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Research article

Mutational Screening of Dihydrofolate reductase and Dihydroptereoate synthase Target Genes in *Plasmodium falciparum* Isolated from Out-patients with Febrile Conditions

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ABSTRACT

Sulfadoxine-pyrimethamine (SP) is no longer recommended for the treatment of uncomplicated falciparum malaria due to its increasing failure rate, but is still being used as preventive treatment during pregnancy and in chemoprevention. SP targets either dihydrofolate reductase (DHFR) and dihydroptereoate synthase (DHPS) involved in folate biosynthesis in the parasite. This study screened for mutation in SP target genes, dhfr and dhps in *Plasmodium falciparum* among out-patients in Awka South, Anambra State. Blood samples were obtained from 210 patients aged 5-64 years with febrile conditions. The blood samples were screened for malaria parasites using blood smear stained with giemsa. The dhfr and dhps genes were amplified using PCR and sequenced using dideoxy chain termination method. The overall prevalence of Plasmodium falciparum malaria in this study was 74.8 % (157/210) out of which 19.1 % (30/157) had high parasite count of ≥ 4000 asexual parasite /µL of blood. Out of this number, 14.6% [23/157] with high yield and purity of ≥50 ng/µL and ≥1.7 respectively were considered good for downstream PCR.The result showed that dhfr alone was detected in 8/23 [34%], dhps alone was seen in 7/23 [30%] while both dhfr and dhps genes were seen in 4/30 [13%]. In all [8/23] parasites where dhfr was detected, mutations at Asn-108, Ile-51 and Arg-59 were present. Additional mutation was seen in 75% [6/8] at position Leu-164. Gly-581, Phe-436 and Thr-613 mutations were seen in all isolates where dhps were detected. Rare Leu-164 and Thr-613 mutants were seen in dhfr and dhps respectively. These mutants may be responsible for SP resistance in this region, suggesting an increased compromise over the efficacy of SP in malaria treatment.

Keywords: *Plasmodium falciparium, dihydrofolate reductase, dihydropteorate synthase, malaria.*

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INTRODUCTION

Malaria as a life-threatening disease is caused by parasites that are transmitted to people through the bites of infected female Anopheles mosquitoes. In 2019, there were an estimated 229 million cases of malaria worldwide with malaria-related deaths at 409, 000 (WHO, 2020). Sulphadoxine-pyrimethamine (SP) was the second-line treatment drug while chloroquine (CQ) was the first- line antimalarial for the treatment of uncomplicated *P. falciparum* malaria. Unfortunately, *P. falciparum* developed resistance to both widely used drugs and are not currently recommended singly for the treatment of malaria in the general population (Quan *et al*., 2020). Currently, SP is one of the partner drugs of artemisinin-based combination therapy (ACT) being used for treatment of uncomplicated falciparum malaria and the only drug recommended by the World Health Organization (WHO) for intermittent preventive treatment (IPT) in pregnant women in Sub-Saharan Africa where large number of deaths occur due to malaria in pregnancy (Verity *et al*., 2020). The malaria parasite's resistance to SP is due to point mutations in target enzymes, dihydrofolate reductase (*dhfr*) and dihydropteroate synthase (*dhps*) (Aydemir *et al*., 2018). Several mutations that results in higher levels of resistance to pyrimethamine/sulphadoxin which subsequently diminish the efficacy of SP have been reported in Ghana (*dhfr*-C50R, *dhfr*-N51I, *dhfr*-C59R, and *dhfr*-I164L) (Amenga-Etego *et al*., 2021) Malaysia (*dphs* -Gly-581 and *dphs*- Phe-436) (Lau *et al*., 2013) DR-Congo and Gabon (*dhfr*-S436A, A437G, K540E, A581G, A613S) and (*dphs*-A16V, N51I, C59R, S108T, I164L, T185) (Aydemir *et al*., 2018; Boukoumba *et al*., 2021). In Nigeria, while most reports involved epidemiological studies, two recent studies on mutational analysis of *plasmodium falciparum*-resistant genotype were reported (Kayode *et al*., 2021; Quan *et al*., 2020). Lack of this

molecular approach in Nigeria hampers constant surveillance of these haplotypes that should be done in the country to detect any change in prevalence. Hence this study investigated mutations in SP target genes, *dhfr* and *dhps* in *Plasmodium falciparum* among out-patients in Awka South, Anambra State.

MATERIALS AND METHODS

Blood samples were collected from 210 outpatients aged 5-64 years; 92 males and 118 females from three different hospitals in Awka, Anambra State, Nigeria after obtaining ethical clearance (ANSUTH/AA/ECC/33) and informed consents and in accordance with the Declaration of Helsinki (Goodyear *et al*., 2007). The sample size was determined based on previously reported *Plasmodium falciparum* prevalence rate of 83.8 % in the same locality (Nwonu *et al*., 2009). Cochran formula (1951) was used and calculation was done at 95.0 % confidence interval with 0.05 precision (Cochran *et al*., 1951). Patients who reported to the hospital with history of fever (auxillary temperature >37.5^{0}C) and complained of malaria symptoms like night fever, chills, headache and muscle pains were included.

Parasite density: Blood samples collected from patients were prepared for thick and thin films. Briefly, 3.0 % giemsa solution in buffered water of pH 7.2 was used to stain both the thick and the thin films for 30 minutes. The slides were then rinsed off gently with clean running water and allowed to air-dry. The slides were examined under the light microscope at x1000 magnification. The thick films were used to determine the parasite densities while thin films were used to identify the parasite species and infective stages (Chessbrough, 2005). Those confirmed positive for the *Plasmodium falciparum* species were further used for the study. Estimation of Parasite count was achieved by counting asexual parasites as described by Greenwood and Armstrong (1991) (Greenwood and Armstrong, 1991).

Extraction of *Plasmodium falciparum* genomic DNA: Genomic DNA was extracted using rapid DNA extraction from archive blood spots on filter paper as described by Bereczky *et al.* (2005) (Bereczky *et al*., 2005). Extraction was achieved by carefully cutting out about 3 - 5mm dried blood sample into an eppendorf tube containing 125 µL of absolute methanol, making sure that the whole paper-cut was soaked into the methanol. Thereafter, the medium was incubated at 37^{0}C for 1 h and centrifuged at 10,000 rpm for 10 min. The solvent was carefully decanted and the paper was allowed to air dry in the tube at room temperature for 15 min with the lid open. 100 µL of sterile water was added in each tube and heated at 99^{O}C in a heating block for 15 min. While heating, the paper was smashed in the water with the micropipette tips to release the DNA. Thereafter tubes were centrifuged again at 10,000 rpm for 10 min and supernatant (~65µL) was collected and stored for PCR use. The purity and yield of the supernatant collected (extracted parasite DNA) were measured using Nanodrop 1000 Spectrophotometer. The DNA yield was measured in concentration (ng/µL) and purity was measured by the absorbance ratio at 260 - 280nm.

PCR amplification and analysis of *dhfr* and *dhps* genes: Amplification of *dhfr* gene was done using specific primers: 5′-ATGATGGAACAAGTCTGCGACGTTTTCGAT-3′ (forward primer, ST1L) and 5′- TTCATTTAACATT TTATTATTCGTTTTCTT-3′ (reverse primer, ST2L). The PCR conditions were programmed using Applied Biosystem, 2720 Thermal Cycler USA in a 20 µL reaction mixture. Initial denaturation was done for 5 min at 94^{0}C, denaturation at 94^{0}C for 30 s, annealing at 52^{0}C for 60 s, 65^{0}C for 60 s extensions for 25 cycles with the final extension of 5 min at 65^{0}C (Tahita *et al*., 2015). Amplification of *dhps* gene was done using specific primers: 5′- GTATTTTTGTTGAACCTAAACGTG-3′ (dhps-1 forward primer) and 5′-CCACAATATTTTTATTTTCATTTTG-3′ (dhps-2R reverse primer). The PCR conditions were programmed using Applied Biosystem, 2720 Thermal Cycler, the USA in a 20 µL reaction mixture. Initial denaturation was done for 5 min at 94^{0}C, denaturation at 94^{0}C for 30 s, annealing at 54^{0}C for 60 s, 72^{0}C for 60 s extensions for 25 cycles with the final extension of 5 min at 72^{0}C (Tahita *et al*., 2015). PCR products were purified using QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). Sanger sequencing was performed using a Genetic Analyser 3130 xl sequencer from Applied Biosystems and BigDye terminator version 3.1 cycle sequencing kit. Detection of mutation was done using MEGA X version through comparative analysis with the wild type sequence with GenBank accession numbers; XM_001351443 and Z30654 for *pfdhfr* and *pfdhps*, respectively. Translations of nucleotides were done using Expassy tools.

Agarose gel electrophoresis: About 10 µL of each final amplification products were loaded in the wells on the 2 % agarose gel together with 5 µL of a 1 kb DNA marker (DNA ladder) and allowed to run in 2% TAE buffered electrophoresis at 100 volts for 30 minutes. Thereafter, gel was visualized under ultraviolet transillumination to confirm the presence of the DNA fragments.

Statistical analysis: Frequencies were compared using chi-squared tests, and continuous variables were compared using Student's t-tests or Mann-Whitney U-tests, as appropriate. All reported p-values are for two-tailed tests and were considered statistically significant if less than 0.05.

RESULTS

Out of 210 blood samples screen for *plasmodium falciparum* 157 representing 74.8% were positive. Those not considered positive included those with *plasmodium vivax* and those without any detected parasite. Table 1 represents the gender and age range of the sampled patients. Males represented 43.8% [92/210] while females represented 56.2% [118/210] of the sampled patients. Patients aged 05-14 and 55-64 represented the highest percentage 21% [44/210] each, followed by aged 45-54 at 19% [40/210]. The least percentage was seen in aged 15-24 at 5.7% [12/210]. Table 2 shows age-specific prevalence rates of *falciparum* malaria amongst studied population. The highest prevalence of 84.4% was seen

in age 35-44, followed by age 45-54 at 82.5%. The least prevalence was 63.3% seen in age 55-64.

Table 1:
Gender and age of patients

Characteristics		Number	percentage (%)
Gender	Male	92	43.8
	Female	118	56.2
	Total	210	100
Age	05-14	44	21.0
	15-24	12	5.7
	25-34	38	18.7
	35-44	32	15.2
	45-54	40	19.0
	55-64	44	21.0
	Total	210	100

Samples with parasite counts of ≥4000 /µL representing 19.1% [30/157] were selected for *dhfr* and *dhps* PCR amplification. Out of this number, 14.6% [23/157] with high yield and purity of ≥50 ng/µL and ≥1.7 respectively were considered good for downstream PCR. Out of the 23 samples, *dhfr* alone was detected in 8/23 [34%], *dhps* alone was seen in 7/23 [30%] while both *dhfr* and *dhps* genes were seen 4/30 [13%], table 3 and figure 1 and 2. In all [8/23] parasites where *dhfr* was detected, mutations at Asn-108, Ile-51 and Arg-59 were present. Additional mutation was seen in 75% [6/8] at position Leu-164. Gly-581, Phe-436 and Thr-613 were seen in all isolates where *dhps* were detected, table 3. There was no type of mutation seen in both genes.

Table 2:
Age-specific prevalence rates (*n*=210)

Age group (years)	Number	Positive	Negative	Prevalence (%)
5-14	44	35	9	79.5
15-24	12	9	3	75.0
25-34	38	25	13	78.1
35-44	32	27	5	84.4
45-54	40	33	7	82.5
55-64	44	28	16	63.6
Total	210	157	53	74.8

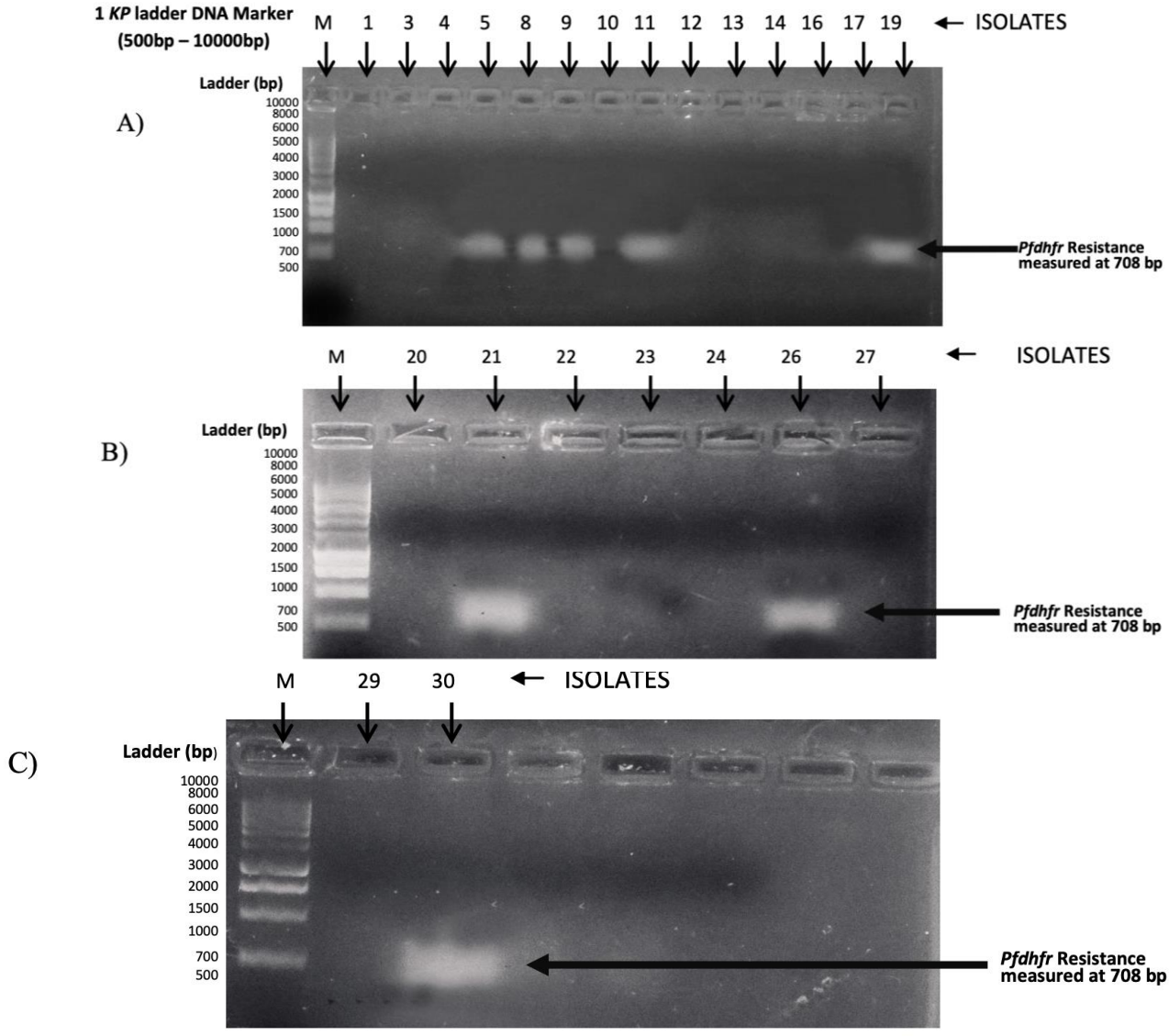

Plate 1
Photographs of agarose gel showing amplified *dhfr* genes

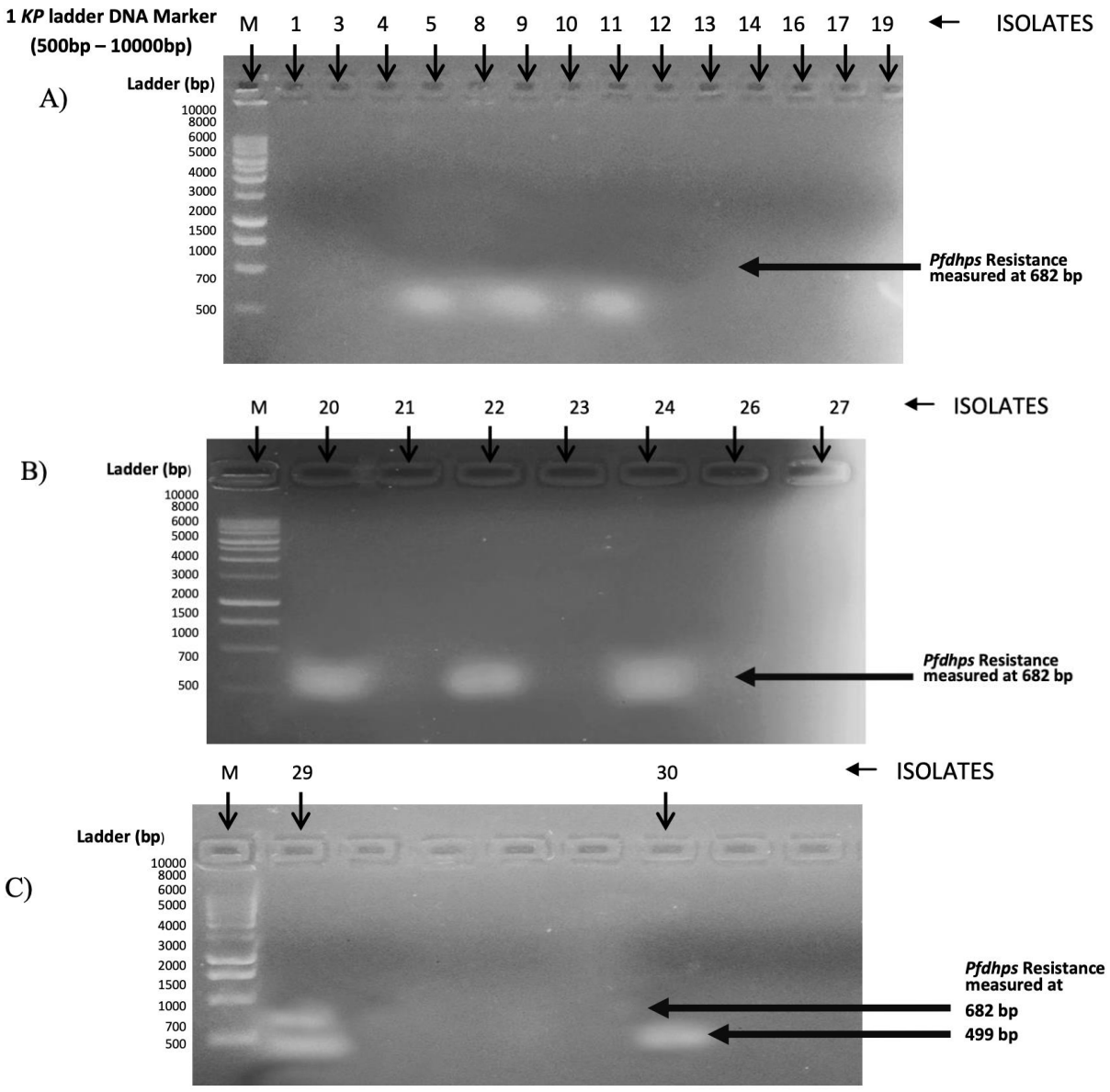

Plate 2
Photographs of agarose gels showing amplified dhps genes

Table 3:
Detection and mutational analysis of *dhfr* and *dhps* genes (*n*=23)

	dhfr	*dhps*	*dhfr & dhps*
Detection	8/23 [34%]	7/23 [30%]	4/30 [13%]
Mutation	108 [Ser-Asn	581 [Ala-Gly]	
	51 [Asn-Ile]	436 [Ser-Phe]	
	59 [Cys-Arg]	613 [Ala-Thr]	
	164 [Ile-Leu]		

DISCUSSION

This study screened for sulfadoxine-pyrimethamine target genes in *plasmodium falciparum* isolated from out-patients aged 5-64 years with febrile condition in Awka South, Anambra State, Nigeria. Out of the 210 outpatients, higher percentage number was seen in females representing 56.2%, while males represented 43.8%. This implies that females may be more prone to malaria infection. In a similar study conducted in Ilorin, Nigeria, out of the infected population, 49% were females while 28% were males (Kolawole *et al*., 2017). This may be due to higher number of females screened as they as they have better good-health seeking behavior than males (Obasi *et al*., 2019). In contrast, in a cross-sectional study performed in Ibadan, Southwestern Nigeria, the number of positive patients with *plasmodium falciparum* was higher in males than in females at 60.2% and 50.9% respectively (Awosolu *et al*., 2021). Again, in Pakistan, an endemic region for malaria, males (11.46%) were more prone than females (6.99%) (Tareen *et al*., 2012). This predominance malaria prevalence in males could be justified by the fact that males have more exposures to malaria vector than females due to

their activities such as working in fields at peak biting times or migrating to areas of high endemic area for work.

Children and aged patients were shown to be the most vulnerable groups in this study. Similar results were obtained elsewhere (Allen *et al*., 2009; Kolawole *et al*., 2017; Olasehinde *et al*., 2019). The main burden of malaria and malaria-related deaths are mostly borne by young children, accounting for over 64% of all malaria related deaths worldwide (WHO, 2015). In a recent publication using the Global Burden of Disease Study 2019, the result showed that malaria episodes of children under 5 years accounted for >30% in Central, Western and Eastern Sub-Sahara Africa (Liu *et al*., 2021). Hence, interventions for children under 5 years are seriously required especially in developing African countries. Overall prevalence rate of *plasmodium falciparum* was seen at 74.8%. This high prevalence rate was observed in other parts of Nigeria; 45.86% in Ogun State (Olasehinde *et al*., 2019), 72% in Ilorin (Kolawole *et al*., 2017), 55% in Ibadan (Awosolu *et al*., 2021). In other *plasmodium falciparum* endemic regions, the prevalence were found to be lower; 18.45% in Quetta, Pakistan (Tareen *et al*., 2012), 2.2% in India (Kumar *et al*., 2012), 5.6% in Guinea Bissau (McGregor *et al*., 2021). These prevalence rates may be a gradual progressive step towards malaria eradication. Based on the recent annual global malaria report published by the World Health Organization (WHO), estimated 229 million malaria cases in 2019 in 87 malaria-endemic countries were observed, representing a decline of 9 million cases from the year 2000. However, they were higher than the 218 million estimated malaria cases for the baseline year 2015 reported at the Global Technical Strategy (GTS) for malaria 2016–2030 (Al-Awadhi *et al*., 2021).

SP target genes, dihydrofolate reductase *dhfr* alone was detected in 34% of the *plasmodium falciparum*, dihydropteroate synthetase *dhps* alone was seen in 30% while both *dhfr* and *dhps* genes were seen in 13%. The high detection of *dhfr* imply that *dhfr* may be more involved in SP resistance. The presence of 5 mutations, 3 in the *dhfr* gene and 2 in the *dhps* gene were found to be associated with treatment failure (Allen *et al*., 2009). To further corroborate the high detection of *dhfr* gene in this study, Gatoon *et al*., 2004 revealed that apart from linking treatment failure to mutations in these genes, presence of mutation in *dhfr* appears to be more important in causing treatment failure than mutation found in *dhps* (Gatoon *et al*., 2004). It was observed that mutations were detected at positions Asn-108, Ile-51 and Arg-59 with additional one at position Leu-164 in *dhfr* gene. Normally associated with low level pyrimethamine tolerance both in vitro and in vivo is a change from wild-type Ser108 to Asn108 (S108N) in *pfdhfr*. This is a basal amino acid substitution which is characterized to have a tenfold increased risk of SP therapeutic failure (Lozovsky *et al*., 2009) and must be present before additional mutation can occur (Plowe *et al*., 1997). Accumulation of other mutations including *pfdhfr*-C50R, *pfdhfr*-N51I, *pfdhfr*- C59R, and *pfdhfr*-I164L that resulted in higher levels of resistance to pyrimethamine and subsequently diminished the efficacy of SP in vivo were reported in Ghana (Amenga-Etego *et al*., 2021). A different mutation was obtained in national drug therapeutic efficacy testing study (DTET) for monitoring antimalarial efficacies of ACT in the treatment of uncomplicated malaria, involving five states in Nigeria which are part of the sentinel locations for the National Malaria Elimination Program of the National Ministry of Health in Nigeria. In the study, greater percentages of the infected children haboured Asn-108, Ile-51 and Arg-59 mutants. Unlike this study, none of the children was infected with parasites habouring the mutant Leu-164 allele (Kayode *et al*., 2021). This implies that mutant Leu-164 may be key to SP resistance involving pyrimethamine in Nigeria. In the same study, only Gly-581 and Phe-436 were also detected in *dphs* genes similar to this study, but in contrast additional Thr-613 mutant was seen in our study implicating the allele as most relevant in sulphadoxine resistance. Elsewhere, mutation at position Phe-436 has been reported as novel (Lau *et al*., 2013). This study observed that females, children and elderly were more prone to *plasmodium* infection in this region. There was increased prevalence in triple mutations with Leu-164 and Thr-613 mutants seen in *dhfr* and *dhps* respectively. These mutants may be responsible for SP resistance in this region.

Author's contributions:

DOO and EED: Conceptualization, methodology and writing. SCO and SC: Review and editing. TNO and CFE: Investigation and resources, JNE: Formal analysis. All authors have read and approved the final article.

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