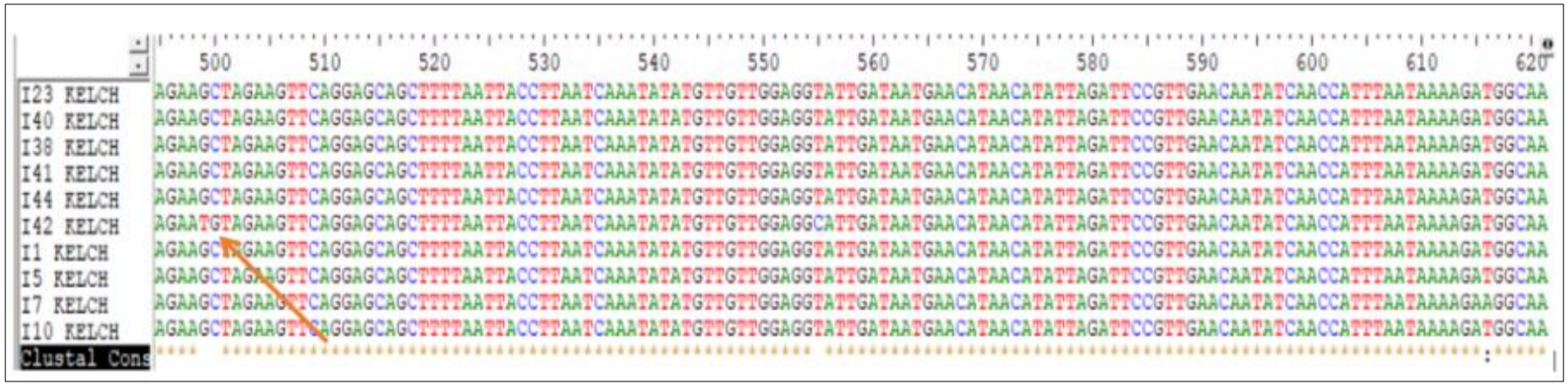

Figure 2C Nucleotide sequence alignment showing point mutation of sample I42 at position 500, GC was replaced with TG

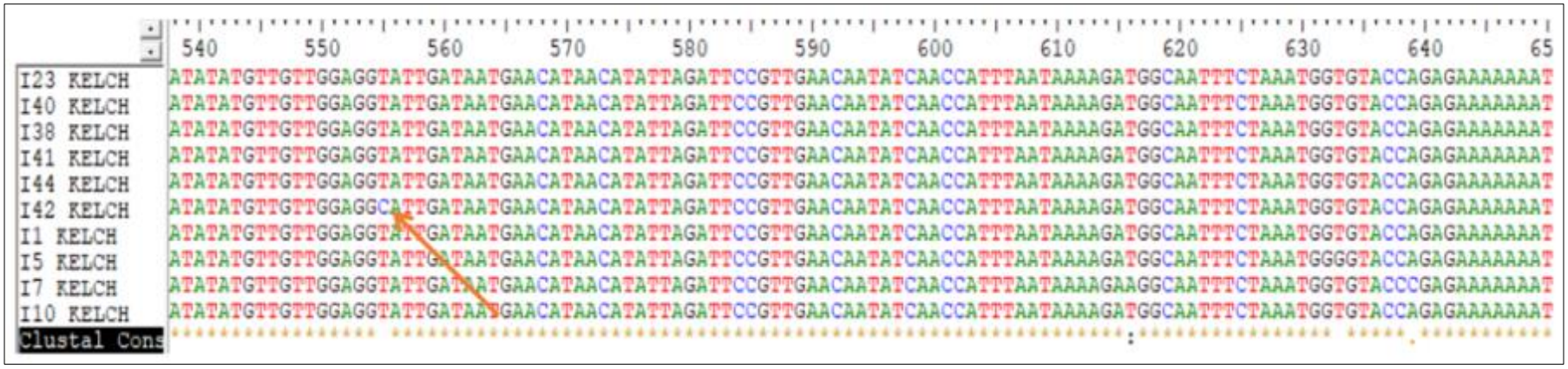

Figure 2D Nucleotide sequence alignment showing point mutation of sample I42 at position 555, T was replaced with C

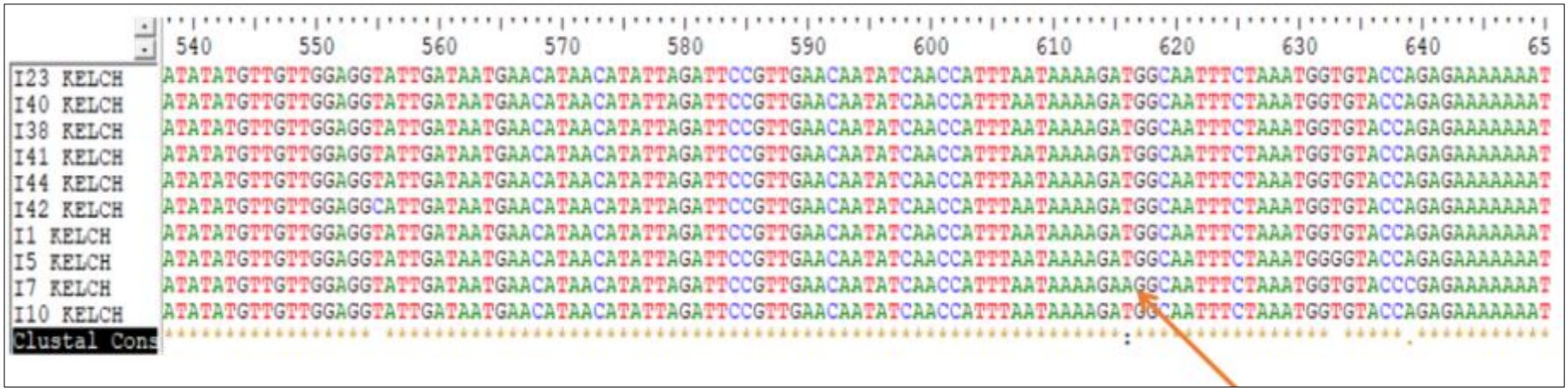

Figure 2E Nucleotide sequence alignment showing point mutation of sample I7 at position 616, T was replaced with A

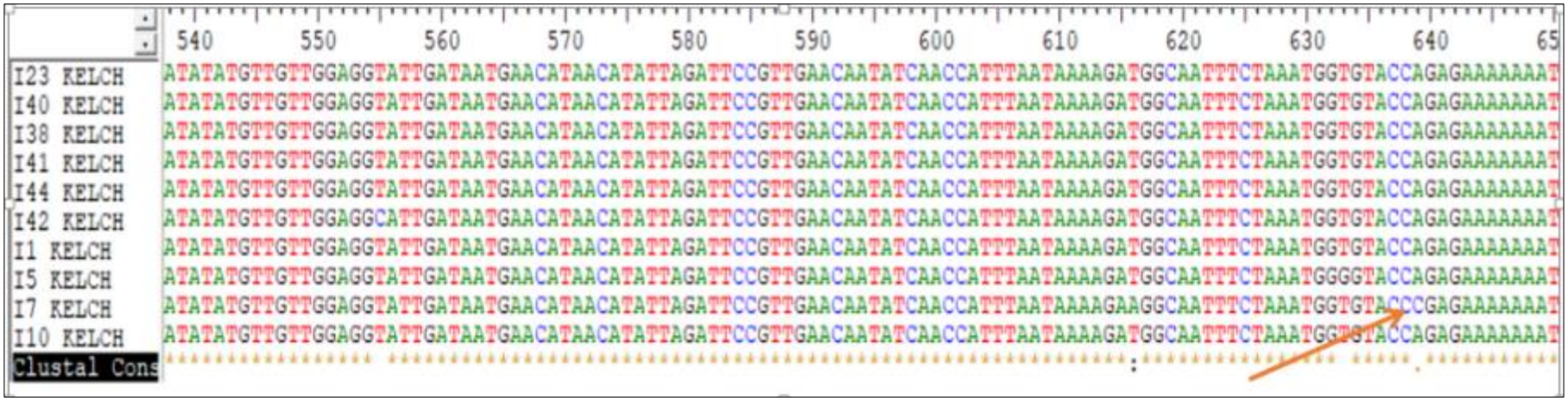

Figure 2F Nucleotide sequence alignment showing point mutation of sample I7 at position 638, A was replaced with C

The nucleotide sequences above were converted to protein sequences where a point mutation was detected in one (1) out of 20 *Pfkelch* 13 gene sequenced representing 5% (1/20) of the study samples. From the result, sample I5 at position 40 showed a point mutation where D (Aspartic acid) replaced V (Valine) as

shown by the red arrow on the protein sequence alignment. The results of the sequences obtained following the conversion of the nucleotide sequences to protein sequences is presented in figure 3.

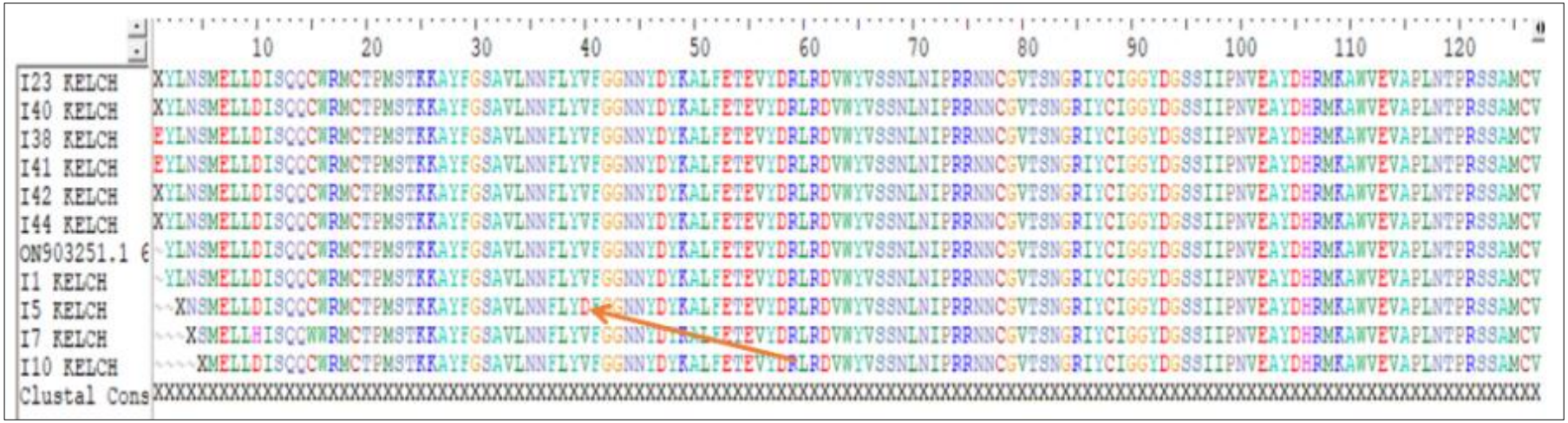

Figure 3 Sequence alignment of Kelch 13 protein sequences showing a point mutation in sample I5 where D (Aspartic acid) replaced V (valine) at position 40 on the protein sequence

4. Discussion

Out of the 90 RDT positive samples amplified by PCR, results from table 2 indicated that, only 53.3% (48/90) tested positive for *P. falciparum* by the PCR method. The remaining 42 (46.7%) samples may be false positives by the RDT method or as a result of low parasitemia and/or poor DNA quality after DNA extraction. This position of positive result by microscopy and RDT but negative by PCR is at variance with a report submitted by [20] where a false negative rate of 19.4% and 7.4% was reported for microscopy and the RDT respectively when PCR showed positive on same samples. False negatives by any diagnostic method is a big public health problem because there is a part of the population that returns home from a health facility without a correct diagnosis and treatment, not complying with the rule "fast and correct diagnosis and treatment with confirmed presence of the parasite". This could have important implications in health, transmission, and possible mortality. Accurate diagnostic methods are the basis for an adequate disease control and avoidance of occurrence and spread of antimalarial drugs resistance.

Results from Figure 1 which is an Agarose gel electrophoresis film of representative samples of the amplified Kelch 13 gene showed lane 1-12 having a band size of 848pb, corresponding to the helix domain of the *Pfkelch13* gene. Lane I is a 100 bp molecular size marker (Bio Labs inc, 100 - 1500 bp ladder). This band size of the gene (*PfKelch 13*) corresponds to other reports on the same gene [9, 7, 21, 22, 23].

The nucleotide sequence alignment result revealed six (6) Single Nucleotide Polymorphisms (SNPs) which are point mutations. The sequenced result revealed a mutant gene at position 40 where Aspartic acid replaced valine. This study detected *V118D, V128G, A500C, G555G, M616L* and *G638G* polymorphisms. This result is not consistent with the already validated mutant genes responsible for Artemisinin resistance detected in the Thai-Myanmar and Thai-Cambodia border regions which have since spread widely across South East Asia [24]. According to research by [9, 24], some of the validated mutant genes in the Thai-Myanmar and Thai-Cambodia border regions include: *F446I, N458Y, M476I, Y493H, R539T, I543T, P553L, R561H, P574L, C580Y* and *A675V.* The result of this study is also not in agreement with reports from [7, 8, 22, 25, 26]. Going by the results of the validated and candidate mutant genes above, none of the mutant genes detected in this study is consistent with the validated and candidate mutant genes. This suggest that the detected mutant genes in this study could be further investigated to ascertain whether this mutation is capable of Artemisinin resistance thereby making the genes to serve as a marker for the surveillance of Artemisinin resistance genes in the study area.

5. Conclusion

Malaria-endemic populations with genetically polymorphic *P. falciparum* strains as seen in this study could increase the complexity or multiplicity of infection within such communities. This could result in the emergence of more virulent or drug-resistant forms of the parasite, posing a threat to eradication of the disease. The is due to the fact that genetically diverse malaria strains could respond differently to treatment. Decreased efficiency of hemoglobin uptake due to K13 mutation may contribute to reduced artemisinin activation and subsequent resistance of the Artemisinin based drugs by the mutant parasite. The detection of *P. falciparum* gene polymorphisms in this study (though in low frequency) could serve as important marker for assessing differences in malaria transmission intensity and antimalarial resistance trends in the study area when widely checked and further investigated. Conclusively, the need for continuous surveillance following the detection of mutant gene in this study population is recommended in order to have a wider picture of the parasite diversity for effective malaria treatment and control.

Compliance with ethical standards

Acknowledgments

We thank all the Staff in the Department of Microbiology Laboratory, Nasarawa State University, Keffi for the cooperation during the bench work.

Disclosure of conflict of interest

The authors declared no conflict of interest.

Statement of ethical approval

The ethical approval was obtained from the Research and Ethical Committee of the Nasarawa State Ministry of Health, Nasarawa State, Nigeria.

Statement of informed consent

Informed consent was obtained from all individual participants included in the study.

Author's Contributions

The study was designed by Igbawua IN who also conducted the experiments and prepared the manuscript. Ngwai YB supervised the study. Both authors proof-read and approved the article.

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