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Mutant DHFR Prevalence and Pyrimethamine Resistance among Asymptomatic Adolescents in Kwara State

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Abstract
Plasmodium falciparum malaria remains a leading public health problem in sub-Saharan Africa and its control is seriously challenged by drug resistance. Resistance to sulfadoxine-pyrimethamine is mediated by point mutations in genes encoding the target enzymes dihydrofolate reductase (*pfdhfr*) and dihydropteroate synthase (*pfdhps*). Blood from a total of 176 subjects aged 11-19 years from Ilorin East and Irepodun Local Government areas (LGAs) was examined for mutant alleles of the *Plasmodium falciparum* dihydrofolate reductase gene by polymerase chain reaction (PCR) at codon 51 and 108 respectively. Rapid Diagnostic Test kits were used for the malaria test. Of the 176 participants, 73 (41.5%) subjects tested positive for malaria parasite while 95 (54.0%) tested negative. For both N51I and S108N SNP screening, mutant alleles were dominant over the wild type. The Iponrin community (Ilorin East LGA) recorded the highest percentage of mutant alleles 14 (33.33%) of the N51I SNP, while the Igbonla community (Irepodun LGA) had the least number of mutant alleles 8 (19.04%). On the other hand, the Alakuko community recorded the highest number of alleles 14 (31.80%) for S108N mutant while the Igbonla community (Irepodun LGA) had the least number of mutant alleles 8 (18.18%). There was widespread pyrimethamine resistance among the studied population and the malaria parasite remains persistent among the studied population. Therefore, there is a need for monitoring antimalarial drug resistance in Nigeria for prompt management of the antimalarial drug resistance menace.

Keywords: Malaria parasite, drug resistance, prevalence, mutant alleles

Introduction

Malaria remains an important cause of morbidity and mortality worldwide, particularly in sub-Saharan Africa [1]. In 2019, an estimated 227 million malaria cases were reported; but in 2020, malaria estimated cases were 241 million. This increase was attributed to the interruption in control measures caused by the outbreak of COVID-19. In 2019, the total number of deaths due to malaria was 558 000; this increased in 2020 to 627 000 [1]. The manifestations of *Plasmodium falciparum* are roughly divided into three clinical forms: asymptomatic infection, mild malaria, and severe malaria. Asymptomatic malaria is the presence of asexual parasites in the blood without showing any symptoms of illness, hence, they do not usually seek treatment for infection. Therefore, they constitute a reservoir of parasites,

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ready for transmission through a female anopheles mosquito [2]. Presently, malaria poses an enormous burden to societies, especially in developing countries. In Nigeria, over 12.0% of the gross domestic product (GDP) is expended on malaria annually [3]. In 2021, World Health Organization (WHO) recommended the use of RTS, S/ASO1 malaria vaccine among children which showed 30% effectiveness, falling short of the WHO standard (75% threshold) [4]. Therefore, in the absence of an effective malaria vaccine, chemotherapy remains the mainstay for the treatment and prevention of the disease [5]. The main cause of the worsened malaria situation recorded in recent years has been the spread of drug-resistant parasites. This has led to rising malaria-associated mortality, even though overall child mortality has fallen [6].

Drug resistance of *Plasmodium falciparum* has become a worrisome situation for the control and elimination of malaria. A prolonged anti-malarial monotherapy may select for drug-resistant parasite strains and may provide a chance to spread the resistance to sensitive parasite populations in endemic areas under favourable conditions for parasite transmission [7]. Over two decades, the loss of Chloroquine (first-line antimalarial drug) efficacy led to a shift toward sulphadoxine-pyrimethamine (SP) – a second-line antimalarial medicine, which targets two enzymes in the folate synthesis pathway. Sulphadoxine, a combination of antifolates with sulfadoxine, target the parasite's dihydropteroate synthase (DHPS); pyrimethamine, on the other hand, targets dihydrofolate reductase (DHFR) [8]. Although these two drug regimens are recommended as monotherapy for use in the management of asymptomatic/uncomplicated *P. falciparum* in the population; resistance to this drug gives rise to the introduction of artemisinin-based combination therapy (ACT) as the current drug of choice for the treatment of malaria [9].

Mutations in the dihydrofolate reductase (*dhfr*) and dihydropteroate synthase (*dhps*) genes of *Plasmodium falciparum* are associated with resistance to anti-folate drugs, most notably sulphadoxine-pyrimethamine (SP). The level of resistance to SP has been shown to increase with the accumulation of mutations at codons 16, 51, 59, 108, and 164; as the *pfdhfr* inhibits its activity, the parasite becomes resistant to pyrimethamine. While mutations at codons 436, 437, 540, 580 and 613 of *pfdhps* reduce the substrate binding capacity and confer resistance to sulphadoxine [10–12]. The S108N, C59R, and N51I mutations in the *Plasmodium falciparum* gene that encodes dihydrofolate reductase confer resistance to pyrimethamine and are common in Africa [3, 13–15]. Despite this, SP is used for intermittent preventive treatment of malaria in infants, children, and pregnant women in some African countries (WHO 2014, 2019). For instance, in Nigeria, the use of SP clears existing parasite and prevent subsequent parasite infection. Studies show that the drug prevents spontaneous abortion, reduced maternal anaemia, improved fetal birth weight and reduced placental parasitemia [16].

The prevalence of resistant phenotypes that are responsible for SP treatment failure is greater than 50% in several sub-Saharan African countries [14]. To adequately address the threat of intensifying SP resistance, particularly in Nigeria, it has become necessary to establish a system that will enable quick and accurate mapping of SP-resistant malaria parasites [17]. Molecular epidemiological methods based on the detection of mutations in the parasite molecules targeted by SP have been used for mutation surveillance. To this end, this study seeks to determine the prevalence of *pfdhfr* gene mutations among selected asymptomatic individuals in rural communities of Ilorin East and Ifelodun Local Government areas in Kwara State, Nigeria.

Materials and Methods

Study Area

The study was carried out in rural communities of Kwara state which cut across two Local Government Areas (LGAs), Ilorin East (Apado and Alakuko) and Ifelodun (Iponrin and Igbonla) respectively. Four communities - Apado (8°32'15'', 4°32'40''); Alakuko (8°37'46'', 4°25'50''); Iponrin (8°38'53'', 4°49'7''), and Igbonla (8°11'5'', 4°50'23'') were randomly selected from the LGAs for the study. The study areas have natural endowments in the areas of agriculture, tourism and solid minerals [18].

Study Design, Study Population and Sampling

A stratified random sampling technique was adopted for this study. In this method, participants were grouped into four age group strata (11-12, 13-14, 15-16, and 17-18) years. From these strata, participants were chosen by simple ballot paper. Equal numbers of participants were chosen in each stratum.

Questionnaire Administration
A structured questionnaire was administered to voluntary participants, and demographic information (sex, age, and locality) was collected from the participants. The respondents were guided on how to fill out the questionnaires appropriately.

Inclusion Criteria
Participants included in the study were within the age range of 11-18 years, they were asymptomatic individuals who reside in either of the two Local Government Areas. Before their recruitment, participants attended a health sensitisation programme where they were thought about the need for regular health checks irrespective of their current health status. All participants were willing to participate in the study. However, individuals who undergo a regular health check and those who did not meet the inclusion criteria were excluded from the study. One hundred and seventy-six asymptomatic people were recruited for the study.

Ethical Consideration
Ethical approval (NERC/ASN/2014/011) was obtained from the research and ethical committee, faculty of life sciences, University of Ilorin. Informed consent was obtained from all participants before inclusion in the study. In addition, all participants were told about their right to withdraw from the study at any time.

Collection of Blood Samples
Finger prick samples of capillary blood were collected from the subjects onto a filter paper strip (Isocode Stix®; Schleicher and Schuell, Ecquevilly, France) [19]. The samples were dried and stored at room temperature in individual zip-lock polyethylene bags before DNA extraction and PCR study.

Malaria Diagnosis
Blood obtained from participants was analysed on the spot with an RDT (Rapid Diagnostic Kit) kit for malaria (CareStart™ Malaria HRP2 from Access Bio, Inc, England). Drops of blood samples were placed in sample holes and a wash buffer was added to another hole. Two lines, both at the control and test side of the test instrument indicate positive for *P. falciparum* malaria while a line only at the control side indicates negative for *P. falciparum* malaria. The test was carried out according to the manufacturer's instructions.

Molecular Analysis
DNA Extraction
From each filter paper, one blood spot was punched out using Acu-punch skin biopsy punchers (Acuderm Inc, USA) and transferred into a 5-mL polystyrene tube. Subsequently, 2 ml of EasyMAG lysis buffer (bioMérieux, Marcy-l'Étoile, France) was added to the tubes after which they were put on a roller bank for 30 min for thorough lysis. After lysis, the fluid was transferred to EasyMAG vessels and magnetic bead silica was added to each well according to the manufacturer's instructions. DNA was extracted with DBS 1.0 protocol in the NucliSENS EasyMAG. Positive controls (blood spots from diluted *P. falciparum* 3D7 or FCR3 *in vitro* culture), and negative controls (blood spots of uninfected erythrocytes obtained from the Dutch Blood Bank), were included. Samples of extracted DNA were stored at −20 °C until further use.

Detection of dhfr Gene Mutation
The PCR assays for *dhfr* mutation included a primary and semi-nested reaction to enhance specificity, and sequencing was performed to determine the mutations. In the first reaction, 5.0 µL of DNA extract was amplified in a final volume of 25 µL containing 2.5 µL of reaction buffer, 25mM dNTPs, 0.75 units of Taq DNA polymerase, and 0.5µM of forward and reverse. In the first reaction, a 654-base pair (bp) portion of the *dhfr* gene was amplified by primers DHFR forward primer - (5-TTTATATTTTCTCCTTTTTAT-3) and DHFR reverse primer - (5-ACGCTAACAGA AATAATTTGA TACTC-3). For the second reaction, 3µl of amplified product from the first PCR product was added to the second PCR mixture where a 581-bp fragment containing codons 51 and 108 was amplified. PCR products were subjected to electrophoresis on 1.5% agarose gels and visualized by trans-illumination with ultraviolet light after staining with SYBR® Green. Amplicon sequence was determined using an ABI PRISM 3100 Genetic Analyser (Applied Biosystem, Foster City, California, USA) [17]. Sequence results were analysed using the Vector NTI Advance™ version 11.0 software.

Data Analysis
Statistical analysis was carried out using GraphPad Instat (GraphPad Software Inc., San Diego, USA). Frequencies were compared using Fisher's exact tests and two-sided $p<0.05$ were considered statistically significant.

Results

A total of 176 asymptomatic participants from four different communities of Ilorin East and Ifelodun LGAs were screened for *Plasmodium falciparum*. Of the 176 participants screened for infection, 73 (41.5%) tested positive for malaria infection while 95 (54.0%) tested negative (Table 1). The highest infection was found in persons aged 11-12 years; also, this age category recorded the highest number of malaria parasite count, 50 (50.0%) (Figure 1). On the other hand, the least number of infections occurred in persons aged 17-18 years, with a total number of 1 (1.3%).

Table 1. Malaria RDT result across studied age groups

Age group (years)	+ve	- ve	Invalid	Inconclusive	No result
11 -12	50 (50.0%)	46 (42.9%)	49 (48.5%)	2 (4.5%)	1 (1.1%)
13 -14	15 (20%)	26 (23.1%)	2 (4.5%)	1 (1.1%)	0
15 -16	7 (9.3%)	15 (14.9%)	0	0	0
17 -18	1 (1.3%)	8 (7.9%)	0	0	0
Total	**73 (41.5%)**	**95 (54.0%)**	**4 (2.2%)**	**3 (1.7%)**	**1 (0.6%)**

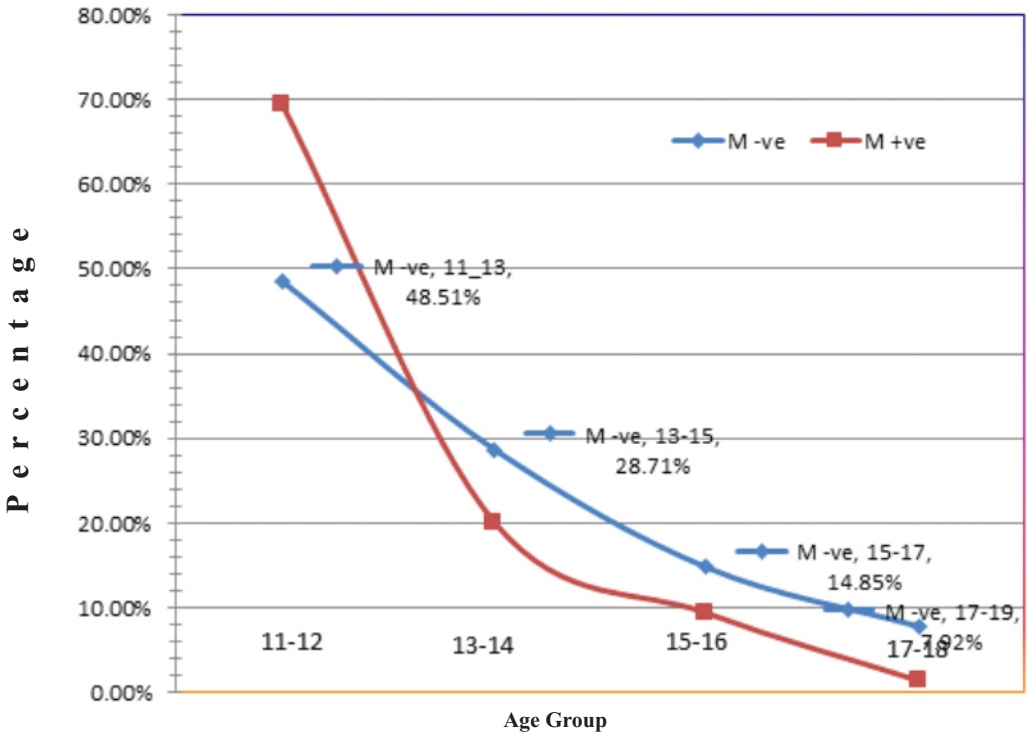


Fig. 1: Malaria parasite plot across age categories of studied participants

Gene Amplification Results

Plasmodium falciparum genes of the studied population were screened by polymerase chain reaction (PCR) and amplified for the presence of a mutation in the dihydrofolate reductase synthase (DHFR) gene of the parasite at codons 51 and 108. The distribution of the mutant alleles and the wild-type alleles of the DHFR gene across the studied communities are depicted in table 2 and table 3.

Table 2. Distribution of Wild type (N51) and Mutant type (51I) DHFR Gene across studied Communities

DHFR Gene	Gene Position	Apado Community	Alakuko Community	Iponrin Community	Igbonla Community	Total
N51 (Wild)	51	7 (20.58%)	10 (29.41%)	6 (17.64%)	11 (32.35%)	34 (44.73 %)
51I (Mutant)	51	11 (26.19%)	9 (21.42%)	14 (33.33%)	8 (19.04%)	42 (55.26%)

Table 3. Distribution of Wild type (S108) and Mutant type (108N) DHFR Gene across studied Communities

DHFR Gene	Gene Position	Apado Community	Alakuko Community	Iponrin Community	Igbonla Community	Total
S108 (Wild)	108	9 (28.12%)	9 (28.12%)	7 (21.87%)	7 (21.87%)	32 (42.10%)
108N (Mutant)	108	11 (25.0%)	14 (31.80%)	11 (25.0%)	8 (18.18%)	44(57.89%)

For N51I, at codon 51, wild-type alleles (N51) accounted for 44.73% (34) while mutant alleles accounted for 55.26% (42). Of this, the Igbonla community (Ifelodun LGA) had the highest percentage of wild-type alleles, 32.35% (11) while the Iponrin community (Ilorin East LGA) had the lowest percentage of wild-type alleles 17.64% (6). On the other hand, the Igbonla community (Ifelodun LGA) recorded the least number of mutant-type alleles (51I), 19.05% (8), while the Iponrin community (Ilorin East LGA) recorded the highest mutant type alleles with 33.33% (14) (Figure 2).

In the case of S108N, at codon 108, Apado and Alakuko communities (Ilorin East LGA) recorded the same percentage of wild-type alleles (S108), 28.12% (9). Similarly, the Iponrin community (Ilorin East LGA) and Igbonla community (Ifeoludun LGA) had the same percentage of wild-type alleles. For mutant alleles (108N), the Alakuko community (Ilorin East LGA) had the highest mutant alleles 31.80% (14) while the Igbonla community (Ifeoludun LGA) recorded the least number of mutant alleles 8 (18.18%) (Figure 3)

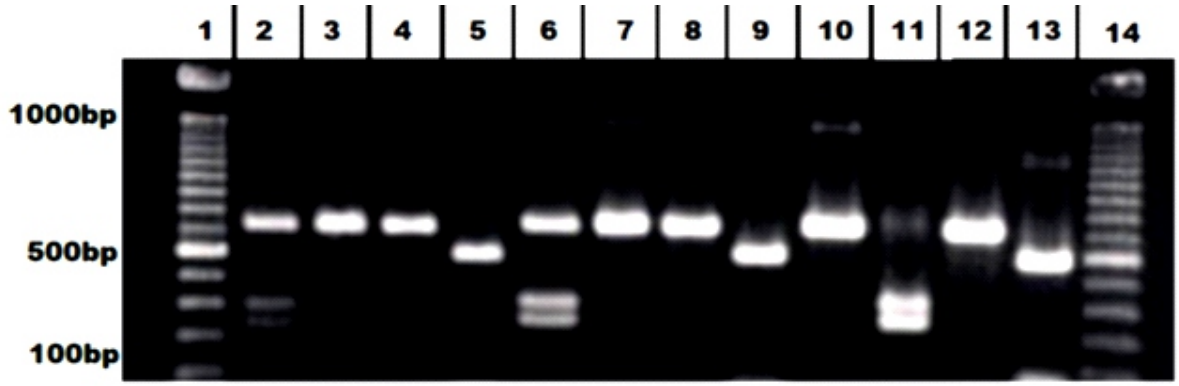


Fig. 2: 1.5% Agarose gel electrophoresis analysis of nested PCR from the primary amplification of the 654bp of *Plasmodium falciparum Dihydro Folate Reductase* (*pfdhfr*). Lanes 2-10 and 12-13 show the expected amplicon band size of 100bp for the N51I mutant *pfdhfr* while lanes 7 and 9 were not amplified. Lane 1 is the DNA ladder (1kb+).

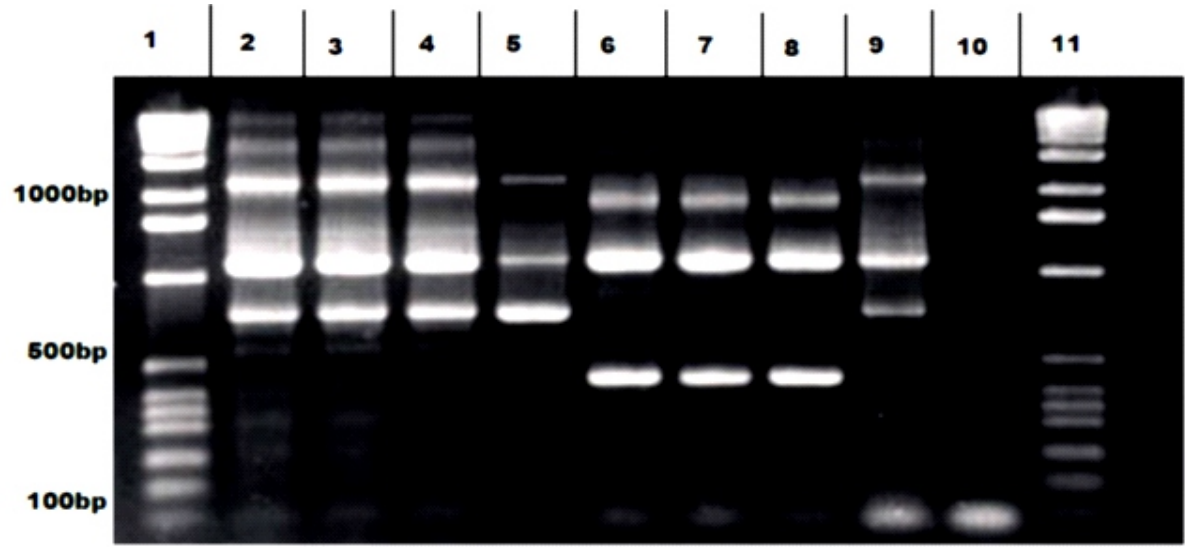


Fig. 3: 1.5% Agarose gel electrophoresis analysis of nested PCR from the primary amplification of the 581bp of *Plasmodium falciparum Dihydro Folate Reductase* (*pfdhfr*). Lanes 2-9 show the expected amplicon band size of 441bp for the S108N mutant *pfdhfr* while lane 10 was not amplified. Lane 1 is the DNA ladder (1kb+).

Discussion

Plasmodium falciparum malaria remains a leading health problem in Africa and its control is seriously challenged by drug resistance. Nigeria, an endemic region of the disease continues to record the highest burden of the disease compared to the rest of the world [1]. Asymptomatic individuals are often left untreated, but they continue to transmit the disease, thereby making malaria infection difficult to control [20]. One hundred and seventy-six asymptomatic participants from two Local Government Areas (LGAs) of Kwara state were screened for *Plasmodium falciparum* malaria. More than 40% tested positive for malaria infection across the two LGAs which is an indication of continuous malaria transmission in the country. Our study aligns with some reported surveys in Nigeria [21, 22] where a high prevalence of malaria infection was recorded. The high negative malaria infection among the participants could be attributed to the extensive utilization of insecticide-treated mosquito nets and chemotherapy in the studied population. In Nigeria, the use of ITNs is gaining more ground, as observed by some researchers [23]. In the world malaria report by the World Health Organization in 2021, globally, a total of 229 million insecticide-treated nets (ITNs) were delivered by manufacturers [1]. According to the report, more than 90% of the total was delivered to countries in sub-Saharan Africa. Of the total delivered, Nigeria was among other African countries that received the highest number of ITNs. However, despite the availability of insecticide-treated mosquito nets in the country, malaria infection remains one of the public health problems affecting the population across different age groups [24]. This observation is not surprising because, in stable transmission areas, a high prevalence of malaria infection is often reported. Therefore, we recommend a longitudinal study involving both *dhfr* and *dhps* genes to determine the distribution of these genes and their effect on SP efficacy in the control of malaria among asymptomatic individuals.

Age-related prevalence of malaria infection in this study shows that persons aged 11-14 years had the highest prevalence of malaria infection which agrees with previous studies [25, 26]. A study on the west coast of Africa showed that a high prevalence of malaria infection usually observed in children <5 years is shifting to older age groups [27]. This could be a result of adequate protective care given to the <5 years age group, this age group failed to develop an adequate immune response to malaria parasites [28]. The high prevalence of infection observed among persons aged 11-14 years could be attributed to the fact that most people in this age group are not directly under the care of their parents/caregivers. Hence, the necessary protective care for them did not receive adequate monitoring by their parents/caregivers. Another explanation for this is the effect of drug

resistance to the malaria parasite.

The widespread drug-resistant *Plasmodium falciparum* has led to a rise in malaria-associated mortality, especially in sub-Saharan Africa. Resistance to sulphadoxine-pyrimethamine (SP) has evolved worldwide caused by point mutations that accumulate at multiple sites in *dhfr* and *dhps* genes [11]. The identification of *pfdhfr* as the central determinant of pyrimethamine *P. falciparum* malaria provides a molecular marker that could be used for the surveillance of resistance and evaluation of drug treatment and prophylaxis policies [29]. This method provides an avenue to monitor the trends and distribution of appropriate resistant genes and haplotypes, thereby ensuring that threats to the available malaria drug regimen are identified and necessary measures are taken at the right time. These measures would aid the national malaria control programmes to reduce the prevalence of malaria to zero levels. In this study, the molecular method was applied to screen 75 participants who tested positive for *P. falciparum*. Positions 51 and 108 of the parasite *dhfr* gene were examined for single nucleotide polymorphism (N51I and S108N), and the results obtained from this study indicated a high prevalence of mutant alleles over wild-type alleles.

Several studies have reported pyrimethamine resistance in Nigerian regions. Widespread mutant S108N alongside other markers were reported in Ogun State. [6] Their study which comprises subjects within 1-15 age groups revealed the prevalence of mutant alleles in the participants over wild type, as in the current study. Similarly, the dominance of mutant (73%) alleles over wild-type occurred among pregnant women in Lagos. [12] Furthermore, the result obtained from this study is in agreement with a comparative study in Enugu, Benin, Borno, and Ibadan [15]. Also, in other parts of southwestern Nigeria, the prevalence of mutant alleles was higher compared to wild-type alleles with a ratio of 90:10 [8]. The three distinct *pfdhfr* mutations (N51I, C59R, and S108N) have been reported in many African countries [7, 13, 14]. However, only two *pfdhfr* mutations (N51I and S108N) were observed in all the study communities, this has been reported from asymptomatic individuals but with higher prevalence compared to this current study [30, 31]. While S108N is involved in the initial low-level pyrimethamine resistance, N51I and C59R increase the resistance level as the prevalence increases in the population [32]. The persistent occurrence of SP-resistant *P. falciparum* in each population could be an indication of continuous use of SP for the treatment of uncomplicated malaria infection. In all four communities, the complete absence of C59R and I164L could be due to the slow development of resistance to SP in Kwara State, Nigeria. A possible reason for the weak selective pressure is the greater use of alternative antimalarial drugs such as artemisinin-based combination therapy (ACT), improved acquired immunity, and use of non-conventional treatment regimens by most of the participants. The I164L mutation which originated from Southeast Asia and South America [11] has not been reported in Nigeria; however, C59R has been reported in Nigerian and other African countries where malaria infection is endemic [11]. The evidence from this study shows the widespread low-level pyrimethamine resistance in the studied population. However, since SP was not monitored among the participants over a long period, further research to ascertain why C59R was not observed among the population is required.

Conclusion

Our study showed the existence of drug resistance among asymptomatic young adults who tested positive for malaria infection in rural communities in Kwara State, North-Central Nigeria. Treatment for asymptomatic individuals, should be encouraged to truncate the transmission of malaria parasites in the country.

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Declarations

Author's Contribution

JOS, OO and OKA conceptualized the study. JOS, OO, and OKA designed the study. OKA participated in fieldwork and data collection. JOO, OKA, and OGO performed the data analysis; JOO, OKA, and OGO interpreted the data. JOO, OKA, and OGO prepared the first draft of the manuscript, reviewed by JOS, OO, and OGO. All authors contributed to the development of the final manuscript and approved its submission.

Disclosure of Conflict of Interest

The authors declare that they have no competing interests

Disclosure of Funding Source

Not applicable

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