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Molecular distribution of *Plasmodium falciparum* antimalarial drug resistance genes in Plateau State, North Central Nigeria

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Malaria elimination activities are facing new threats, such as the global COVID-19 pandemic, which may deprioritize efforts to track the emergence of resistance to antimalarial drugs. This study aimed to determine the profile of genes for antimalarial drug resistance in Plateau State, North-central, Nigeria.This cross-sectional analytical study was conducted from January to September 2018 by screening 3114 asymptomatic participants for *Plasmodium falciparum* malaria using Giemsa-stained thick and thin smears. Total genomic DNA was extracted from the blood of participants using Quick-DNATM Miniprep (Zymo Research) extraction kit to perform Polymerase Chain Reaction (PCR) and detect *Plasmodium falciparum* multidrug resistance 1 (Pfmdr1), *P. falciparum* dihydrofolate reductase (Pfdhfr), *P. falciparum* dihydropteroate synthase (Pfdhps) and *P. falciparum* K13 propeller (PfK13) genes from the isolates obtained. The prevalence of malaria observed was 13.2%. Of the 206 samples tested for the genes, 31(15.0%) were positive for Pfdhfr gene, 124(60.2%) were positive Pfdhps gene, 29(14.1%) were positive for the Pfmdr1 gene while 82(39.8%) were positive for the PfK13 propeller gene. There was a significant association between the drug resistance genes and senatorial zones (p=0.012). The Pfdhps gene was the most prevalent gene encountered (p= 0.002). Drug selection pressure could be accelerating the advent of drug resistance genes to Artemisinin Combination Therapies in North-central Nigeria particularly on Sulphadoxine Pyrimethamine (SP) in Plateau State's Southern Senatorial zone. The high prevalence of the Pfdhps gene requires close tracking for the use of Sulphadoxine Pyrimethamine for intermittent prophylactic treatment of malaria. Genotyping studies are urgently needed to investigate the situation further.

Key words: Malaria, COVID-19 pandemic, *Plasmodium falciparum*, Nigeria.

INTRODUCTION

As arguably the most important disease caused by a parasite in humans, malaria still poses a major threat of global proportions. The disease leads to as many as 600 000 deaths per year especially in very young children and is a major source of morbidity and mortality (Gething et al., 2016). In 2017, it was estimated that 217 million cases (95% CI: 219-285 million) occurred worldwide (WHO, 2018a, b). The African region accounted for 92% of the world's malaria cases, with 15 countries in Sub-Saharan Africa and India alone contributing to nearly 80% of the global burden. Among these, five countries, including Nigeria, were responsible for almost 50% of the global malaria cases (WHO, 2020). The World Health Organization (WHO) reports that Nigeria continues to hold the position as the largest contributor, with 25% of the global burden of the disease. If steps are not taken to delay the advent and proliferation of resistant strains to ACTs, they may be facing a similar fate to chloroquine and sulfadoxine-pyrimethamine which will jeopardize the gains made in the fight against malaria (WHO, 2020).

The selections of malaria parasites with mutations that confer the ability to resist the effect of antimalarial agents endanger malaria control and elimination globally (Tuedom et al., 2021; Benjamin et al., 2020). This ability not only threatens the case management of malaria but is further aggravated by intervention disruptions due to COVID-19.

Currently, resistance has been documented for all classes of antimalarial drugs including those that were administered as monotherapies such as Chloroquine. Resistance to chloroquine has been shown to be caused by the *Plasmodium falciparum* chloroquine transporter gene which increases the capacity in parasites to rapidly remove the drug at a rate that prevents the drug from reaching levels required for inhibiting the polymerization of haem (Foley and Tilley, 1997; Tse et al. 2019).

Decreased sensitivity to anti-folates such as Sulphadoxine-Pyrimethamine (SP) is caused by mutations in the parasite dihydrofolate reductase (dhfr) and dihydropteroate synthase (dhps) genes. These mutations can increase resistance in SP by more than 100-fold in comparison to wild-type (Issa et al., 2022). In a similar fashion, since Artemisinin-based Combination Therapies (ACTs) have become the standard treatment of malaria as first-and second-line drugs for uncomplicated *Plasmodium falciparum* on WHO recommendation (White, 1999; Arrow et al., 2004; WHO, 2020), they have become a vital component in the accomplishments of global malaria control and have also recently come under attack in the Greater Mekong Subregion (GMS) in Southeast Asia (Arya et al., 2021).

The threat of resistance to ACTs in the GMS is nearly reminiscent of the fate of Chloroquine and Sulphadoxine-Pyrimethamine which began in Southeast Asia and spread westward into Africa and America (Ashley, et al. 2014; Packard, 2014; Blasco et al., 2017; Zhao et al., 2020).

To investigate the presence of antimalarial drug resistance in North-central Nigeria, we screened asymptomatic adults and children aged 12 months to 70 years microscopically to confirm *Plasmodium falciparum* infection and to obtain isolates to be analysed for drug resistance.

METHODS

Study area

Plateau, North-Central Nigeria is located between latitude 9°10'N 9° 45' E with an estimated population of about 3.5 million people. The altitude ranges from 1,200 m to a peak of 1,829 m above sea level with a mean temperature of 18 and 22°C although this can be as high as 27°C in the southern part of the state. The mean annual rainfall of the area varies between 131.75 cm in the southern zone to 146 cm in the Northern zone. The highest rainfall between August and September can reach 160 to 230 cm. Nine communities from the three senatorial zones of Plateau State were stratified into the lowland and highland and sampled for asymptomatic malaria. Figure 1 shows the map of study area.

Study design

Three geographical zones identified as the Northern, Central and Southern zones in the state were used to stratify the primary sampling unit using a multistage sampling technique was utilised for this analytical cross-sectional study. Primary or secondary health facilities from nine communities- Du, Vom, Dadin Kowa, Pankshin, Dakwak, Wokkos, Shendam, Yelwa-Shendam and Purdel were identified from the three zones and used to collect the blood of asymptomatic study subjects in a community outreach.

Sampling was carried out between January and September 2018 corresponding to the dry and rainy seasons of the year.

Sample size

Sample size determination was carried out using the formula:

$N= z^2 p (1-p)/e^2$, z=1.96 at 95% level of confidence, p=0.48 a prevalence of 48.1% from Okoli and Solomon (2014), e=0.05, margin of error, N =384, N =400 (Nearest Hundred).

Blood specimen collection

Venepuncture was performed for 3,114 participants to obtain 4 ml of blood samples to be used for DNA extraction for PCR procedure. Screening for *P. falciparum* infection was performed by examining

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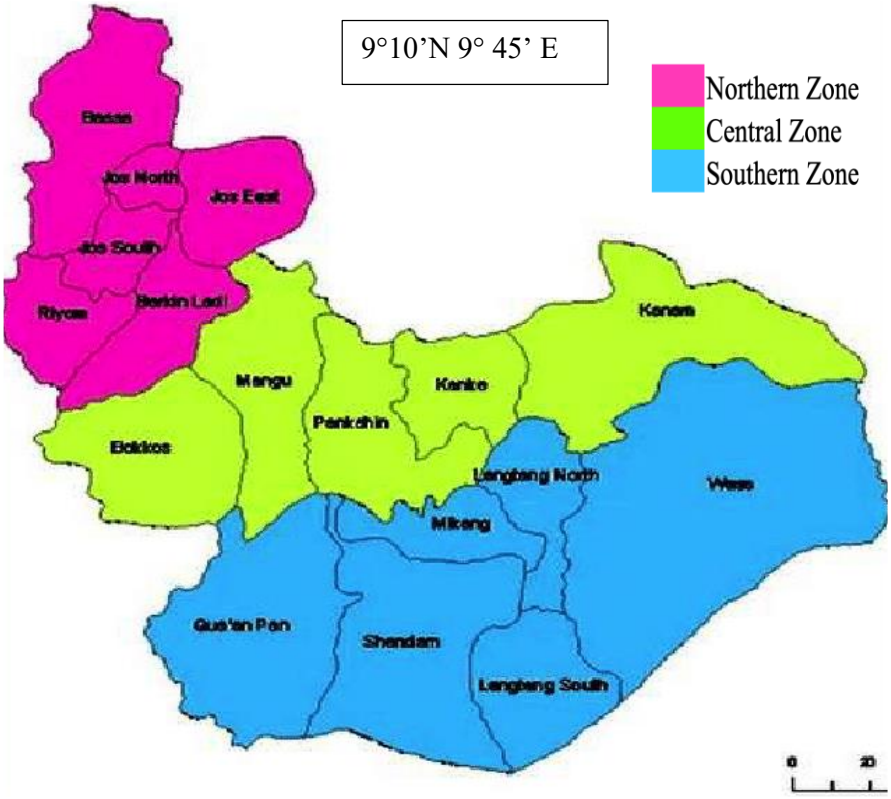

Figure 1. Map of study area.
Source: Adapted from Chollom et al., 2016

thick and thin smears prepared then stained with 10% Giemsa.

Once the stained slides were air dried, they were examined using a 100X objective lens by placing a drop of immersion oil on the stained blood smear under a CX31 light microscope (Olympus, New York, USA),

DNA extraction and PCR for resistant genes

Total genomic DNA from 412 samples was extracted using Quick-DNA™ Miniprep (Zymo Research, California, USA) following the manufacturer's instructions to obtain 75 µl of eluted DNA. The quantity and the purity of the extracted DNA were determined using the NanoDrop One spectrophotometer (ThermoFischer Scientific, Massachusetts, USA) as instructed manufacturer.

DNA samples that had the required purity and concentration were then taken for amplification to perform the detection of the drug resistant genes under study. Table 1 shows the primers used for the amplification of the antimalarial drug resistance genes.

Thermocycling conditions for PfMDR1 gene

The cycling conditions for the first nested experiment to detect the PfMDR1 gene included an initial denaturation at 95°C for 4 min, activation at 95°C for 30 s, annealing temperature was 45°C for 1 min, extension was at 68°C for 2 min this stage was set at 35 cycles. The final extension was at 68°C for 5 min.

The product of the first nest experiment for the Pf MDR1 gene was used as the template for the second nested experiment. The cycling conditions for the first nested experiment to detect the PfMDR1 gene included an initial denaturation at 95°C for 4 min, activation at 95°C for 30 s, annealing temperature was 45°C for 1 min, extension was at 68°C for 2 min this stage was set at 33 cycles. The final extension was at 68°C for 5 min making the total number of cycles for the experiment 35 cycles.

Thermocycling conditions for PfDHPS Gene

The cycling conditions for the first nested experiment to detect the PfDHPS gene included an initial denaturation at 95°C for 4 min, Activation was at 95°C for 30 s, annealing temperature was 47°C for 1 min, and extension was at 68°C for 2 min this stage was set at 35 cycles. The final extension was at 68°C for 5 min.

The product of the first nest experiment for the Pf DHPS gene was used as the template for the second nested experiment. The cycling conditions for the experiment to detect the PfDHPS gene included an initial denaturation at 95°C for 4 min, Activation at 95°C for 30 s, annealing temperature was 44°C for 1 min, and extension was at 68°C for 2 min this stage was set at 33 cycles. The final extension was at 68°C for 5 min making the total number of cycles 35.

Thermocycling conditions for Pf DHFR Gene

The cycling conditions for the first nested experiment to detect the Pf DHFR gene included an initial denaturation at 95°C for 4 min, Activation at 95°C for 30 s, annealing temperature was 49.1°C for 1 min, and extension was at 68°C for 2 min this stage was set at 35 cycles. The final extension was at 68°C for 5 min.

The product of the first nest experiment for the Pf DHFR gene

Table 1. Primers used for the amplification and detection of *Plasmodium falciparum* genes.

Gene	Direction	Nest 1 primer	Nest 2 primer
Pf	F	TTAAAATTGTTGCGTTAAAA	TTAAATGGTTTGGGAAAACCA
	R	CCTGTTGTTGCCTTAAACTTC	CACAATGAACTCAATCATGAC
MDR1	F	AGAGAAAAAAGATGGTAACCTCAG	CAGGAAGCATTTTATAATATGCAT
	R	ACCACAAACATAAATTAACGG	CGTTTAACATCTTCCAATGTTGCA
Dhps	F	TGCTTAAATGATATGATACCCGAATATAAG	GTTGAACCTAAACGTGCTGT
	R	TCCACCTGAAAAGAAATACATAAAT	TTCATCATGTAATTTTTGTTGTG
Dhfr	F	TTCTCCTTTTTATGATGGAACAAGT	GTTGAACCTAAACGTGCTGT
	R	ATATTTGAAAATCATTTGGATGTATAG	TTCATCATGTAATTTTTGTTGTG
K13	F	CGGAGTGACCAAATCTGGGA	GCCAAGCTGCCATTCATTTG
	R	GGGAATCTGGTGGTAACAGC	GCCTTGTTGAAAGAAGCAGA

was used as the template for the second nested experiment. Initial denaturation was at 95°C for 5 min, activation at 95°C for 30 s, annealing at 46.4°C for 1 min, initial extension at 68°C for 1 min. This was repeated for 33 cycles and the final extension was at 68°C for 5 min making the total number cycles 35.

Thermocycling conditions for K13 propeller gene

The cycling conditions for the first nested experiment to detect the Pf K13 Propeller gene included an initial denaturation at 95°C for 4 min, Activation at 95°C for 30 s, annealing temperature was 53.8°C for 1 min, and extension was at 68°C for 2 min this stage was set at 35 cycles. The final extension was at 68°C for 5 min.

The product of the first nest experiment for the Pf K13 Propeller gene was used as the template for the second nested experiment. Initial denaturation was at 95°C for 5 min, activation at 95°C for 30 s, annealing at 51°C for 1 min, initial extension at 68°C for 1 min. This was repeated for 35 cycles and the final extension was at 68°C for 5 min.

Statistical analysis

Data were collected in Microsoft Excel while SPSS software (IBM, SPSS, Armonk, USA) was used for the analysis to determine descriptive statistics of variables from the study. Continuous variables will be analysed using the students't-test and one-way Analysis of variance while associations were determined using the chi-square distribution to determine the strength of associations.

Ethical approval

With permission number JUTH/DCS/IREC/127/XXXXXI/405, the Institutional Review Board of the Jos University Teaching Hospital gave its ethical approval for the conduct of this study. Each subject gave their consent, which was recorded using the ELDACAP electronic data capture application.

RESULTS

Of the 3114 participants screened for malaria in this study, 412(13.2%) were positive. These asymptomatic subjects were screened during the dry and rainy seasons of the year to collect samples representative of yearly seasonal variations. Of that total number, 1683(54.1%) of the samples were collected from the dry season of the year, while 1431(45.9%) were collected during the rainy season. Of these, 986(31.7%) were from the northern zone, 1077(34.6%) were from the central zone and 1051(33.8%) were from the southern zone.

Of the 3114 participants screened for malaria, 1102(35.4%) were male while 2012(64.6%) were female participants. Of the total number of male participants screened, 554(34.9%) of those tested in the dry season were male while 1129(67.1%) were female. Similarly, 548(38.3%) of those screened in the rainy season were male while 883(61.7%) were female.

Age distribution of participants

The mean age of participants was 29.6 ± 22.9 years (95% CI 28.7-30.4). The mean age of male participants was 25.1 ± 24.7 years (95% CI 23.5-26.6) while the mean age for female participants was 32.0 ± 21.4 years (95% CI 31.0-33.0) with the female participants significantly older than their male counterparts ($p<0.05$) (Table 2).

Distribution of *Plasmodium falciparum* resistant genes

A total of 206 samples had a DNA purity of between 1.8 to 2.0 and were tested to identify the presence of four genes indicative of resistance to antimalarial drugs. The prevalence of drug resistance genes in this study was 32.3%.

Of the 206 samples that were tested (Plate 1), 31(15.0%) were positive for the Pf DHFR gene (Plate 3), 124(60.2%) were positive for Pf DHPS gene (Plate 2), 29(14.1%) were positive for Pf MDR gene (Plate 5) while 82(39.8%) were positive for the PfK13 Propeller gene (Figure 2, Plate 4). The Pf DHPS gene was the most prevalent gene encountered in our study, $p=0.002$ (Table 4).

Table 2. Age distribution of male and female participants.

Parameter		95% Confidence interval		
Sex	**Mean Age ± SD (Years)**	**Lower Limits**	**Upper Limits**	**pValue**
Male	25.1± 22.9	23.5	26.6	0.001
Female	32.0 ± 21.4	31	33	
Total	29.6 ± 22.9	28.7	30.4	

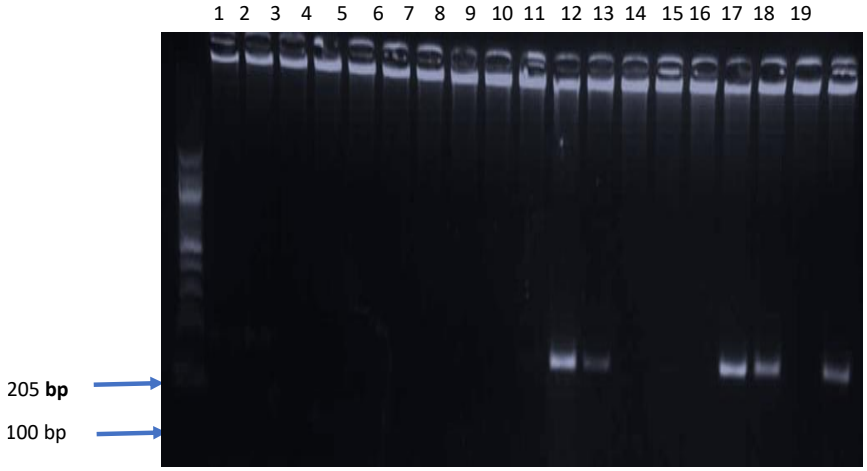

Plate 1. Gel electrophoresis Image of *P. falciparum* infection.

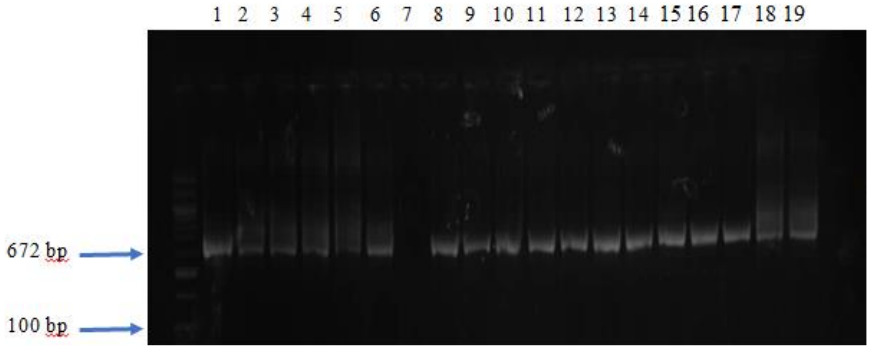

Plate 2. Gel electrophoresis Image of *Plasmodium falciparum* DHPS infection.

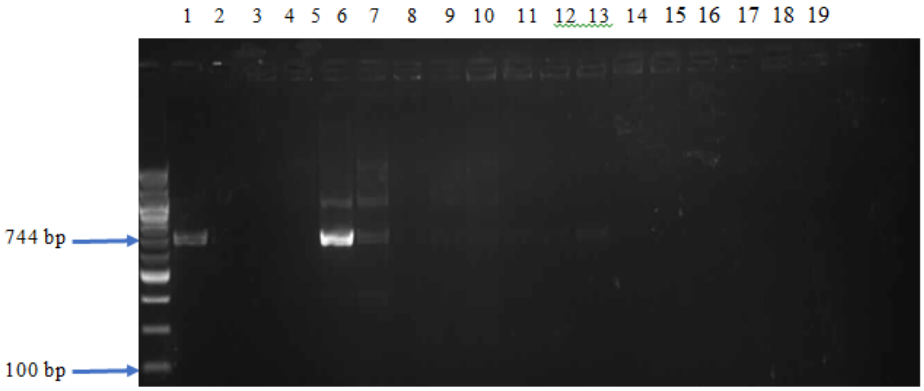

Plate 3. Gel electrophoresis image of *Plasmodium falciparum* DHFR gene at 2% agarose gel.

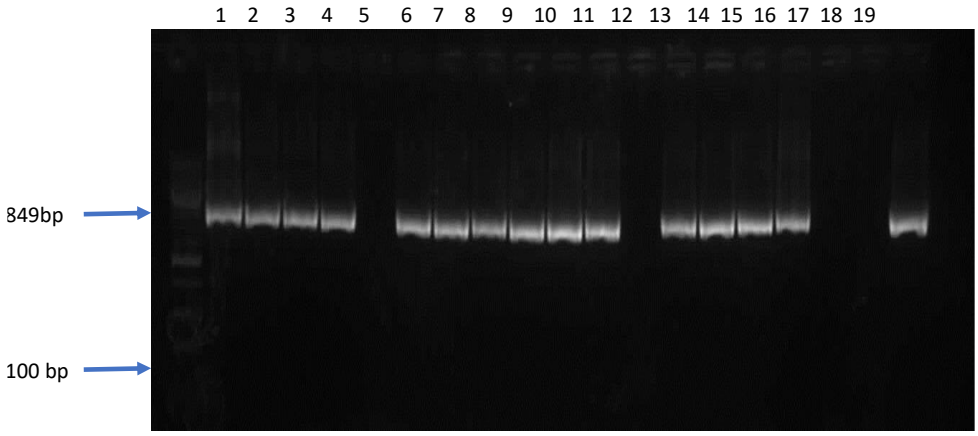

Plate 4. Gel electrophoresis image of *Plasmodium falciparum* K13 Propeller gene at 2% agarose gel.

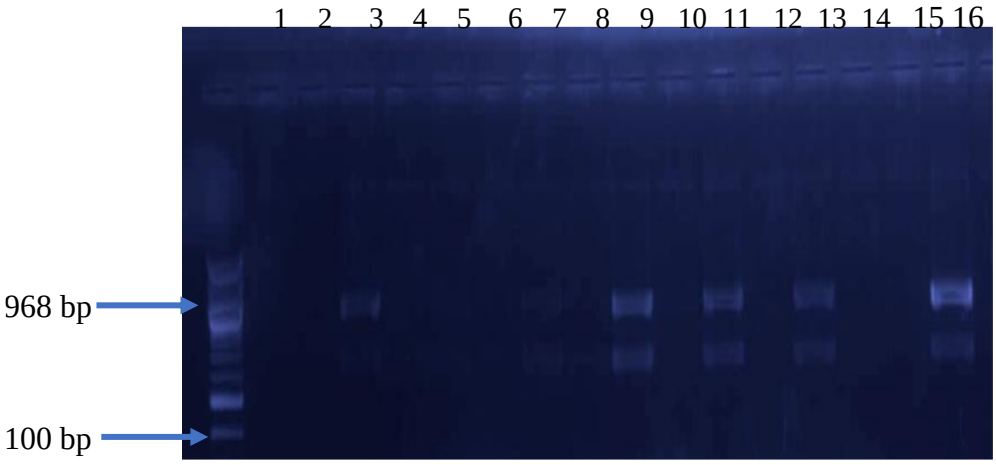

Plate 5. Gel electrophoresis image of *Plasmodium falciparum* MDR 1 Gene at 2% agarose gel.

Of these samples that were tested, 56(27.2%) were from the Northern zone, 62(30.1%) were from the Central zone while 88(42.7%) were from the Southern zone. The positive results for any of the genes for antimalarial resistance observed in this study showed that the sites from Jos-South site of the Northern senatorial zone accounted for 90(33.8%) of all positive results for any of the resistant genes. The sites in Pankshin in the central senatorial zone accounted for 72(27.1%) of positive results while the sites in Shendam accounted for 104(39.1%) of all the positive cases observed in the study (Figures 3 and 4). There was a significant association between a positive result for any of the genes for antimalarial resistance and the senatorial zone from which the sample was obtained (χ^2=8.793, df=2, p=0.012) with the sites in the southern zone being the most likely to have a positive result for resistance to any of the genes studied (Table 3).

DISCUSSION

The malaria prevalence found in this study is lower than that reported by Benjamin et al. (2020), who documented 23.7% prevalence from Kaduna in Northwest Nigeria, and Awosolu et al. (2021), who claimed 55% prevalence from Southwestern Nigeria. The mean age of study participants who were female was substantially greater than the mean age of study participants who were male ($p<0.05$). This conclusion aligns with the findings of Reddy et al. (2020), which suggested that women seeking healthcare is a positive behavior for overcoming societal barriers that hinder equity and access to healthcare services. This propensity has been demonstrated to be a potent mechanism that may be used to improve the results of interventions for malaria within communities since these women can be taught about control and prevention strategies such as the use

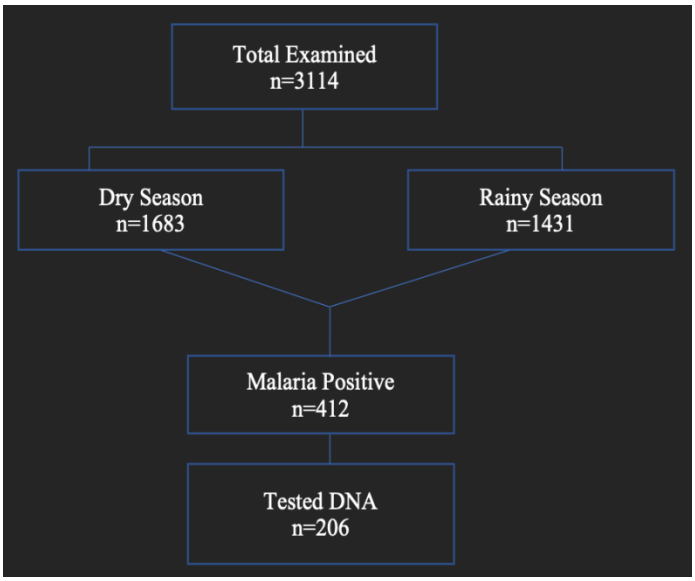

Figure 2. Schematic flowchart funnel of the sample testing for *P. falciparum* drug resistance in Plateau State.

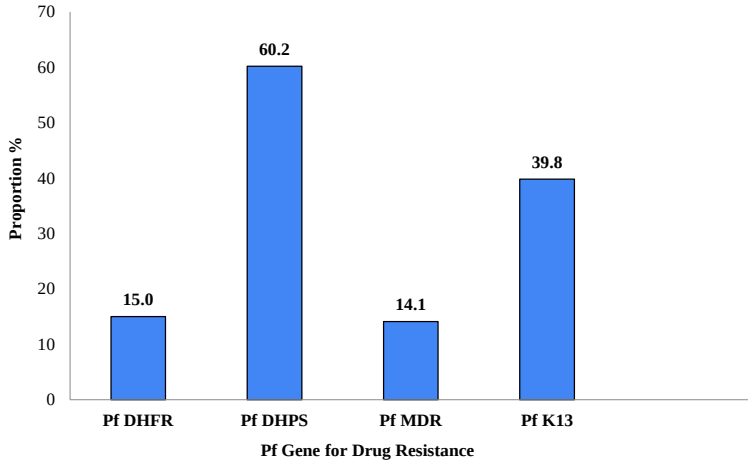

Figure 3. Proportion of *P. falciparum* genes resistant to antimalarial drugs.

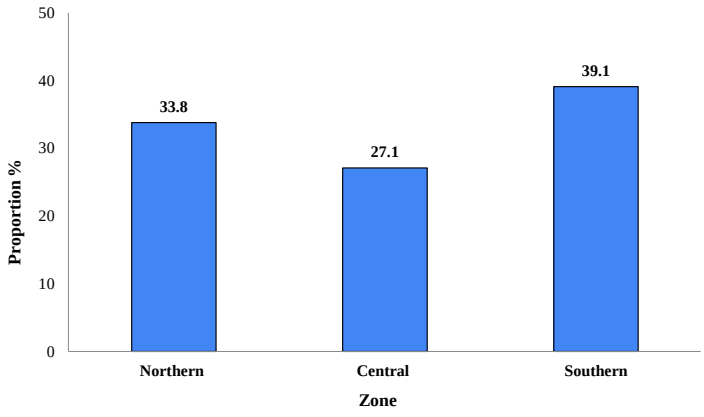

Figure 4. Proportion of drug resistance in *P. falciparum* genes from the three zones of Plateau State.

Table 3. Distribution of malaria cases by zone in Plateau State.

Site	Positive	Negative	Total	Pvalue
	n(%)	n(%)	n(%)	
Northern zone	88(21.4)	898(33.2)	986(31.7)	0.012
Central zone	147(35.7)	930(34.4)	1077(34.6)	
Southern zone	177(42.9)	874(32.4)	1051(33.8)	
Total	412(13.2)	2702(86.8)	3114(100.0)	

Table 4. Proportion of *P. falciparum* drug resistance genes by zone in Plateau State.

P. falciparum gene	Positive		p value
	Zone	n(%)	
MDR 1	Northern	13(23.2)	0.052
	Central	5(8.1)	
	Southern	11(12.5)	
DHFR	Northern	16(28.6)	0.574
	Central	4(6.5)	
	Southern	11(15.0)	
DHPS	Northern	37(66.1)	0.002
	Central	36(58.1)	
	Southern	51(57.9)	
K13	Northern	24(42.9)	0.509
	Central	27(43.5)	
	Southern	31(35.2)	

of long-lasting insecticide nets and seeking attention of community health care workers to receive testing and treatment for sick children who may be infected with malaria parasites. A previous study by Pam et al. (2019) raises the concern that the age distribution of male participants in this present study is similar to the age group of male members of rural communities they studied with the highest propensity for night-time outdoor activities that could sustain residual malaria transmission. Pam et al. (2019) opined that residual transmission of malaria can limit the benefit of control efforts that target indoor resting populations of Anopheles mosquito vectors. Effective control strategies in such communities will therefore require a robust consideration for how to provide education and awareness to this demographic to improve the uptake and effectiveness of the deployed interventions. Drug-resistant genes to malaria were more prevalent in the southern zone which is found in the lowlands of the Plateau where the prevalence of malaria among asymptomatic subjects was also higher. The prevalence of asymptomatic malaria agrees with another study by Aju-Ameh et al. (2017) in the Benue valley in North-central Nigeria.

The southern zone in Plateau area has been previously reported to have a higher prevalence of malaria than the highlands as climatic determinants of relatively higher temperatures and humidity with other factors provide conditions suitable for the breeding of vectors that maintain transmission in the area (Mafuyai et al., 2022; Nanvyat et al., 2017a, b). Considering the meteorological factors of a locale and the role of altitude in driving malaria transmission is a critical component of tailoring elimination strategies that will improve program outcomes for the elimination of malaria (Nanvyat et al., 2018, Dabaro et al., 2021). As pointed out by Blasco et al. (2017) as infection rates increase, drug pressure from the use of antimalarial drugs in a locality will lead to the development and emergence of drug-resistant genes that confer resistance to parasite strains. Subpar and fake antimalarial medications also present a threat to the long-term efficacy of ACTs. An estimate reported by Kaur et al. (2016) showed that up to 50% of artesunate sold in Southeast Asia, may be fraudulent.

With the use of ACTs, which are more expensive, it is a nticipated that the situation will get worse in other countri es where malaria is endemic (Kaur et al., 2016).

The observation in this study of no significant association between the presence of the Pf DHFR K13 propeller or MDR1 gene and the three study zones in this present study may be indicative of a similar drug usage

pattern that does not significantly differ across the areas for antifolates, ACTs and the previous use of chloroquine which is strongly associated with the MDR1 gene which reflects the current trend of Nigeria adopting the use ACTs as the first-line therapy for the treatment of uncomplicated malaria (Ajogbasile et al., 2022; Ikegbunam et al., 2021, Tuedom et al., 2021). Tuedom et al., (2021), posit that the widespread use of SP as an intervention for intermittent preventive therapy in pregnant women and Seasonal Malaria Chemoprevention (SMC) in children may be linked to the high occurrence of Pf DHFR and PfDHPS resistant genes for SP.

The high frequency of the Pf DHPS gene observed in this study differs from what was reported by Benjamin et al. (2020) in Kaduna State where there was a higher frequency in the Pf DHFR gene. Since the mutations that occur in these two genes are associated with decreased parasite sensitivity to Sulphadoxine-Pyrimethamine and other antifolates, the prevailing mechanism for resistance may be different but ultimately leads to the same outcome of reduced efficacy. This finding requires vigilance so that resistance caused by the presence of Pf DHFR and Pf DHPS genes does not jeopardise the use of SP in combination with amodiaquine for routine SMC in children.

Until recently, it was thought that drug resistance against artemisinin was still a long way off, however; since resistance to ACTs was on the western border of Thailand, there is mounting evidence for the emergence and westward spread of artemisinin resistance (Ouji et al., 2018). The result of this present study raises concern that the presence of PfK13 propeller gene requires close monitoring of ACTs to ensure it is notcompromised on the Plateau and consequently Nigeria. The distribution of the K13 gene in this study was not strongly associated with any of the three zones considered indicating that the use of ACTs is ubiquitous in distribution. A recent study by Ikegbunam et al. (2021) reported not finding any validated mutations like those found in Southeast Asia in the K13 isolates they observed from the analysis of samples collected from febrile children in 2004 and 2015. Their findings may be related to the study population of children rather than adults since the latter group can cultivate attitudes and practices toward taking antimalarial drugs that promote the emergence of resistance. This is very similar to another study by Ajogbasile et al. (2022) that profiles the K13 gene in Nigeria where they identified 13 polymorphisms that had not been previously unreported elsewhere in the world and were different from the previously validated mutations reported in Asia and South America in association with the slow clearance of artemisinin. While none of the mutations in isolates from the K13 gene that have been reported by some researchers in Nigeria includes themutations implicated in causing resistance to ACTs, there is a need to exercise caution and closely monitor these mutations so that they do not become mutations that cause resistance (Ajogbasile et al., 2022; Ikegbunam et al., 2021; Abubakar et al., 2020). It is critical to track the emergence of mutations in the K13 gene and the effectiveness of current tools in our malaria control arsenal cannot be overemphasized as a viable strategy to elongate the usefulness of ACTs in our war against malaria. Although the sequencing of the samples from this study is pending, it will be interesting to determine whether the K13 isolates observed in this study have validated mutations of significance or not.

Conclusion

Climatic factors and altitude are playing a role in driving drug-selection pressure by influencing malaria transmission in Plateau State. The advent of mutations to drug resistance to Artemisinin Combination Therapies in North-central Nigeria and particularly on Sulphadoxine Pyrimethamine SP in the southern senatorial zone of Plateau State require strategies that actively involve a monitoring plan using validated molecular tools and cataloguing potential mutations that may become significant in the future.

The high prevalence of the Pfdhps gene will also require keeping an eye on the efficacy profile of Sulphadoxine Pyrimethamine for Seasonal Malaria Chemoprevention of malaria in young children according to WHO treatment guidelines. Further investigation and sequencing studies are urgently needed to shed light on the situation in this part of Nigeria.

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CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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