Original Research Article

Molecular Epidemiology of *Plasmodium falciparum* Infections using PCR-based assays in Jos, Nigeria - Cross-Sectional study

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ABSTRACT

Background: Malaria remains a significant health threat globally, with *Plasmodium falciparum* being the predominant and lethal parasite in Africa. Nigeria is still faced with ongoing cases of asymptomatic malaria, hindering effectivecontrol measures.

Aim: The aim was to generate epidemiological data that will provide good background and guide strategies for driving malaria control efforts, research, and resource allocation in the region.

Place and Duration of Study: This study was conducted in Jos, Plateau State, Nigeria, where the samples were originally collected within about 16 monthsbetween October 2019 and January 2021.

Methodology:A cross-sectional molecular epidemiological study was conducted using 136 microscopically screened 2 plus (++) and above positive malarial whole blood samples obtained in EDTA bottles from two hospitals in Jos, Plateau State, Nigeria. The DNA extraction was performed according to the manufacturer's instructions using Zymo Research extraction kits. *Plasmodium genus* and *Plasmodium falciparum* were detected in the samples using the PCR method and gel electrophoresis.

Results: In the results, using PCR techniques, 47.8% (65/136) of the total malaria-positive samples collected were confirmed for the presence of the *Plasmodium genus*. Out of these 65 positive samples, 63 were found to be *Plasmodium falciparum*.

Conclusion: This study demonstrates that *Plasmodium falciparum* remains the predominant malaria species in Jos, Plateau State, comprising approximately 96.9% (63/65) of the malarial cases. This indicates that only about 3% of malaria cases affecting the residents of Jos, Plateau State might be caused by the other four species of malaria parasites (*Plasmodium vivax, Plasmodium malariae, Plasmodium ovale,* and *Plasmodium knowlesi*).

Keywords: Epidemiology,PCR, Plasmodium falciparum,Malaria

1. INTRODUCTION

1.1 Malaria and Laboratory Diagnosis

Malaria is a serious and widespread disease caused by parasites called Plasmodium, with different species like *Plasmodium falciparum* and others [1]. Sadly, more than half of all malaria deaths occur in countries like Nigeria, the Democratic Republic of the Congo, Tanzania, and Niger, emphasizing the urgent need for strong control measures [2,3]. In Nigeria, there are still cases of asymptomatic malaria (where people have the disease, but show no

symptoms), making it tough to control and so contribute to the spread of the parasites [4]. Another challenge is that the parasites can change, becoming more dangerous or resistant to drugs [5]. Also, cerebral malaria, the most severe form of the disease which involves neurological complications, is of particular concern due to its high mortality rate [6]. It is also important to know that malaria shows various symptoms, from fever to serious problems like anaemia and even death if not treated [7]. The 2021 statistics reveal a big global health problem, with about 247 million cases and 619,000 deaths from malaria [1].

Although there are many laboratory methods for diagnosing malaria, the most common include microscopic diagnosis, rapid diagnostic tests (RDTs), and molecular diagnosis using mainly polymerase chain reaction (PCR). The microscopic diagnosis of plasmodium malaria is considered the gold standard for laboratory confirmation. However, its accuracy relies on the quality of reagents, the microscope, and the expertise of the laboratorian [8]. This can make it less reliable than the PCR method [9], potentially leading to false positive results [10].

1.2 The Prevalence of *Plasmodium falciparum* and Its Complications

Plasmodium falciparum remains one of the most prevalent and deadly malaria parasites in Africa [11,7], representing a major public health threat even though between 2005 and 2017 there was a noticeable reduction in its burden, marking progress in control efforts [12]. The clinical manifestations of falciparum malaria are diverse, ranging from asymptomatic parasitemia to life-threatening complications such as cerebral malaria, which involves symptoms like seizures, impaired consciousness, and potentially irreversible coma [13]. Other severe manifestations precipitated by *Plasmodium falciparum* include respiratory distress, acute kidney injury, severe malarial anaemia, hypoglycemia, and more [13]. Molecular studies have identified major subpopulations of *Plasmodium falciparum* in sub-Saharan Africa, underscoring the importance of deciphering the genetic diversity of this adaptable parasite [14]. One major salient feature of *Plasmodium falciparum* is its proficient ability to evade its host immune defences through antigenic variation and cytoadherence mechanisms at multiple stages of its pathogenic life cycle [15]. Because of this ability to evade the immune system, prompt diagnosis along with urgent commencement of artemisinin-based combination therapy is crucial, especially for travelers returning from high-transmission areas where *Plasmodium falciparum* predominates [14].

1.3 Significance of the Study

Epidemiological data for diseases is a major component of the strategies for control, management, treatment and elimination. Malaria is not left behind. The world Health Organization Malaria Report for 2022 captures Nigeria, in general, and Plateau State in particular [3]. The report relies on epidemiological data using RDT and Microscopy. The use of Molecular diagnostic tools for malaria epidemiology places this study in a unique class, because of the established superiority of PCR over the RDT and microscopy.This study on*Plasmodium falciparum*infections using molecular tools in Jos, Plateau State, Nigeria, is significant as it will provide crucial

epidemiological data validation. This will drive malaria control efforts, research, and resource allocation where they are most needed to reduce the burden of the disease in the locality, in particular.
Therefore, the study was designed and executed to achieve the following objectives:

1. Deploy the use of molecular tools in detecting malaria cases in Jos.
2. Establish the level of *Plasmodium falciparum* infection in Jos
3. Examine the relatedness of the hospital-based diagnosis to the molecular approach.

2. MATERIAL AND METHODS

2.1 Materials

Malaria-positive human blood samples, EDTA containers, Genomic Lysis Buffer, Beta-mercaptoethanol, Zymo-SpinTM IICR columns, DNA Pre-Wash Buffer, g-DNA Wash Buffer, DNA Elution Buffer, forward and reverse primers (rPLU5 and rPLU6) for *Plasmodium genus* PCR, One Tag Quick-load 2X Master Mix for *Plasmodium genus* PCR, forward and reverse primers (rFAL-1 and rFAL-2) for *Plasmodium falciparum* PCR, agarose gel TAE buffer, Refrigerator, Thermocycling machine, and Gel imager.

2.2 Sample Size Calculation

The sample size was calculated based on a 95% confidence level (Z-score of 1.96), an expected prevalence of 0.5, and a margin of error of 0.05 using the formula below.

$n = Z2 \times p \times (1-p)/E2$

Where:

n = required sample size
Z = Z statistic for 95% confidence = 1.96
p = expected prevalence = 0.5
E = margin of error = 0.05

Therefore, $n = (1.96)2 \times 0.5 \times (1-0.5) / (0.05)2$

$n = 384$

2.3 Sample Collection and Storage

The target of about 384 samples (2 plus (++) for collection within the period of the research designed was not realized. This was attributed to the season (October and January) which is usually dry and cold in Jos making it less favourable to the malaria vector and records low malaria cases. Large number of samples that were positive but only 1 plus (+) were excluded. One hundred and thirty six(136) clinically screened malarial samples that tested positive at 2 plus (++) level and above were obtained from two hospitals in Jos, Nigeria and placed in EDTA containers.

2.4 DNA Extraction

Before the DNA extraction, beta-mercaptoethanol was added to the Genomic Lysis Buffer at a 0.5% (v/v) dilution. For each blood sample, 100 µl of whole blood was combined with 400 µl of the prepared Genomic Lysis Buffer in a 4:1 ratio and vortexed for 2 minutes, then incubated at room temperature for 5 minutes. The lysate 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

and collection tubes were discarded. The column was transferred to a new collection tube, and 200 µl of DNA Pre-Wash Buffer was added and centrifuged at 10,000 x g for 1 minute. The flow-through was discarded again. Next, 500 µl of g-DNA Wash Buffer was added to the column, centrifuged at 10,000 x g for 1 minute, and the flow through discarded. The column was placed in a clean microcentrifuge tube, 70 µl of DNA Elution Buffer was added, incubated at room temperature for 5 minutes, and then centrifuged at 14,000 x g for 30 seconds. Of the eluted DNA, 30 µl was stored at 4°C for immediate use while the remaining 40 µl was stored at -20°C as a backup. This 6-step extraction process was repeated for 36 blood samples.

2.5 Detection of *Plasmodium genus*

To amplify the 18S rRNA gene specific to the *Plasmodium genus*, forward (rPLU5 - 5′-CCTGTTGTTGCCTTAAACT TC-3′) and reverse (rPLU6 - 5′-TTAAAATTGTTGCAGTTAAAACG-3′) primers were utilized. The 25 µL PCR reaction mixture 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. The thermal cycling conditions were as follows: initial denaturation at 95°C for 10 minutes; 35 cycles of denaturation at 94°C for 1 minute, annealing at 60°C for 2 minutes, and extension at 72°C for 2 minutes; final extension at 72°C for 10 minutes; and hold at 4°C indefinitely. The 1100bp amplified products were visualized by electrophoresis on a 1.5% agarose gel [15-16].

2.6 Detection of *Plasmodium falciparum*

For the amplification of *Plasmodium falciparum*, 5 µl (95µl) of the Plasmodium species' amplicons were used as template with the forward primer rFAL-1 (5′TTAAACTGGTTTGGGAAAACCAAATATATT-3′) and reverse primer rFAL-2 (5′-ACACAATGAACTCAATCATGACTACCCGTC-3’) [21]. The 25µL PCR reaction mixture contained 12.5µl of One Tag Quick-load 2X Master Mix, 1µl each of 10µM forward and reverse primers, 6.5 µl of nuclease-free water, and 4µl of amplicon template. Cycling conditions were as follows: initial denaturation at 95°C for 10 minutes; 35 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 2 minutes, and extension at 72°C for 2 minutes; final extension at 72°C for 10 minutes; and hold at 4°C indefinitely. The 205-base pair (bp) amplified products were visualized by electrophoresis on a 1.5% agarose gel [15,16].

3. RESULTS AND DISCUSSION

3.1 Gel Result for *Plasmodium falciparum*Gene

This study provides crucial insights into the prevalence of *Plasmodium falciparum* within Jos, Plateau State, using molecular tools as shown in Figures 1, 2, 3 and 4. Out of the 136 samples shown to be positive for malaria using the microscopic approach, only 47.8% (65/136) were confirmed positive using the PCR technique as shown in Figure 1. This raises concerns about the accuracy of diagnostic methods in the study area, highlighting the necessity for improved techniques like PCR, which offer higher sensitivity and specificity in malaria detection[8-10].The sensitivity and specificity of RDT have been compared to microscopy and reported to be just

above average [11] and both could not detect over 40% malaria infections [12] compared to the molecular approach. It was concluded that, although WHO recommends their use, these conventional tools cannot be relied upon and so novel tools are required for prompt malaria diagnosis [11,12].
Furthermore, about 96.9% (63/65) of the *Plasmodium genus* detected in this study as presented in Figure 1 were confirmed as *Plasmodium falciparum* using PCR technique. This high percentage aligns with ongoing challenges faced by countries like Nigeria, where asymptomatic malaria cases persist, and impede control efforts [14]. As a result, these cases might contribute significantly to continuous parasite transmission, thereby presenting a substantial obstacle to malaria control efforts. This significant presence of *Plasmodium falciparum* in Jos could have serious consequences. First of all, because *Plasmodium falciparum* is the deadliest malaria parasite and causes more than 90% of malaria-related fatalities worldwide [7, 16]. This emphasizes the importance of tailoring control measures to specifically target *Plasmodium falciparum* and implementing robust strategies to reduce its prevalence. Secondly, the prevalence of *Plasmodium falciparum* in the study area highlights the need for enhanced surveillance and early detection programs. This is because, early identification is imperative to prevent the progression of infections to severe and potentially fatal forms, such as cerebral malaria, acute renal failure, or severe anaemia [15].

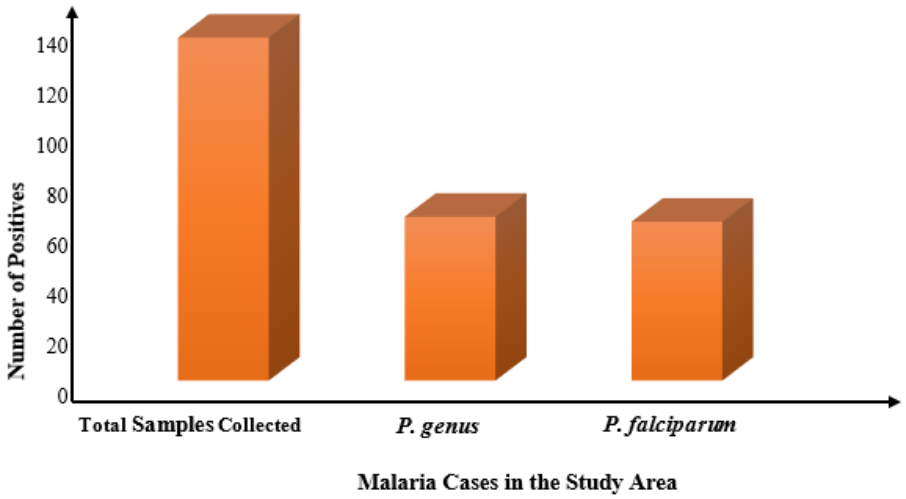

Figure 1 Graphical Distributions of *Plasmodium genus* and *Plasmodium falciparum* in Jos

Although earlier reports indicated some successes in lowering the incidence of *Plasmodium falciparum* malaria in Nigeria between 2005 and 2017[18,19], the recent high prevalence of this fatal Plasmodium species in Plateau State emphasizes the need for ongoing and intensive control efforts [20,21]. This is crucial due to *Plasmodium falciparum*'s high prevalence in African countries like Nigeria, the Democratic Republic of the Congo, Tanzania, and Niger [2], necessitating continuous efforts to combat the illness, especially in these hotspot regions. Additionally, considering the variety of malaria complications such as cerebral malaria, acute renal failure, and severe anaemia that can arise from *Plasmodium falciparum* infections, early diagnosis and prompt

treatment are of paramount importance, particularly for travelers returning from malaria-endemic areas [16]. Therefore, to implement individualized interventions and ensure successful treatments, an understanding of the genetic variety of *Plasmodium falciparum* strains found in sub-Saharan Africa has become essential [5].

Out of 136 microscopically positive samples, 65 were PCR positive for the *Plasmodium genus*, with 63 of those confirmed as *Plasmodium falciparum*.

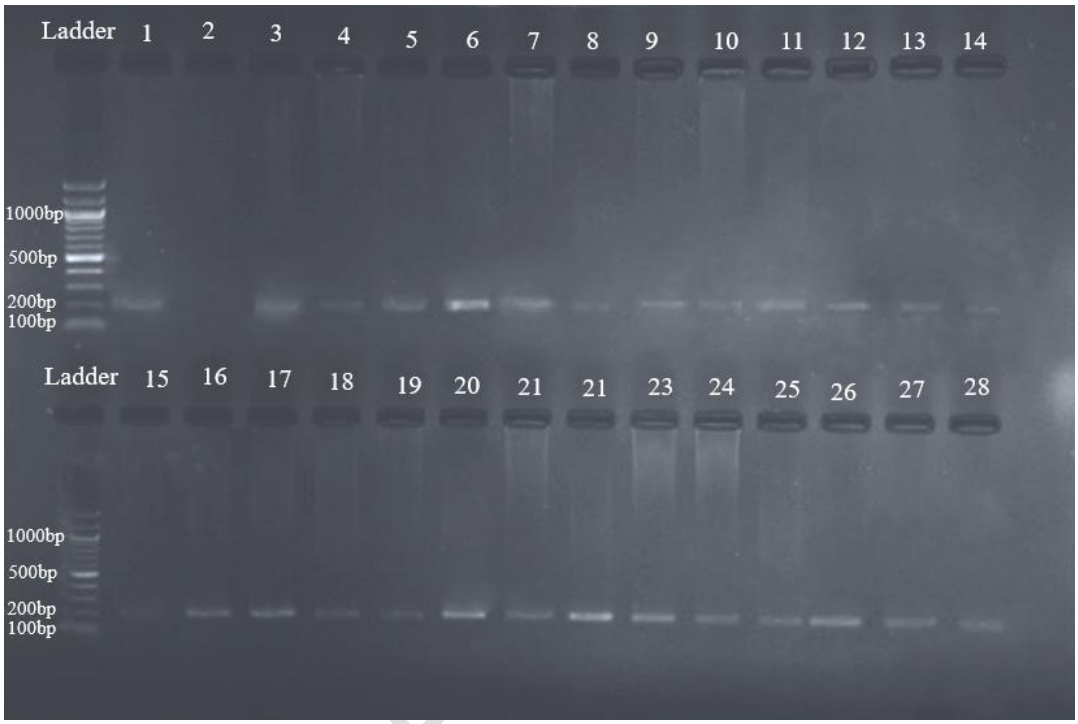

Figure 2 Gel Electropherogram of *Plasmodium falciparum* PCR Amplicons (Samples 1-28)

A 100bp ladder was used. Expected band size of 205 bp.

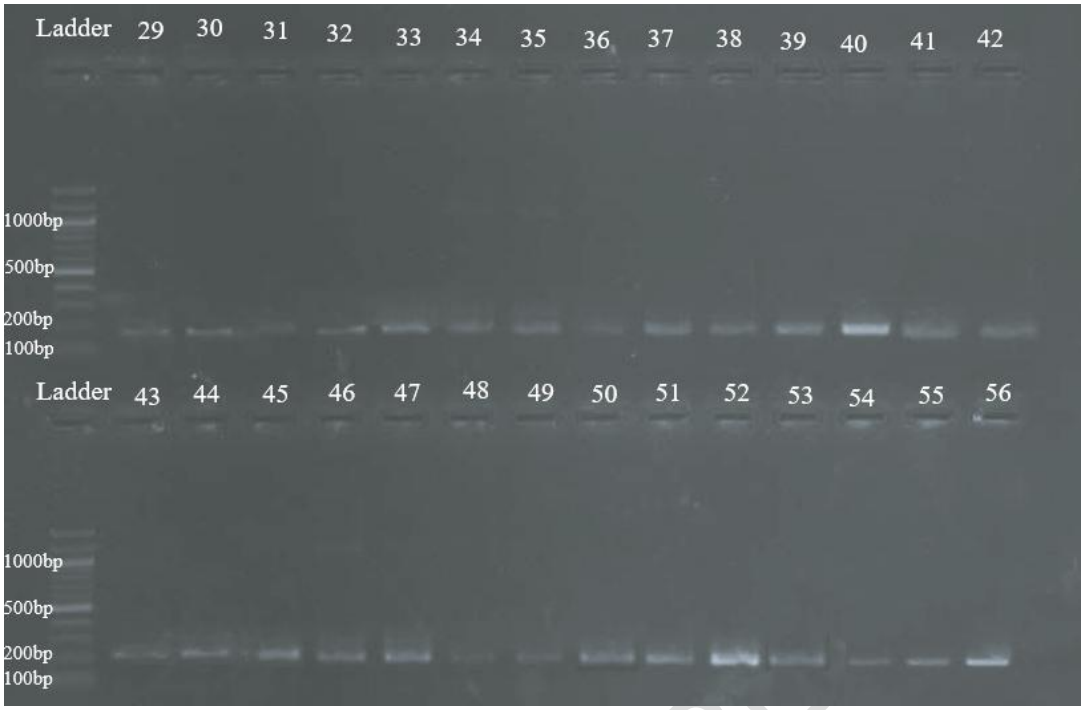

Figure 3 Gel Electropherogram of *Plasmodium falciparum* PCR Amplicons (Samples 29-56)
A 100bp ladder was used. The expected band size of 205 bp.

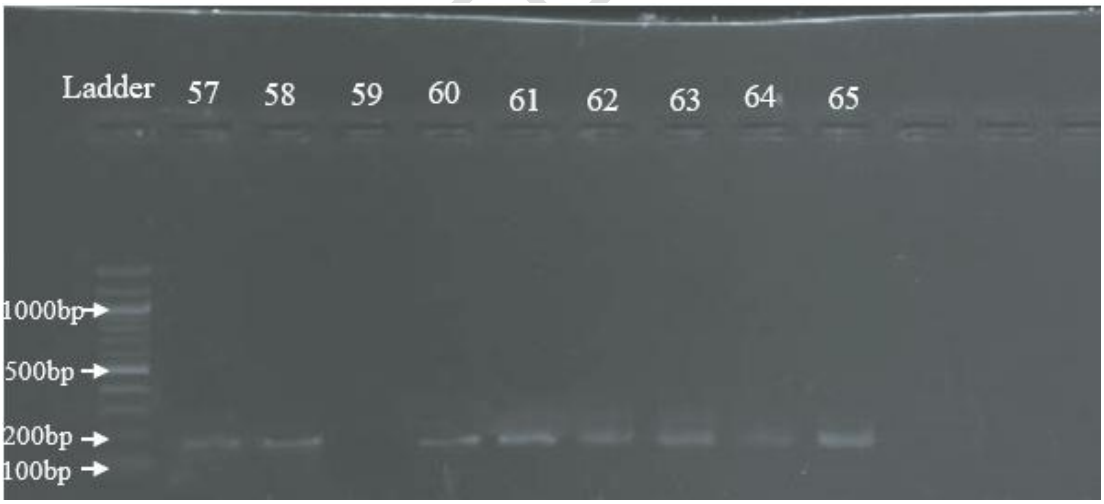

Figure 4 Gel Electropherogram of *Plasmodium falciparum* PCR Amplicons (Samples 57-65); A 100bp ladder was used. The expected band size of 205 bp.

4. Conclusion

Our findings have reaffirmed that *Plasmodium falciparum* remains prevalent in Jos North, Nigeria, providing crucial data to guide malaria control and research efforts aimed at reducing the global severity of malaria. This emphasizes the urgent need for comprehensive control measures, specific

interventions, and continuous research to alleviate the impact of this lethal disease in malaria-burdened regions like Jos. Therefore, it is crucial to comprehend the prevalence, diversity, and complications of *Plasmodium falciparum* infections for effective global malaria combat in Nigeria.

LIMITATIONS OF THE STUDY

The target of about 384 samples (2 plus (++) for collection within the period of the research designed was not realized. This was attributed to the season (October and January) which is usually dry and coldin Jos making it less favourable to the malaria vector and records low malaria cases. Largenumber of samples that were positive but only 1 plus (+) were excluded.The smaller sample size limits the generalizability and precision of the prevalence estimate.

However, going forward, we considered sharing this data with the research and scientific community ahead of a larger sample-sized study with genomic sequences to fully characterize *Plasmodium falciparum* diversity and prevalence in the region. We hope this may spark participation or collaboration in this direction.

CONSENT

Not applicable

ETHICAL APPROVAL

This study obtained ethical approval from the Health Research Ethics Committee of the Hospitalsin Jos with numbers: NHREC/21/05/2005/00677 and NHREC/05/01/2010b. All experiments were performed according to relevant guidelines and regulations for research involving human samples.

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