Genetic Insights into *Plasmodium falciparum* Resistance to Sulfadoxine-Pyrimethamine through a Study of the 540-dihydropteroate synthetase gene Mutations in Jos, Nigeria

ABSTRACT

Aim: This study investigates mutations at codon 540 in the dihydropteroatesynthetase (540dhps) gene that may confer resistance to sulfadoxine-pyrimethamine treatment by Plasmodium falciparum in Jos.

Study Design: The research employs a cross-sectional study design.

Place and Duration of Study: Samples were collected in Jos, Plateau State, Nigeria, from October 2019 to January 2021.

Methodology: One hundred malaria cases underwent assessment, 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 540dhps gene. Sanger sequencing and bioinformatics analyses were performed.

Results: About 50% of the collected samples were positive for the Plasmodium genus, with P. falciparum in 96% of cases. About 16% of P. falciparum samples were successfully amplified for the 540dhps gene. A silent point mutation was discovered at position 10 of the nucleotide sequence of the 540dhps gene which did not result in amino acid changes.

Conclusion: The study highlights the predominant presence of Plasmodium falciparum in Jos, with a substantial 96% prevalence among malaria patients. Unlike the previously reported mutations at codon K540E on the 540dhps gene from other studies, the observed mutation on the nucleotide sequence did not affect the amino acid sequence. Thus, the observed mutation may not be associated with resistance to sulfadoxine-pyrimethamine drugs.

Keywords: *Malaria, Drug-resistance,Single-Nucleotide Polymorphism, Plasmodium falciparum*

1. INTRODUCTION

Malaria remains one of the leading causes of illnesses and deaths globally, surpassing other infectious diseases such as tuberculosis and HIV [1]. In 2020, this widespread parasitic disease affected approximately 241 million individuals, resulting in around 627,000 avoidable deaths [2]. The African continent bears the greatest burden, accounting for approximately 96% of malaria mortality, with about 80% of these deaths occurring in children under five, equating to nearly 750 daily fatalities [3].

The World Health Organization defines severe falciparum malaria as the presence of Plasmodium falciparum parasitaemia alongside clinical or laboratory characteristics without other explanatory causes [4]. Around 30 million pregnant women in sub-Saharan Africa face malaria risk [5], with an estimated prevalence of around 28%. Consequently, Plasmodium falciparum malaria persists as a major public health issue in the region [6], as human malaria parasites develop resistance to medications [7]. Many countries have switched first-line treatment from chloroquine to sulfadoxine-pyrimethamine due to spreading chloroquine resistance and rising clinical failure rates [8]. However, it is important to know that the sulfadoxine-pyrimethamine drug works by jointly inhibiting the malaria parasites' folic acid production through the enzymes DHPS and DHFR [9].

It is also important to note that the resistance to sulfadoxine-pyrimethamine drugs has been traced to polymorphisms in the DHPS and DHFR genes respectively [7]. Also, the advent of the major East African sulfadoxine-pyrimethamine treatment failures was presaged by the discovery of a DHPS double mutant A437G + K540E [10]. It was also reported that the mutations at five DHPS amino acid sites drive changes to the Plasmodium falciparum sulfadoxine binding site, beginning with A437G, followed by S436A, K540E, A581G, and A613S/T, progressively elevating drug resistance [10]. The distinct Plasmodium falciparum DHPS (540dhps) mutant alleles have produced varying sulfadoxine resistance levels globally [11]. Moreover, the accumulation of PFDHFR and 540dhps mutations in vivo has also raised concerns about sulfadoxine-pyrimethamine resistance [12].

To effectively control and prevent malaria, it's important to customize strategies based on the local situation, considering factors like disease patterns, available resources, and local commitment [13, 14]. Research on the genetic variations in the Plasmodium parasite can help improve both current and future antimalarial treatments. That's why we are seeking to understand the specific genetic changes in the 540dhps gene among Nigerian malaria patients in Jos who are resistant to sulfadoxine-pyrimethamine. Thus, this research aims to provide valuable information to support the ongoing efforts to control malaria.

2. MATERIALS AND METHODS

All procedures conducted in this investigation adhered to the guidelines and regulations stipulated by the institutional review board and ethical committee.

2.1 Sample Collection

One hundred malarial samples testing positive for 2 plus and above were obtained in EDTA bottles from Plateau State Specialist Hospital and preserved at 4°C for subsequent DNA extraction.

2.2 DNA Extraction

The Zymo Research extraction kits were used to extract the genomic DNA from 100 µL of each whole blood sample collected, following the manufacturer's instructions. After the extraction, 30µl of the extracted DNA was immediately stored at 4°C for current use, while the remaining 40 microliters (40µl) were archived at -20°C for future use. This process was applied uniformly to all 100 blood samples.

2.3 PCR Amplification of Plasmodium Genus and Plasmodium falciparum

The purified DNA served as a template for amplifying the Plasmodium genus. The amplification targeted the 18S rRNA gene using the forward and reverse primers (rPLU5-5′-CCTGTTGTTGCCTTAAACTTC-3′) and (rPLU6-5′-TTAAAATTGTTGCAGTTAAAACG-3′) [15] with an expected product size of 1100bp. The thermocycling conditions are detailed in [16].

Five microliters (5µl) of the Plasmodium species' amplified product were used for the second round targeting the 18S rRNA gene of Plasmodium falciparum, employing forward and reverse primers 5′-TTAAACTGGTTTGGGAAA ACCAAATATATT-3′ and 5′-ACACAATGAACTCAATCATGACTACCCGTC-3' respectively, with an expected product size of 205bp [15]. The thermocycling conditions are also detailed in [16].

2.4 PCR Identification of 540dhps

Two rounds of PCR (nested PCR) were conducted to amplify the 540 DHPS gene. The nest1 forward and reverse primer sequences were 5′ AACCTAAACGTGCTGTTCAA 3′ and 5′ AATTGTGTGATTTGTCCACAA 3′ respectively, while the nest2 forward and reverse primer sequences were 5′ CCTAAACGTGCTGTTCAAAGAA 3′ and 5′ TTCGCAAATCCTAATCCAATATC 3′ respectively.

2.5 Sequencing and Bioinformatic Analysis of 540 PfDHPS Gene

The Sangar sequencing method at InqabaBiotec laboratory, Pretoria, South Africa, was employed for sequencing the Plasmodium falciparum 540dhps gene. Bioedit software was used for base calling and consensus sequence formation, and NCBI BLAST search tools were used for nucleotide and amino acid sequence similarity search. OmegaX software facilitated sequence alignments.

3. RESULTS AND DISCUSSION

3.1 PCR Results:

Figures 1 to 5 depict gel images of the amplicons resulting from the PCR amplification of the Plasmodium falciparum gene. Specifically, Figure 1 illustrates the PCR products of Plasmodium falciparum gene amplification for samples 1 – 20. Successful amplification for the Plasmodium falciparum gene with the expected size (205bp) was observed in samples 7, 11, 12, and 14. In contrast, the remaining samples tested negative for Plasmodium falciparum.

Figure 1. Gel Image of PCR Products for Samples 1–20 of Plasmodium falciparum

Figure 2 displays the PCR products derived from the amplification of Plasmodium falciparum genes for samples 21 – 40 where Plasmodium falciparum was detected in samples 23, 24, 26, 27, 28, 29, 30, 31, 35, 36, 37, 38, and 40, while the remaining samples tested negative. Furthermore, Figure 3 illustrates the PCR products of Plasmodium falciparum genes amplified for samples 41 – 60. Plasmodium falciparum was identified in samples 41, 42, 43, 49, 50, 53, 54, 55, and 56, while all other samples tested negative. Subsequently, Figure 4 depicts the PCR products of Plasmodium falciparum genes amplified for samples 61 – 80. Plasmodium falciparum was detected in samples 61, 62, 66, 69, 77, 78, 79, and 80, with all other samples testing negative. Likewise, Figure 5 presents the PCR products of Plasmodium falciparum genes amplified for samples 81 – 100, where Plasmodium falciparum was identified in samples 82-86, 88, 90- 95, and 97-100. Conversely, the remaining samples tested negative for Plasmodium falciparum. Finally, Figure 6 provides the gel picture of the amplified PCR products of the 540dhps gene. The PCR amplification revealed that samples 26, 27, 28, 30, 41, 56, 62, and 86 were positive for the 540dhps gene when

screened with a 100bp ladder, and positive control (+Ve control) with a known size of 500bp.

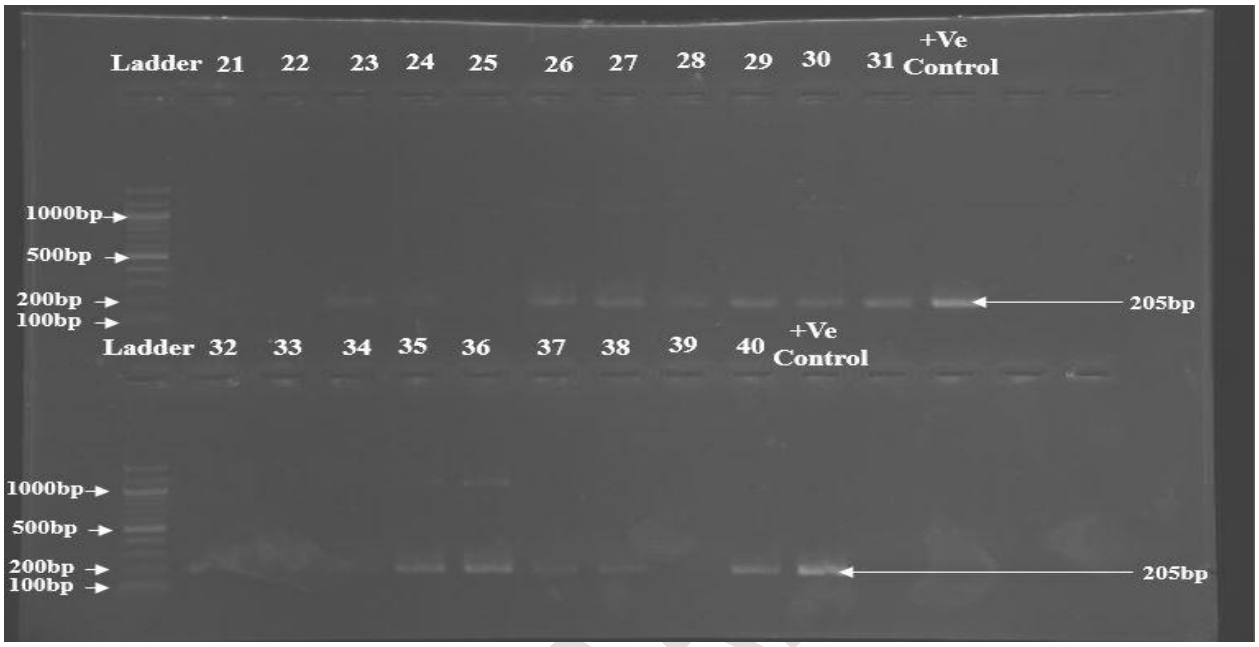

Figure 2. Gel Image of PCR Products for Samples 21-40 of Plasmodium falciparum

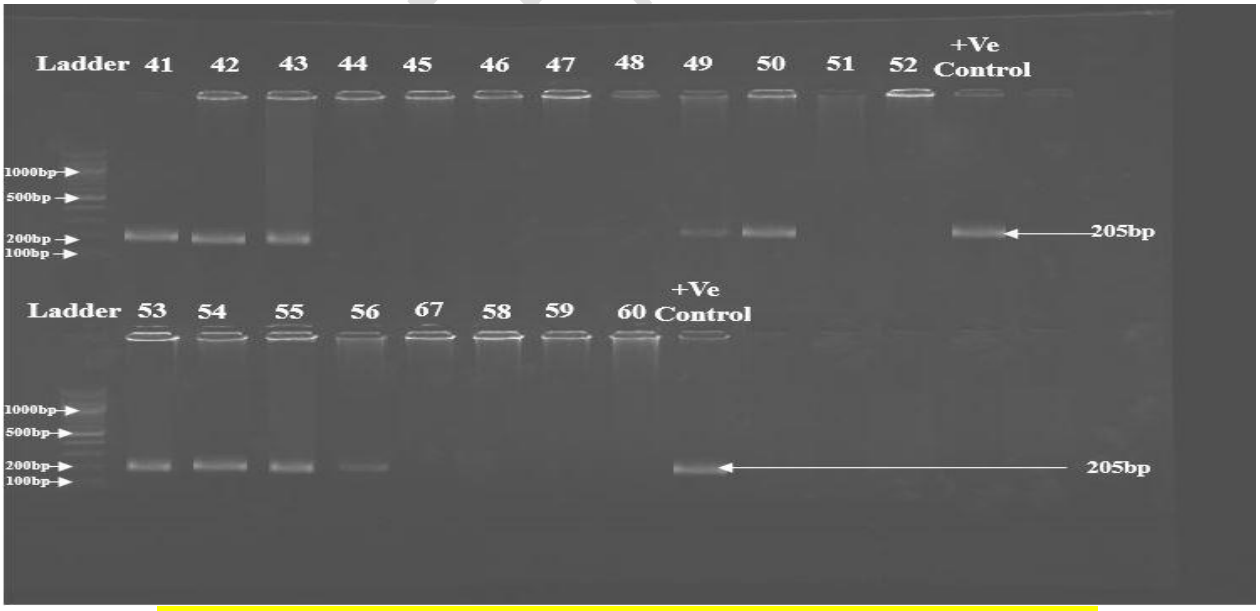

Figure 3. Gel Image of PCR Products for Samples 41-60 of Plasmodium falciparum

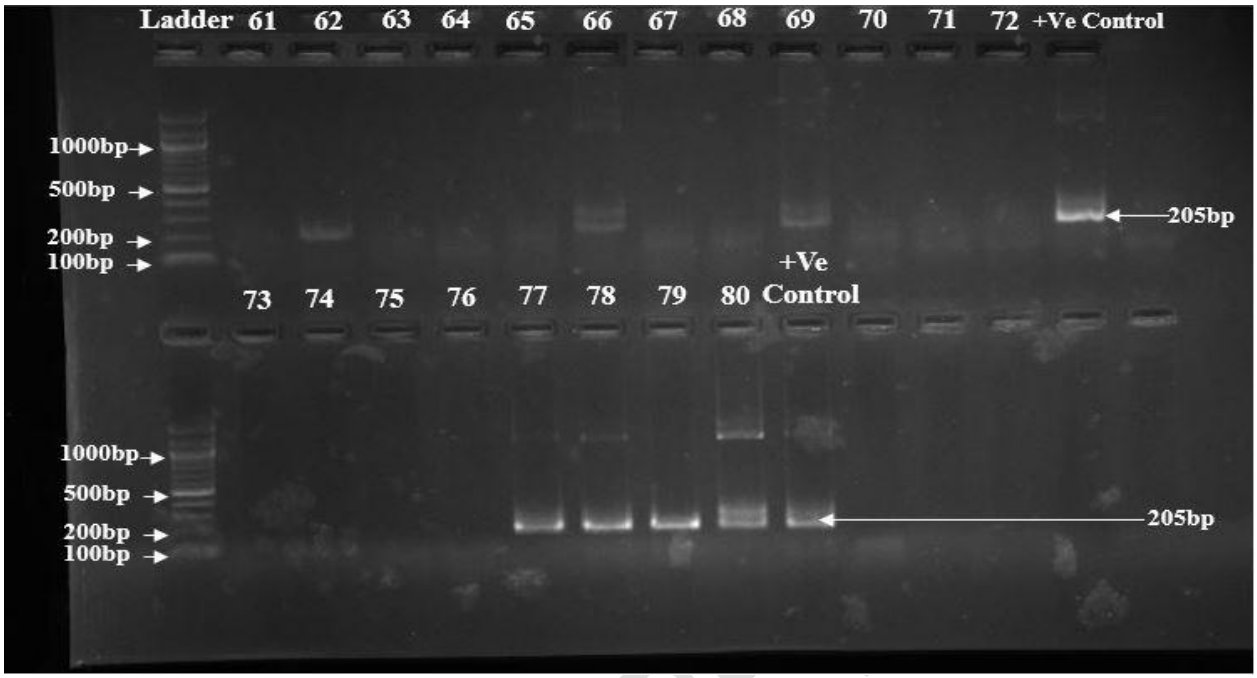

Figure 4. Gel Image of PCR Products for Samples 61-80 of Plasmodium falciparum

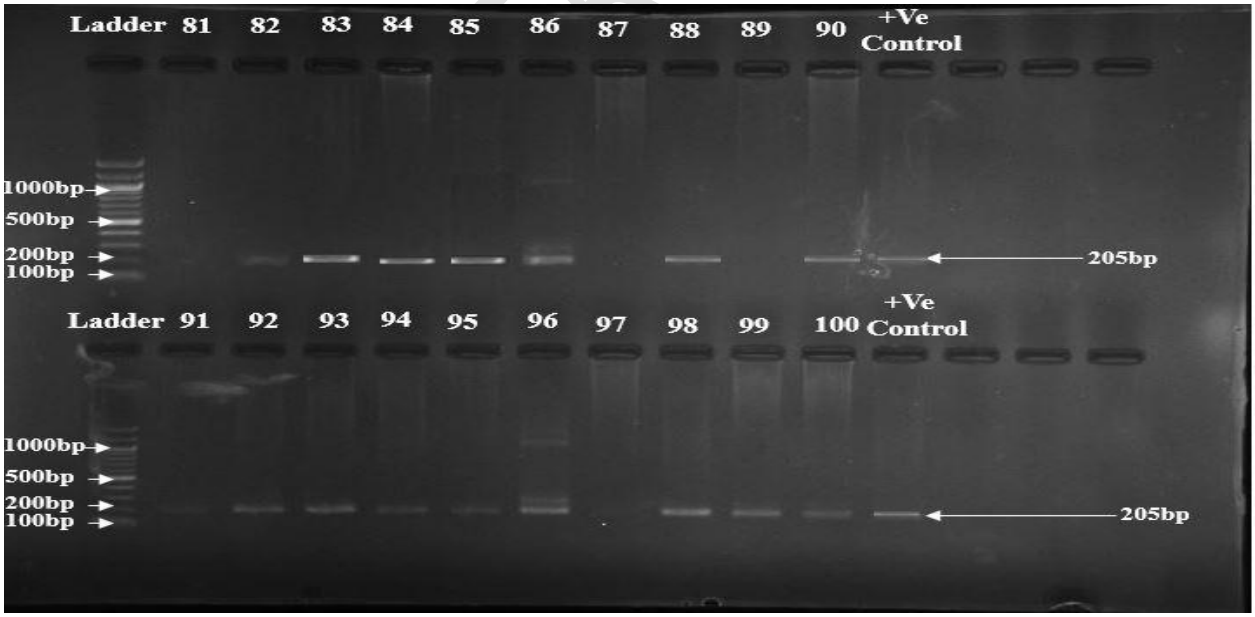

Figure 5. Gel Image of PCR Products for Samples 81-100 of Plasmodium falciparum

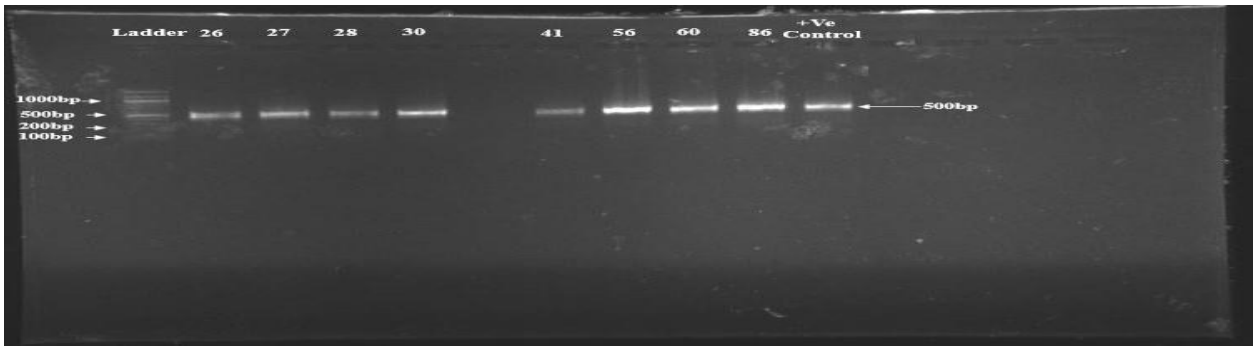

Figure 6. Gel Image of PCR Products of *540dhps* gene

3.2 Analysis of Plasmodium Genus, Plasmodium falciparum, and 540dhps Gene

Among the 100 microscopically screened samples obtained from the Hospital, 50 samples (50%) were confirmed positive for the Plasmodium genus using the PCR technique, while the remaining half tested negative, as detailed in Table 1. Subsequent screening of the 50 positive samples revealed that 48 (96%) were positive for Plasmodium falciparum, with only 2 (4%) testing negative. Further analysis showed that out of the 48 Plasmodium falciparum-positive samples, 8 (16.67%) were detected for the 540DPHS gene, while 40 (83.33%) were negative. These findings align with previous research indicating Plasmodium falciparum as the predominant malaria species in Nigeria. Also, the observed 96% prevalence of Plasmodium falciparum in positive samples is consistent with earlier studies reporting approximately 85% prevalence in Edo and Lagos [17], and an 80% prevalence using both microscopic and PCR techniques [15]. However, the overall 50% false positive rate for the Plasmodium genus in our study raises concerns regarding the potential misdiagnoses and treatments within Nigerian hospital settings. As highlighted by [18], clinical laboratories are susceptible to diagnostic errors, particularly in the pre-analytical phases. Consequently, half of the samples collected for this study may have been inaccurately reported by the hospital. As noted before, false positive results can lead to unnecessary malaria treatment and heightened medication use, thereby increasing the risk of drug resistance [20, 21]. Therefore, accurate diagnostic techniques are crucial to prevent the administration of antimalarials to patients without true infections. Also, employing more precise testing methods for Plasmodium falciparum could enhance treatment accuracy and mitigate the risks associated with incorrect dosing.

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 represents the total sample size, while n denotes the count of samples categorized as either positive or negative, and % signifies the percentage of positive or negative samples.

3.2 Alignment of Nucleotide Sequences for the 540dhps Gene

The results obtained from the query-anchored alignment revealed the substitution of thymine with guanine at position 10 of the nucleotide sequence of the 540dhps gene, as highlighted in yellow in Figure 7. The nucleotide blast search confirmed sequences with 99.53% query coverage and 98.79% identity, signifying identical characters in each sequence and high similarity to the sequences in the database. This affirms that the extracted 540dhps gene sequence closely aligns with those in the database. Although previous research has demonstrated that mutations in the 540dhps gene can result in sulfadoxine-pyrimethamine resistance [11], the parasite's resistance to this drug combination has been associated with mutations that reduce its binding affinity [14]. The accumulation of 540dhps mutations also correlates with increased sulfadoxine-pyrimethamine resistance in vivo [22]. However, the observed mutation at the nucleotide level in this study does not appear to confer resistance. Therefore, this mutation indicates that sulfadoxine-pyrimethamine resistance may not be linked to the variations in the parasites' 540dhps gene, contrary to some earlier research [23].

The NCBI Blast of 540dhps was conducted as a query sequence against sequences with the accession numbers MN373855.1, MN373848.1, MN373846.1, MN373835.1, and MN373834.1, serving as reference sequences. A silent point mutation was identified at position 10 in the 540dhps query sequence compared to homologous reference sequences, where guanine replaced thymine.

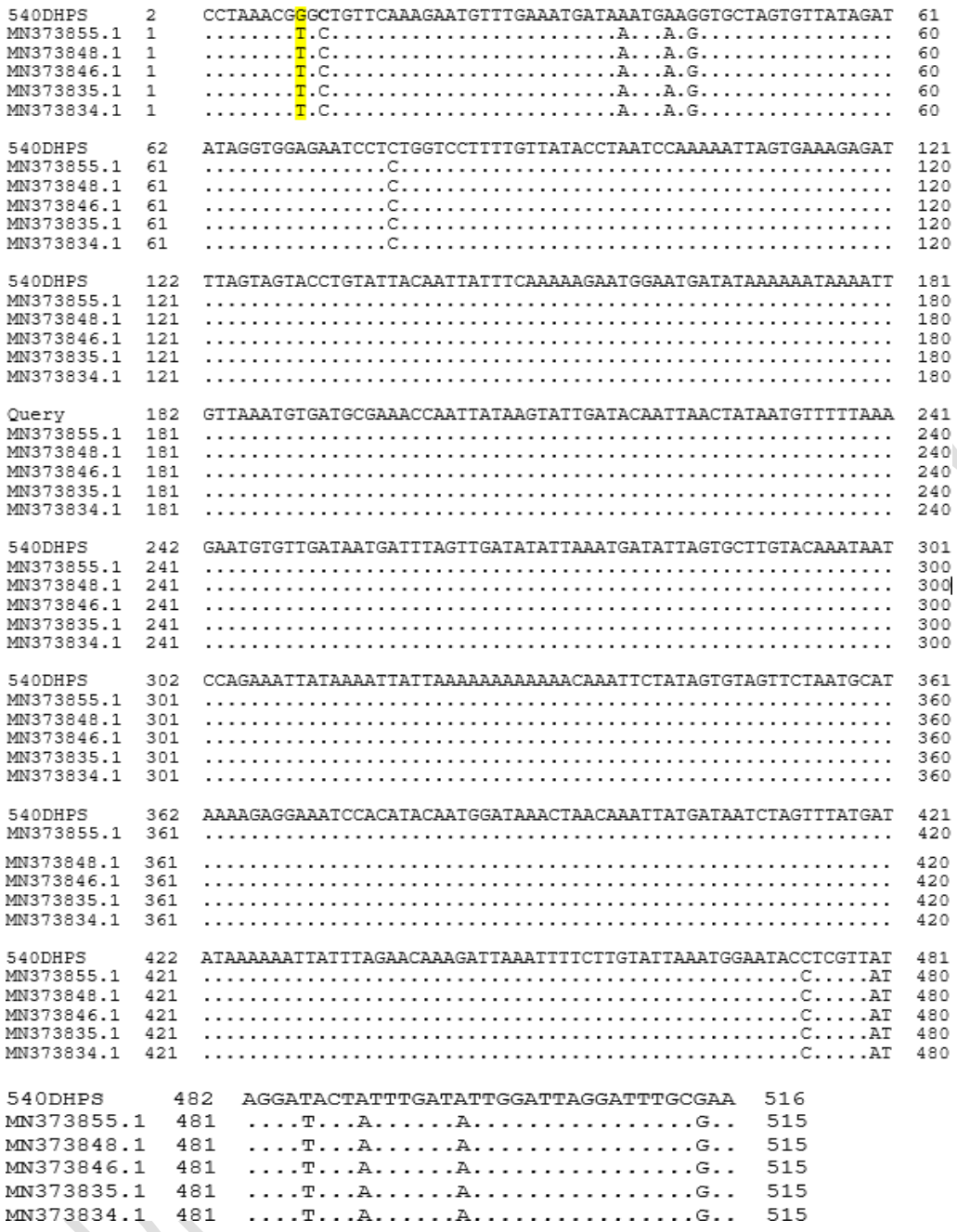

Figure 7: Query-Anchored Alignment Views for Single-Nucleotide Polymorphism (SNP) Detection

3.3 Analysis of Amino Acid Sequences in the 540dhps Gene

Our findings demonstrate that the mutations observed at the nucleotide level in Figure 7 above did not affect the amino acid sequence, as illustrated in Figure 8 below. While some studies indicate that specific mutations in the 540dhps gene can confer resistance to sulfadoxine-pyrimethamine [6, 12, 14, 21], while other studies report a 16.67% prevalence of the K540E mutation [23], however, it was noted that the K540E mutation is less prevalent in West

Africa compared to East and South Africa [23, 24]. Previous regional studies have reported higher K540E prevalence rates, including 38.5% in Saudi Arabia [25], 22.5% in Lagos, Nigeria [26], and a prevalence of 58.7% in Northeast Nigeria [27]. However, these regional disparities underscore geographic variations in mutation patterns linked to SP resistance. The absence of amino acid changes in this study however, suggests that the observed nucleotide alteration in the nucleotide sequence may not be responsible for resistance to the sulfadoxine-pyrimethamine drug.

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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: Alignment of Amino Acid Sequences in the 540dhps Gene
Utilizing MegaX software, all nucleotide sequences—540dhps, MN373855.1, MN373848.1, MN373846.1, MN373835.1, and MN373834.1—were translated and aligned through NCBI Blast.

4. CONCLUSION

Our investigation revealed that *Plasmodium falciparum* stands as the predominant parasite species responsible for malaria infections in humans, making it the most prevalent malaria species in Jos. The identified mutation, observed exclusively at the nucleotide level, does not impact the amino acid sequence. Consequently, this mutation is unlikely to affect the functionality of the protein. Thus, it is plausible that the reported resistance is not linked to a mutation in the 540-dihydropteroate synthetase gene but rather attributed to other factors.

ETHICAL APPROVAL

Ethical clearance, with registration number NHREC/05/01/2010b, was obtained from the Hospital Institutional Review Committee. All experimental procedures strictly adhered to the relevant laws governing the use of human subjects, ensuring ethical compliance throughout the study.

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