Original Research Article

Genetic insights into Plasmodium falciparum resistance to sulfadoxine-pyrimethamine through a study of the 540-dihydropteroate synthetase gene mutations in Jos, Nigeria

ABSTRACT

Aims: To investigate mutations in the 540-dihydropteroate synthetase (*540dhps)* gene, specifically at codon 540, that may confer resistance to sulfadoxine-pyrimethamine treatment by*Plasmodium falciparum* in Jos.

Study design: This cross-sectional stud.

Place and Duration of Study: The samples for this study were gathered in Jos, Plateau State, Nigeria, between October 2019 and January 2021.

Methodology: One hundred malaria cases were assessed, and parasite DNA was extracted from whole blood samples using Zymo extraction kits. PCR and gel electrophoresis identified the *Plasmodium* genus and *P. falciparum*, along with the 540-dihydropteroate synthetase gene. Sanger sequencing and bioinformatics analyses (nucleotide blast, blastx, and query-anchored alignment view with dot of the NCBI tool) were employed.

Results: Results revealed that 50% of the tested samples were positive for the Plasmodium genus, with 96% identified as *P. falciparum*. Point mutations were found in the *540dhps* gene at codon K540E in 16.67% of the samples. Interestingly, while a nucleotide mutation occurred in the DNA's coding region, it did not translate into amino acid changes, indicating no likely impact on the protein's functionality.

Conclusion: This study highlights the prevailing presence of Plasmodium falciparum in Jos, with a significant 96% prevalence among malaria patients. Contrary to the previously reported mutations at codon K540E on 540dhps, the mutation observed in the nucleotide sequence in our findings did not affect the amino acid sequence, suggesting that this mutation may not be associated with the literature previously reported on resistance to

sulfadoxine-pyrimethamine drug.

Keywords: *sulphadoxine-pyrimethamine resistance,Plasmodium falciparum,Mutations, Malaria*

1. INTRODUCTION

Malaria is a leading source of illness and mortality, among other infectious diseases such as tuberculosis and HIV [33]. It is the most widespread parasite-causing illness worldwide with 241 million cases in 2020, resulting in 627,000 fatalities that could have been avoided [34]. The African continent bears the brunt of the disease's toll, with 96% of malaria-related deaths and 80% of these are under the age of five resulting in nearly 750 deaths daily [31].

According to the World Health Organization, severe falciparum malaria is defined as the presence of *P. falciparum* asexual parasitaemia and one or more clinical or laboratory characteristics with no other known reason for their symptoms [11]. There are around 30 million pregnant women in Sub-Saharan Africa who are at risk of malaria [19], and the prevalence of malaria in pregnancy is believed to be around 28%. As a result, *Plasmodium falciparum*malaria continues to be a serious public health issue in Sub-Saharan Africa [3]. This problem endures because human malarial parasites (*Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae,* and *Plasmodium ovale*) develop resistance to anti-malarial medications [18]. Many countries switched their first-line pharmacological treatment from chloroquine to sulfadoxine-pyrimethamine, as a result of the emergence of chloroquine resistance and increased rates of clinical treatment failure [21]. Sulfadoxine and pyrimethamine (SP) work together to stop the malaria parasite from making folic acid by blocking the enzymes dihydropteroate synthetase (dhps) and dihydrofolate reductase (DHFR) in *Plasmodium falciparum* [12].

Resistance to sulfadoxine and pyrimethamine in *Plasmodium falciparum* has been linked to polymorphisms in the dihydropteroate synthase (DHPS) and dihydrofolate reductase (DHFR) genes, respectively [18]. The arrival of major SP treatment failures in East Africa was foreshadowed by the discovery of a DHPS double mutant A437G + K540E[16]. Changes in the sulfadoxine binding site of *P. falciparum* DHPS are caused by mutations at five amino acid sites. The initial step to sulfa drug resistance is a change from alanine to glycine at codon 437 (A437G), which is followed by subsequent changes at codons 436 (S436A), 540 (K540E), 581 (A581G), and 613 (A613S/T), which produce a further increase in drug resistance [16]. Due to the occurrence of distinct *P. falciparum* DHPS (*540dhps*) mutant alleles, the amount of sulfadoxine resistance in *P. falciparum* isolates varies around the world [14]. In vivo, the accumulation of *pfdhfr* and *540dhps* mutations causes an increase in sulfadoxine/pyrimethamine resistance [2]

To be able to control this problem, Malaria control and prevention activities must be tailored to the unique setting in which they will be implemented [17], taking into account local malaria epidemiology, resource availability, and political will [4]. As a result, molecular research into the genetic make-up of these *Plasmodium* species and their polymorphism areas will aid in the improvement of existing anti-malarial medications as well as the development of new drugs for malaria treatment. In the light of these, the goal of this study is to look at polymorphisms in the *Plasmodium falciparum* dihydropteroate synthase gene (*540540dhps*) in malaria patients who are resistant to sulphadoxine and pyrimethamine.

2. MATERIAL AND METHODS

All methods were performed in accordance with the guidelines and regulations provided by the institutional review board and ethical committee.

2.1 Sample Collection

One hundred clinically screened 2 Plus (++) and above positive malarial samples were collected in EDTA bottles from Plateau State Specialist Hospital and stored at 4^{o}C for DNA extraction.

2.2 DNA Extraction

Genomic DNA was isolated from one hundred (100 µL) of each whole blood sample using the Zymo Research extraction kits according to the manufacturer's instruction. Thirty microliters (30µl) of the elusive DNA were transferred to a clean micro-centrifuge tube and stored at 4^{o}C for immediate use, while the remaining forty microliters (40µl) was stored at -20^{o}C as a backup for future use. Each of the 100 blood samples underwent the same process.

2.3 PCR identification of Plasmodium genus

The purified DNA was used as templates for the amplification of *Plasmodium* genus. For the initial amplification targeting the 18S rRNA gene, forward and reverse primers (rPLU5-5′-CCTGTTGTT GCCTTAAACT TC-3′) and (rPLU6 - 5′-TTAAAATTGTTGCAGTTAA AACG-3′) respectively were used and the product size expected was 1100bp.

2.4 PCR identification of *Plasmodium falciparum*

Five microliters (5µl) of the amplified product of *Plasmodium* species was used for the second round of amplification of *Plasmodium falciparum* with forward and reverse primers rFAL-1 (5′-TTAAACTGGTTTGGGAAAACCAAATATATT-3′) and rFAL-2 (5′-ACACAATGAACTCAA TCATGACTACCCGT C-3') with the expected product size of 205bp.

2.5 PCR identification of *540dhps*

Two rounds of nested PCR were performed to detect 540 DHPS gene. The nest1 forward and reverse primer sequences were R2-5′-AACCTAAACGTGCTGTTCAA-3′ and R- 5′-AATTGTGT GATTTGTCCACAA-3′ respectively while the nest2 forward and reverse

primers sequence were K-5′-CCTAAACGTGCTGTTCAAAGAA-3′ and K-5′-TTCGCAAATCCTAATCCAATATC-3′ respectively.

2.6 Sequencing and Bioinformatic Analysis of540 *Pf* DHPS Gene

The *Plasmodium falciparum540dhps* gene was sequenced using Sangar sequencing method at InqabaBiotec Industry, Pretoria, South Africa. The sequence results were edited using Bioedit software, blast search was done using NCBI tools for nucleotide and amino acid sequence alignments, nucleotide sequence alignment using OmegaX software and protein structure model was predicted using Swiss Model from ExPaSy web server.

3. RESULTS AND DISCUSSION

3.1 PCR Results: The gel picture of the amplicons produced by PCR amplification of the *Plasmodium falciparum* gene is represented in Figure 1 – 5. From the electrophorogram, Figure 1 shows the Agarose Gel electrophorogram of PCR products of *Plasmodium falciparum*gene amplification for sample 1 – 20; sample 7, 11, 12, and 14 were positive for *Plasmodium falciparum* at expected band size of 205bp, while the other samples were found to be negative for *Plasmodium falciparum.*

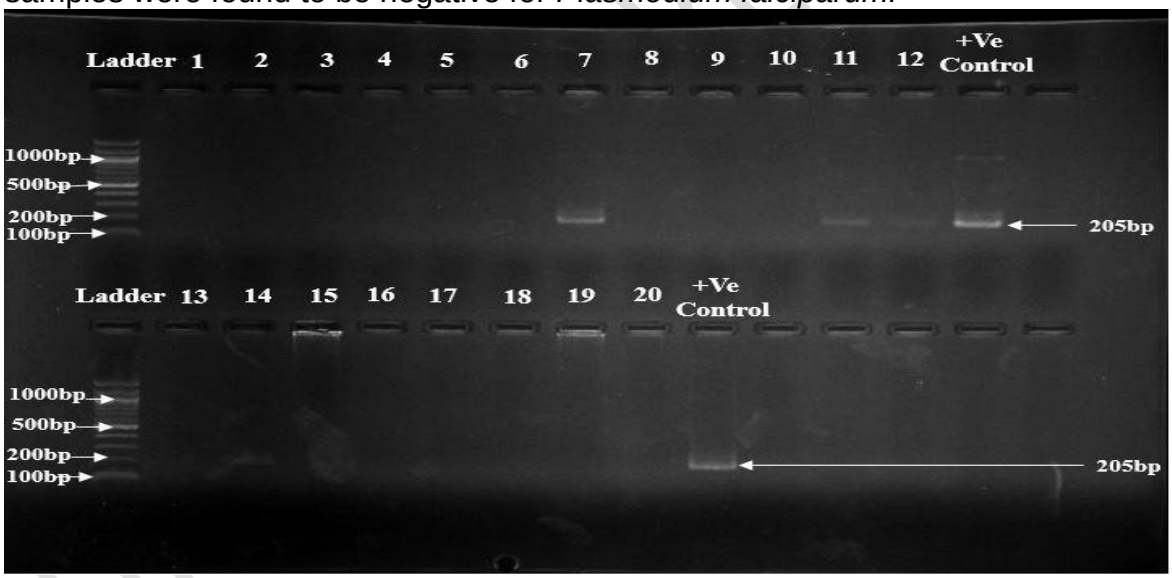

Figure 1. PCR products of *Plasmodium falciparum*Gene Amplification (sample 1 – 20)

Figure 2 presents the PCR products of *Plasmodium falciparum*gene amplification for sample 21 – 40; sample 23, 24, 26, 27, 28, 29, 30, 31, 35, 36, 37, 38, and 40 were positive for *Plasmodium falciparum,* while the other samples were found to be negative.

Also, Figure 3 gives the PCR products of *Plasmodium falciparum*gene amplification for sample 41 – 60; sample 41, 42, 43, 49, 50, 53, 54, 55, and 56 were positive for *Plasmodium falciparum,* while the other samples were found to be negative. Figure 4 presents the PCR products of *Plasmodium falciparum*gene amplification for sample 61 – 80; sample 61, 62, 66, 69, 77,

78, 79, and 80 were positive for *Plasmodium falciparum,* while the other samples were found to be negative. While Figure 5 presents the PCR products of *Plasmodium falciparum* gene amplification for sample 81 – 100; sample 82, 83, 84, 85, 86, 88, 90, 91, 92, 93, 94, 95, 97, 98, 99, and 100 were identified positive for *Plasmodium falciparum* at expected band size of 205bp, while the other samples were found to be negative for *Plasmodium falciparum.* Figure 6 is the PCR products of amplification of *540dhps* gene. The PCR amplification depicted that sample 26, 27, 28, 30, 41, 56, 62, and 86 were positive for *540dhps* gene when screened with a 100bp ladder, and positive control (+Ve control) with 500bp known size used as references for positive samples.

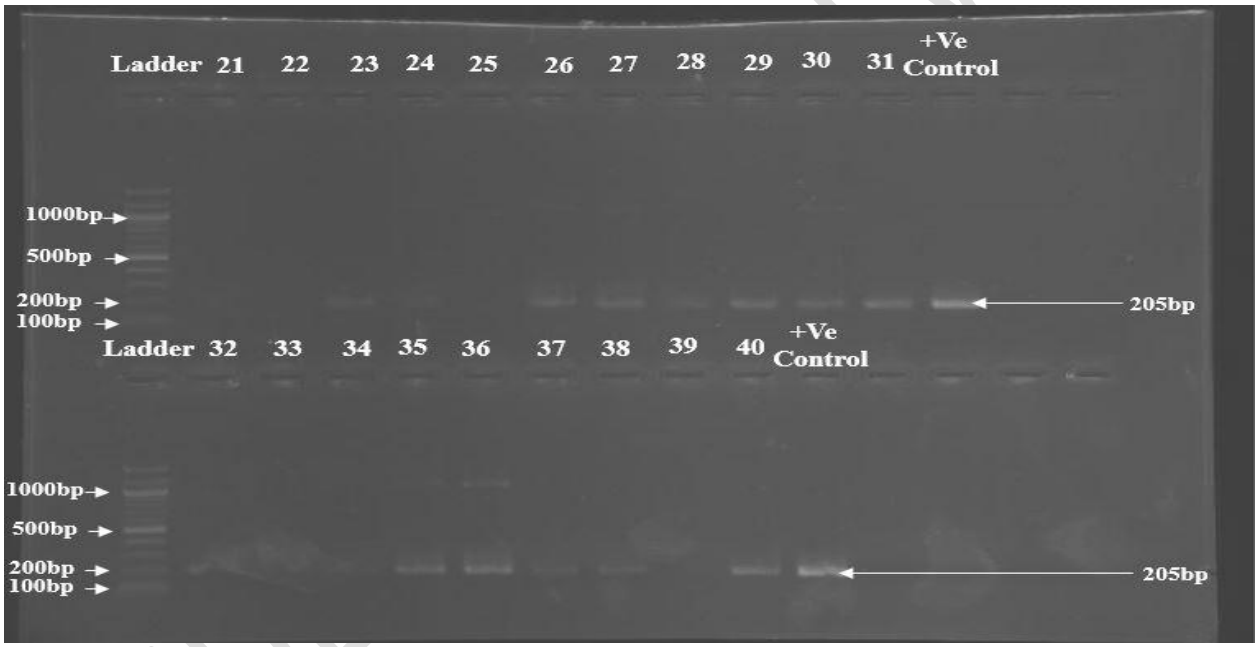

Figure 2. PCR products of *Plasmodium falciparum*Gene Amplification (sample 21 – 40)

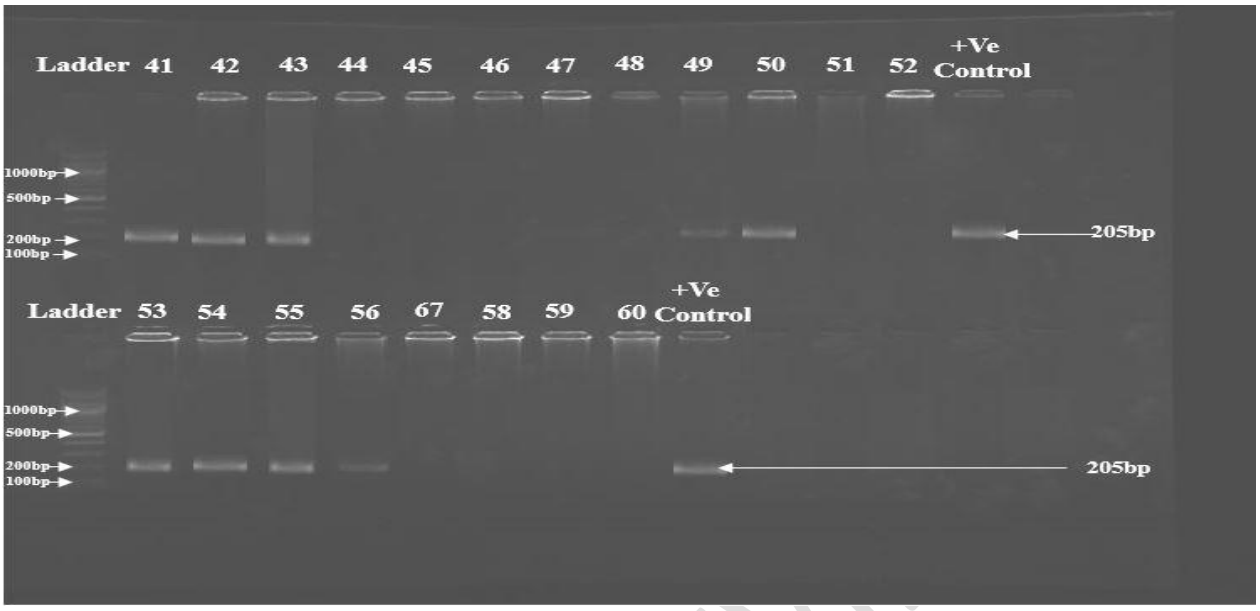

Figure 3. PCR products of *Plasmodium falciparum*Gene Amplification (sample 41 – 60)

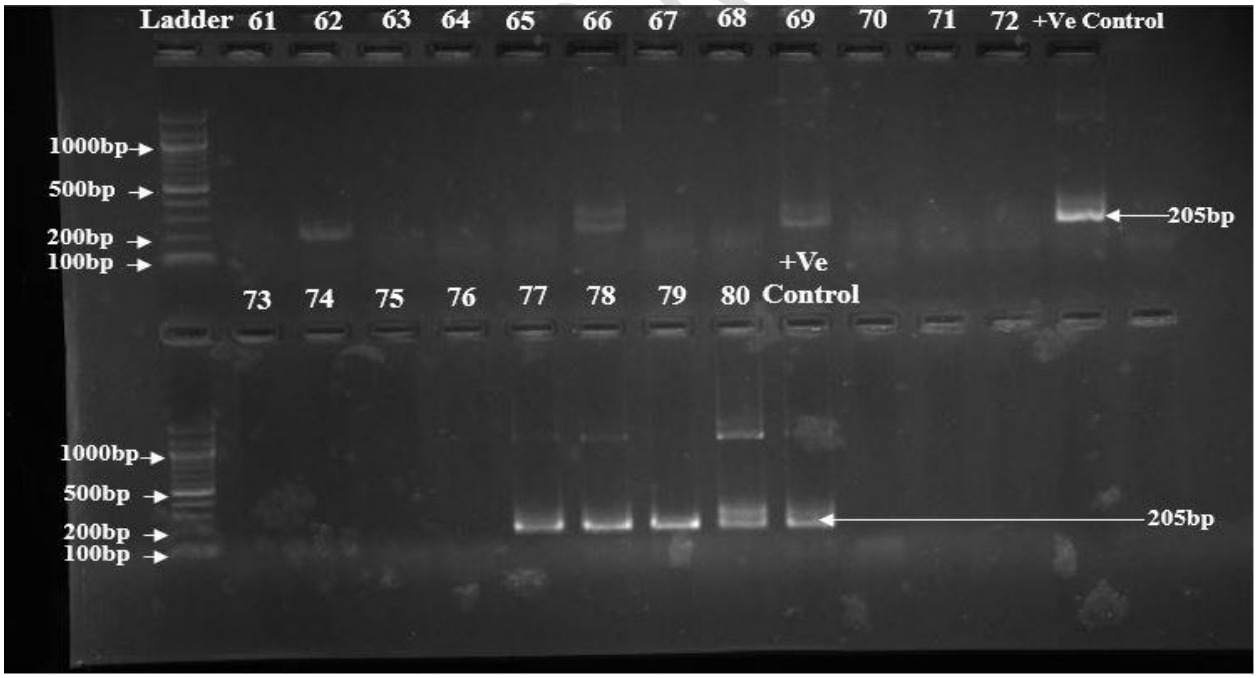

Figure 4. PCR products of *Plasmodium falciparum*Gene Amplification (sample 61 – 80)

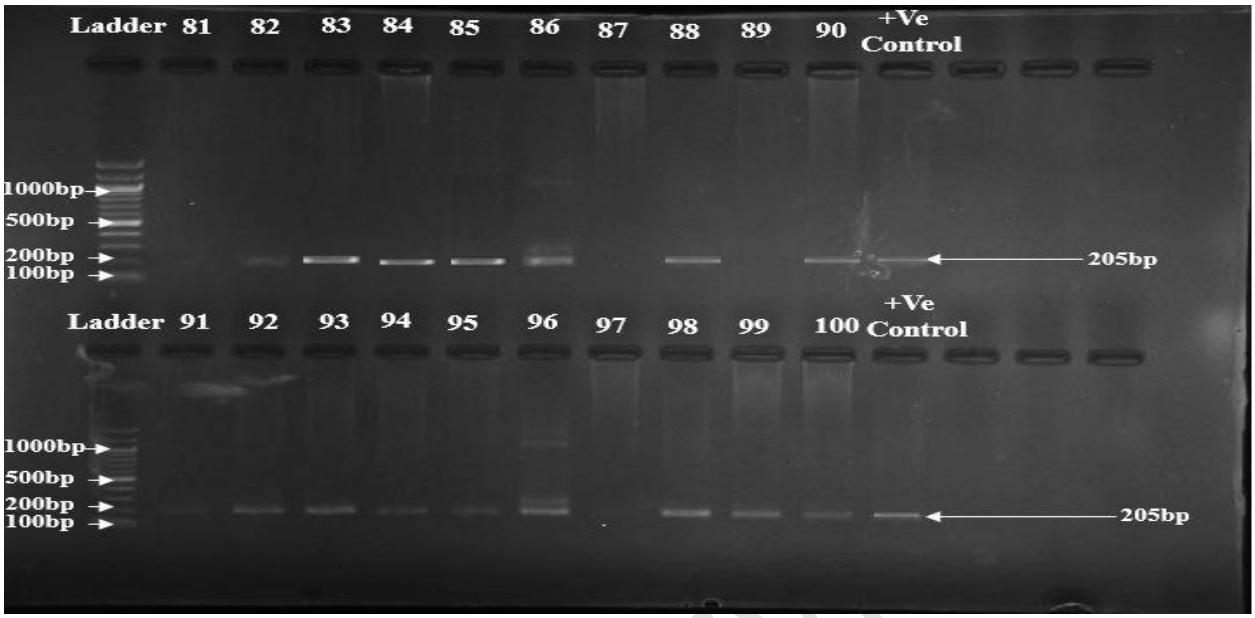

Figure 5. PCR products of *Plasmodium falciparum*Gene Amplification (sample 81 – 100)

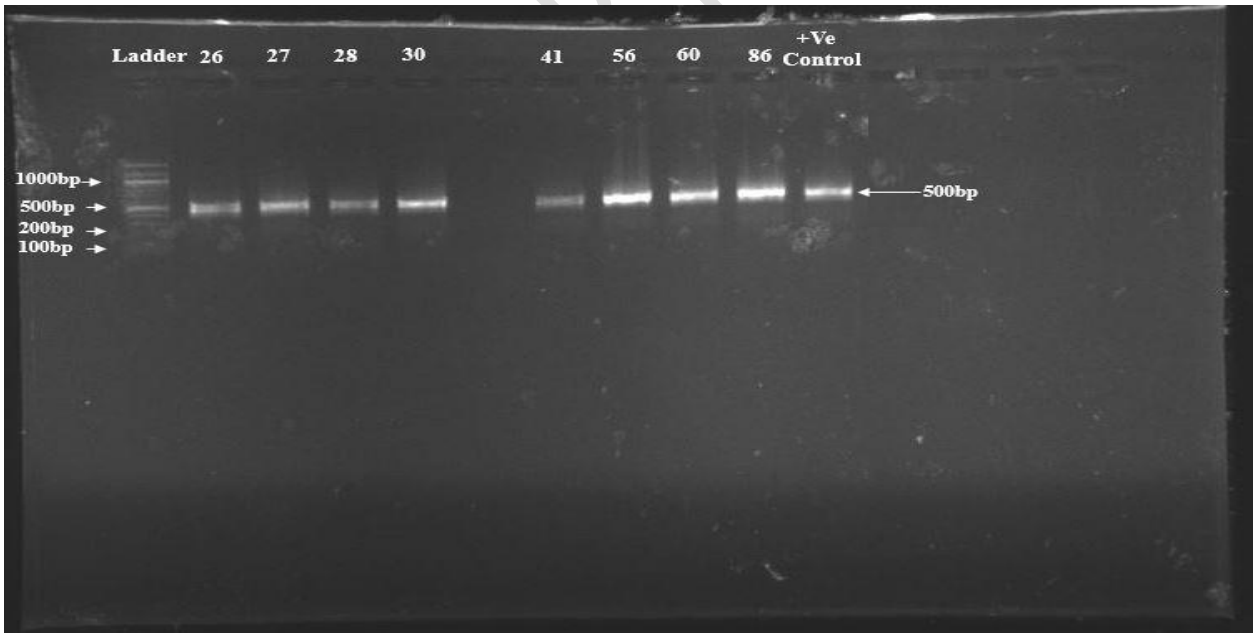

Figure 6. PCR products of Amplification of *540dhps* gene

3.2 Plasmodium Genus and *Plasmodium falciparum* and *540dhps* Gene Analysis

Out of 100 samples collected from the Hospital, 50 (50%) tested positive for plasmodium genus via PCR testing, while 50 (50%) tested negative as shown in Table 1. Screening of the 50 positive samples found 48 (96%) were positive for *Plasmodium falciparum* and just 2 (4%) were negative. Additionally, out of the 48 *P. falciparum*positive samples, 8 (16.67%) tested positive for the 540 DPHS gene, while 40 (83.33%) were negative.

These results corroborate prior research indicating *P. falciparum* is the most prevalent malaria species in Nigeria. The 96% rate of *P. falciparum* in positive samples reflects past studies finding 85% prevalence in Edo and Lagos (Citation 23) and 80% prevalence using microscopy and PCR [25]. Evidently, *P. falciparum* remains the primary plasmodium species causing malaria infections nationally.

However, the overall 50% plasmodium positive rate raises concerns of potential false positive diagnoses. As cited research notes, clinical laboratories are prone to diagnostic errors, often in pre-analytical phases [26]. Half of the samples may have been incorrectly deemed positive. Literature shows false positives lead to unnecessary malaria treatment and increased medication resistance [20, 29]. More accurate diagnostic techniques are vital to avoid giving antimalarials to patients without true infections.

Therefore, while *P. falciparum* was the main species in positive samples, the high 50% positive rate warrants further investigation into possible false positives, as per past research. More precise testing could improve treatment and prevent incorrect dosing risks. Though *P. falciparum* persists as prevalent, better diagnostics are essential to determine true infections.

Table 1: Distribution of *Plasmodium* genus,*P. falciparum*and*540dhps*

Parameters	***Plasmodium* Genus**	***Plasmodium falciparum***	***540dhps*Gene**
Total Sample N (%)	100(100)	50(100)	48(100)

Positives n (%)	50(50)	48(96)	8(16.67)
Negative n (%)	50(50)	2(4)	40(83.33)

N is the total number of samples used; n means the number of samples that are either positive or negative; % mean the percentage number of samples that are positive or negative.

3.2 Nucleotide Sequence Alignments for *540dhps*Gene

The result from the query-anchored alignment showed that guanine replaces thymine at position 10 on the query (*540dhps*) sequence of the DNA indicating a point mutation in the area marked yellow (Figure 7) [1]. The nucleotide blast search result listed sequences with 99.53% query coverage and 98.79% identity, showing that the characters in each of the sequences are identical and thus the sequences are similar [2]. The Qualitative Model Energy Analysis (QMEAN) value of -0.07 also shows agreement of the predicted sequence with the sequence in the database in terms of geometrical structure, long range interactions, and the burial status of the residues [3]. These results affirm that the extracted *540dhps* gene sequence is highly related to the ones in the database.

While this particular mutation does not appear to confer resistance, prior studies have shown that point mutations in *540dhps* can lead to sulfadoxine-pyrimethamine resistance. It has been reported that parasite resistance to this medication combination is linked to point mutations that diminish drug binding affinity [4] and accumulation of *540dhps* mutations leading to increasedsulfadoxine-pyrimethamine resistance in vivo [5].

However, the results of this study also show that sulfadoxine-pyrimethamine resistance is not linked to a variation in the parasite *540dhps* gene, contrary to some prior studies like [6]. A study in Lagos, Nigeria discovered a 22.5% increase in mutant *P. falciparum* isolates harboring sulphadoxine resistance-associated K540E mutations in the dhps genes, resulting in 15% of the parasite population carrying quintuple mutations [7]. This contrasts with the lack of resistance conferred by the silent mutation in this study.

Although a point mutation was identified but the lack of amino acid change and divergence from some previous findings suggests this specific mutation does not confer anti-malarial drug resistance. Therefore, further studies are needed to elucidate the mechanisms of resistance in this parasite population.

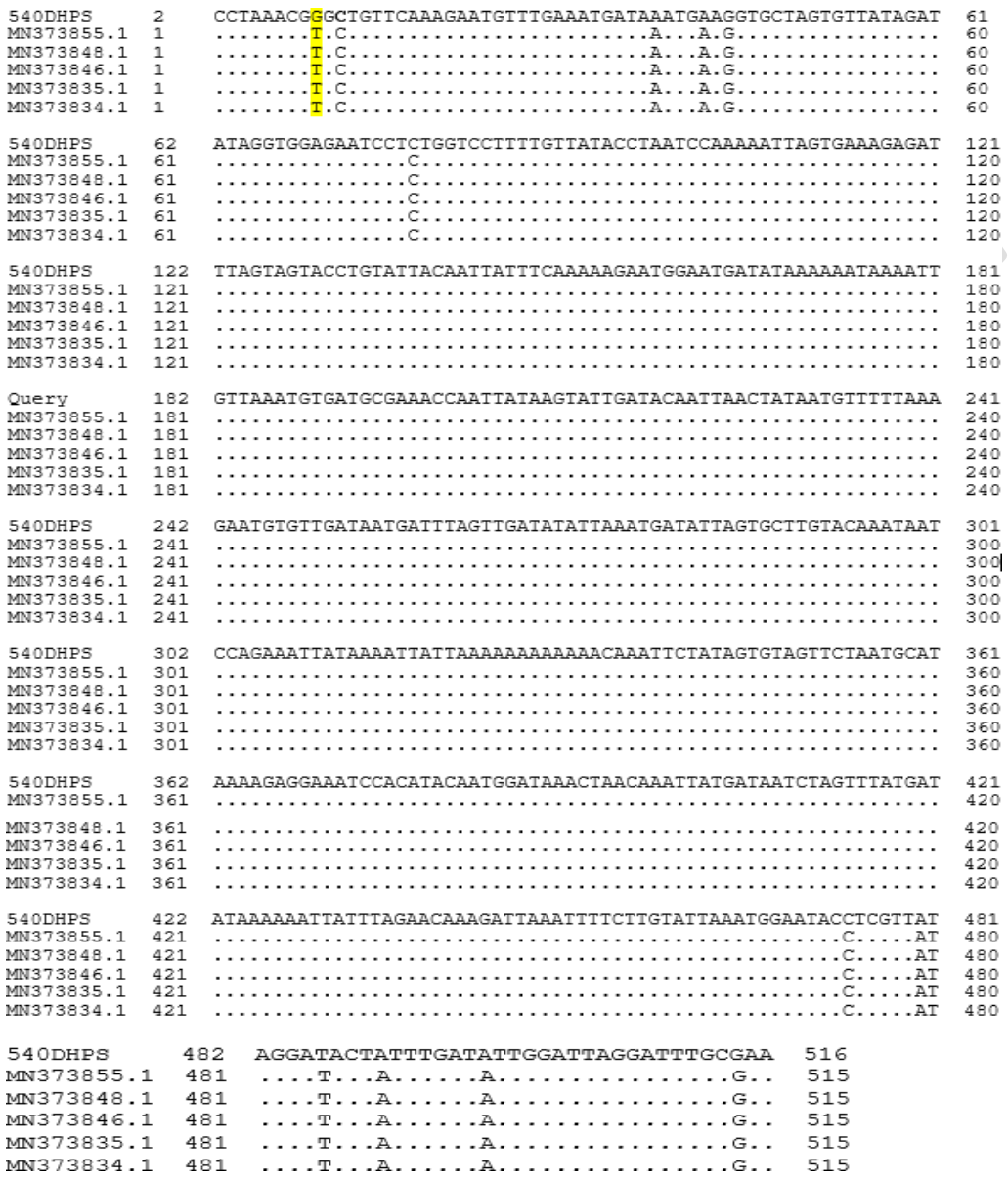

Figure 7. Query-anchored alignment views for finding Single-Nucleotide Polymorphism (SNP)

NCBI Blast *540dhps* is query sequenced with sequences with the following accession numbers MN373855.1, MN373848.1, MN373846.1, MN373835.1,

and MN373834.1, as references sequences. A silent point mutation was identified at position 10 in the *540dhps* query sequence versus homologous reference sequences, with guanine replacing thymine.

3.3 Amino Acid Sequence Analysis of *540dhps* Gene

Our findings showed that the mutations observed at the nucleotide level did carry over to the amino acid level as seen in Figure 8. Although some studies have shown that specific mutations in the *540dhps* gene can confer resistance to sulfadoxine-pyrimethamine (SP) [1-4], the discovered mutation in this study has no influence on the amino acid sequence. Consequently, it has no effect on the protein's functionality.

Although one study found a 16.67% prevalence of the K540E mutation [5], other studies have also found that the K540E mutation is less common in West Africa compared to East and South Africa [6,7]. Prior regional studies found higher K540E prevalence rates of 38.5% in Saudi Arabia [8], 22.5% in Lagos, Nigeria [9], and 58.7% prevalence in Northeast Nigeria [10].

These differences highlight geographic variations in mutation patterns conferring SP resistance. However, the lack of amino acid changes suggests that the reported resistance in the nucleotide sequence may not be attributable to mutations observed at the nucleotides level.

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540DHPS      2    PKRAVQRMFEMINEGASVIDIGGESSGPFVIPNPKISERDLVVPVLQLFQKEWNDIKNKI  181
QEQ07522.1   1    ...A........NEG..........S.................................  60
QEQ07515.1   1    ...A........NEG..........S.................................  60
AQM50543.1   1    ...A........NEG..........A.................................  60
QEQ07502.1   1    ...A........NEG..........S.................................  60
QEQ07513.1   1    ...A........NEG..........S.................................  60

540DHPS      182  VKCDAKPIISIDTINYNVFKECVDNDLVDILNDISACTNNPEIIKLLKKKNKFYSVVLMH  361
QEQ07522.1   61   ............................................................  120
QEQ07515.1   61   ............................................................  120
AQM50543.1   61   ............................................................  120
QEQ07502.1   61   ............................................................  120
QEQ07513.1   61   ............................................................  120

540DHPS      362  KRGNPHTMDKLTNYDNLVYDIKNYLEQRLNFLVLNGIPRYRILFDIGLGFAK  517
QEQ07522.1   121  ..................................P.Y.I...I......  172
QEQ07515.1   121  ..................................P.Y.I...I......  172
AQM50543.1   121  ..................................P.Y.I...I......  172
QEQ07502.1   121  ..................................P.Y.I...I......  172
QEQ07513.1   121  ..................................P.Y.I...I......  172
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Figure 8. Amino Acid Sequence Alignments of *540dhps* Gene

All nucleotide sequences *540dhps*, MN373855.1, MN373848.1, MN373846.1, MN373835.1, and MN373834.1 are translated using MegaX software and aligned using NCBI Blast.

3.4 The Swiss – Model Homology of Protein Structure

The predicted protein structure in Figure 9 has query coverage of 99.53% and sequence identity of 98.79% to the sequence in the database. The Global Model Quality Estimation (GMQE) is 0.95 reveals that the six sequence has the same property and quality structure. Global Model Quality Estimation (GMQE) shows the reliability of the predicted structure, it is expressed as a number between 0 and 1. The higher the GMQE value, the higher the structure's reliability. Qualitative Model Energy Analysis (QMEAN) shows a statistically significant improvement over nearly all quality measures describing the ability of the scoring function to identify the native structure and to discriminate good from bad model. The QMEAN of the model is -0.07 indicating that the query sequence agrees with the native sequence, with a solvation of 0.92 showing that the amino acids within the query and native

protein have the same attraction and association. Solvation potential describes the burial status of the residue.

The energy gap between the native fold and the average of an ensemble of misfolds in units of the ensemble's standard deviation is defined as the Z-score of a protein. The Z-score is frequently used to assess the capacity of knowledge-based potentials to distinguish the native fold from other options. For a highly reliable prediction, the Z – score should be 0. Figure 9 shows that the Z-score of the predicted structure is less than 1 (<1). This is an indication that the predicted protein structure is highly reliable.

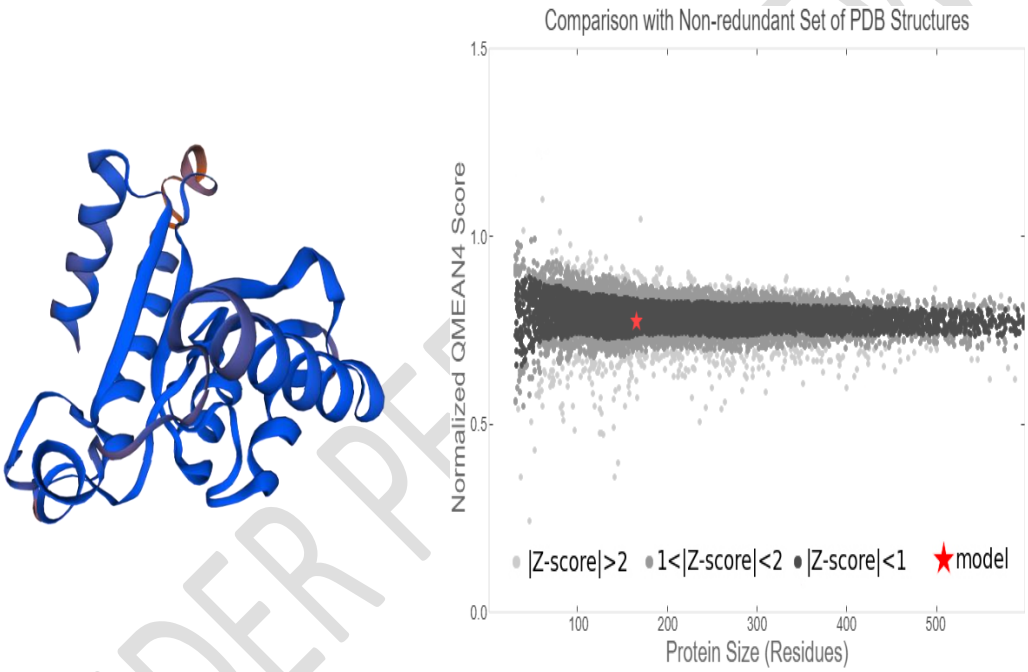

Figure 9 Predicted *540dhps* Protein Model.

The predicted protein structure displays high confidence metrics including 99.53% coverage, 98.79% identity, GMQE 0.95, QMEAN -0.07, solvation 0.92, and Z-score <1 versus native, indicating reliable structure prediction.

4. CONCLUSION

It was identified from the outcome of our investigation that *Plasmodium falciparum* was the most prevalent responsible for malaria infection in humans so, *Plasmodium falciparum* is the most common malaria species in Jos. The discovered mutation has no influence on the amino acid sequence, and consequently has no effect on the protein's functionality. It's possible that

the reported resistance isn't attributable to a mutation in the 540-dihydropteroate synthetase gene, but rather to other factors mentioned in the literature.

CONSENT

Informed consent was not obtained for this research because we (the authors) do not have direct contact with the patients. We only obtained positively screened blood sample from the hospital's laboratory. We ensured that the privacy of the individuals labeled on the EDTA bottles are fully protected and will never be shared with anybody or group.

ETHICAL APPROVAL (WHERE EVER APPLICABLE)

Ethical clearance was obtained from the Hospital Institutional Review committee with registration number NHREC/05/01/2010b. 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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