

***Plasmodium falciparum* Resistance in Selected Areas of Niger State, Nigeria: Chloroquine and Sulphadoxine/Pyrimethamine Treatment Outcome and Predictive Value of Molecular Markers**

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Abstract

Malaria is a public health problem affecting two third of the world population with high mortality among children and pregnant women. A study of the determination of drug-resistance molecular markers in *Plasmodium falciparum* infection was carried out in three malaria endemic local government areas of Niger State, Nigeria between October 2018 and September 2019. The objective of the study was to determine the prevalence of molecular markers of resistance to chloroquine and sulphadoxine/pyrimethamine. *P. falciparum* infected red blood cells (RBC) were confirmed using microscopy, and deoxyribonucleic acid extraction from *P. falciparum* positive RBC specimen was done using chelex extraction method. Nested polymerase chain reaction followed by Restriction Fragment Length Polymorphisms (PCR/RFLP) were used for the detection of *Plasmodium falciparum* chloroquine resistance transporter (*pfcr*t), *P. falciparum* multidrug resistance 1 (*pfmdr* 1), *P. falciparum* dihydrofolatereductase (*pfdhfr*) and *P.falciparum* dihydropteroate synthase (*pfdhps*). The results revealed well characterized molecular markers of *P. falciparum* resistance to the 4-aminoquinolines ($p<0.05$) while the antifolate drugs revealed high prevalence($p<0.05$) of resistance: 41%, 60%, 51% and 47% of *P. falciparum* isolates at codons N86Y, K76T, S108N, N51I and A437G respectively. The prevalence of resistance of isolates to these antimalarial drugs were comparatively high. Therefore,

strategies that reduce multiple-strain of malaria infections should be implemented in order to improve antimalarial drug efficacy and reduce rate of spread of drug resistance.

Key words: Molecular Markers, *Plasmodium falciparum*, Chloroquine and sulphadoxine/pyrimethamine resistance, Nigeria

Introduction

Malaria which occurs in over 106 countries is one of the most severe public health problems affecting about 200 million people globally and it is a leading cause of avoidable death especially among children and pregnant women in tropical countries (CDC, 2012).

Malaria used to be rife all over the world but since the 1950's it been eliminated from many regions including Europe, North America and Carribean (Sarah, 2011). However, it continues to be a huge social, economic and health problem particularly in the tropical countries, especially in Nigeria according to Demographic Health Surveys (DHS,2011). Malaria fever certainly arose in Africa and spread within Africa as nomadic lifestyle was abandoned for agriculture which accidentally created favourable habitats for mosquitoes to breed (Weiming *et al.*,2014). Trading and migration eventually brought malaria to China, India and Europe. By the medieval era, malaria was endemic throughout Africa, Europe, the Balkans to Southern Sweden and Southern Asia and parts of Russia (Sarah, 2011)

The classic symptom of malaria is cyclical occurrence of sudden coldness followed by rigor and then fever and sweating lasting for four to six hours, occurring every two days in *P.vivax* and *P.ovale* infections, while every three days for *P.malariae* and *P. falciparum*(*ref*). There could be recurrent fever every 36-48 hours or a less pronounced and almost continuous fever. Malaria has been found to cause cognitive impairments, especially in children(*ref*). It causes widespread anaemia during a period of rapid brain development and direct brain damage. This neurologic damage results from cerebral malaria to which children are more vulnerable. Cerebral malaria is associated with retina whitening, which may be a useful clinical sign in distinguishing malaria from other causes of fever (MacCormick *et al.*, 2014).

Severe malaria is almost exclusively caused by *P.falciparum* infection, and usually arise 6-14 days after infection. If untreated severe malaria can result to coma and death among young children and pregnant women. Splenomegaly, severe headache, cerebral ischemia, hepatomegaly (enlarged liver), hypoglycaemia, and hemoglobinuria with renal failure may occur. Renal failure is a feature of black water fever, where haemoglobin from lysed red blood cells leaks into the urine. Severe malaria can progress extremely rapidly and cause death within hours or days (WHO, 2013). In the most severe cases of the disease, fatality rates can exceed 20%,

even with intensive care and treatment. In endemic areas, treatment is often less satisfactory and the overall fatality rate for all cases of malaria can be as high as one in ten. Over the long term, developmental impairments have been documented in children who have suffered episodes of severe malaria (WHO, 2014).

Chloroquine was discovered by a German, Hans Andersag, in 1934 which he named resochin. In 1945, chloroquine replaced quinine as the first-line treatment for malaria due to low cost, straightforward synthesis and low toxicity. The first resistance to chloroquine was documented in 1957. The onset of chloroquine resistance marked the beginning of a new chapter in the history of malaria (Akshawa *et al.*, 2012). The emergence of drug resistance, widespread resistance to available insecticides, wars and massive population movements, difficulties in obtaining sustained funding from donor countries and lack of community participation made the long-term maintenance of the effort to eradicate malaria untenable. Completion of eradication campaign was eventually abandoned. The goal of most current National Malaria Prevention and Control Programs and most malaria activities conducted in endemic countries is to reduce the number of malaria-related cases and deaths. To reduce malaria transmission to a level where it is no longer a public health problem is the goal of what is called malaria control (CDC, 2016). According to Prugnolle and Yalcindag (2012), drug resistance is the reduction in the effectiveness of a medication in treating a disease or a condition. This is very rampant in the management of malaria including preventive therapy in its target population.

The genetic complexity of *P. falciparum*, particularly its ability to crossmate and generate mutant variants, makes it a successful pathogen. Genetic variants are involved in pathogenicity and in immune responses and have led to the emergence of resistance against virtually all available anti-malaria drugs including chloroquine and sulphadoxine/pyrimethamine. Such variants are under strong selective pressure, but analyses of sequence variation in gene segments that are not directly subjected to selection permit the study of inter population diversity of the parasite and its evolutionary origin (Pameta *et al.*, 2013).

Microsatellites (MS) are molecular markers that can provide valuable information on population structure in malaria parasite and are common in the *P. falciparum* genome. They can be defined as nucleotide repeats ranging between 2 and 6 base pairs (bp), one MS can be found in every kilo-base genome-wide. MS are highly polymorphic due to variation of length of these multiple alleles, and more neutral than single nucleotide polymorphisms (SNPs) among parasite isolates. Fortunately, mutations that are associated with drug resistance in *P. falciparum* provide a unique window to identify allelic associations because these must have occurred within the past 50 years (Daniel *et al.*, 2012). These drug resistant genotypes are characterized by reduced diversity around the major resistance alleles. In Asia and some African countries, microsatellite markers with multiple alleles flanking genes associated with drug resistance such as *dhf* and *pfcr* were investigated and allowed typing of

specific haplotypes flanking these regions thus enabling identification of drug resistance origin as well as tracing spread of these mutations (Okombo *et al.*, 2014). However, there have been no supporting literature of such study in Nigeria despite the high mortality, morbidity and public health concern of malaria in the country. Therefore, this study was aimed to determine the prevalence of molecular markers associated with *P. falciparum* resistance to chloroquine and sulphadoxine/pyrimethamine in three malaria endemic local government areas of Niger state, Nigeria.

Materials and Methods

This study was carried out in three Local Government Areas of Niger State, North Central Nigeria, located between latitude 8°00'-11°30'N and longitude 4°00'-8°00'E while sharing international boundary with the Republic of Benin to the West and inter-state boundaries with six Nigerian states. A descriptive cross sectional study was designed for this research work while subjects were selected randomly. The study population were Kambaris of Niger State who are adversely rural, uneducated, and underdeveloped. The target population were school pupils and community members in selected local government areas where 1,264 samples were screened for malaria and only 100 confirmed *Plasmodium falciparum* positive samples were used for molecular detection of mutations in drug targets.

Polymerase Chain Reaction for Detection of *Pfcr*t and *Pfmdr*1 Genes

The detection of mutations responsible for chloroquine resistance was performed by amplifying sequences marking the *Plasmodium falciparum* chloroquine resistance transporter (*pfcr*t) and *Plasmodium falciparum* multidrug resistance 1 (*pfmdr*1) genes using nested polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP) according to previously described procedures (WHO, 2013). Primers used for *pfcr*t (K76T) and *pfmdr*1 (N86Y) primary amplification included curiously procuring template pattern (Crtp) 1 & 2, Mdr1 and Mdr2 and their sequences. The primary PCR components in a final volume of 15µl was buffered by 25mM MgCl₂ and 50mM deoxynucleotide triphosphate (dNTPs), 10µm of each of the primer (Crtp1, Crtp2, Mdr1 and Mdr2), 5µl mM deoxynucleotide triphosphate (dNTPs), 10µm of Taq polymerase and 2µl of DNA samples were then added. The cycling protocol for *pfcr*t was as follows: initial denaturation of 94°C for 3 minutes followed by 45 cycles of 94°C for 30 seconds, 60°C for 1 minute and 72°C for 15 minutes. For *pfmdr* 1 the cycling protocol was initial denaturation at 95°C for 5 minutes followed by 45 cycles of 95°C for 30 seconds, 45°C for 30 seconds, 65°C for 45 seconds and 72°C for 15 minutes.

Nested Polymerase Chain Reaction and Restriction (PCR) Fragment Length Polymorphism (RFLP) for *pfcr* and *Mdr* 1 mutation-specific detection

Secondary PCR was conducted by using the forward primer Crtd 1 and the reverse primer Crtd2. Two (2) µl of 10x dilution of primary PCR was used in a follow-up, nested, allele specific PCR amplification to detect the codon for *pfcr* K76T. The PCR stages for these diagnostic amplifications were initial denaturation at 94°C for 3 minutes followed by 45 cycles at 94°C for 30 seconds, 56°C for 30 seconds and 60°C for 1 minute and a final extension at 72°C for 15 minutes.

Purified genomic DNA from *P. falciparum* clones 3D7 (Chloroquine sensitive) and Dd2 (Chloroquine Resistant) were used as positive controls and water and uninfected blood spots on filter paper were used as negative controls. After amplifications, 20µl of the amplicons was incubated overnight at 50°C with mutation-specific restriction enzyme *Apo* I. In the PCR products, the DNA sequence was cleaved at the wild type (76K) codon site (if present) into two fragments, while the mutant alleles (76T) codon found in chloroquine-resistant *P.falciparum* were not cut. The digested products were separated by electrophoresis in a 2% agarose gel containing ethidium bromide and DNA was visualised by ultraviolet trans illumination. Similarly, amplification of codon 86 of the *pfmdr* 1 gene was carried out using the following primers: *Mdr* 1 and *Mdr* 2 for the primary PCR reactions and *Mdr* 3 and *Mdr* 4 for the secondary reactions after which restrictions with *Apo* 1 or *Afl*III was done. DNA fragments were compared by size and with the PCR products generated from genomic DNA of the 3D7 and Dd2 strains (used as references for susceptible and resistant genotypes respectively). Thermocycling conditions for *pfmdr*1 secondary reaction were initial denaturation at 95°C for 3 minutes, followed by 25 cycles at 95°C for 30 seconds, 45°C for 30 seconds and 65°C for 45 seconds and a final extension at 72°C for 15 minutes.

Polymerase Chain Reaction (PCR) Assay for *dhfr* and *dhps* genes

A nested PCR described by Pearce *et al*(2003) was used to amplify fragments of the *dhfr* and *dhps*. The detection of mutations responsible for sulphadoxine and pyrimethamine resistance was performed by amplifying sequences marking the *dhfr* and *dhps* genes using nested PCR followed by restriction fragment length polymorphism. The 15µl *dhfr/dhps* final PCR component consisted of buffer 10x, 50mM dNTPs, 25mM MgCl₂, 10µM of each of the primer, 5µ/µl of Taq polymerase and 2µl of template DNA. The cycling protocol was as follows: an initial denaturation of 95°C for 5 minutes, 40 cycles of 30 seconds at 95°C, 40 seconds at 52°C, 1 minute at 72°C and elongation at a final cycle of 10 minutes at 72°C.

Subsequently, a nested PCR was performed to increase the yields of the specific amplicons, 3µl of the primary PCR product was used in a reaction volume of 15µl with corresponding primers, the PCR mixture was the same as the primary reaction.

The cycling condition was previously described (Pearce *et al.*, 2003). The nested PCR products were confirmed by electrophoresis on a 2% agarose gel stained with ethidium bromide along with a set of controls.

The RFLP was performed by incubating the PCR products according to specification with corresponding restriction enzymes (*Alu* 1 for *dhfr* S108N/T; *Ava* II for *dhps* A437G; TSP 5091 for and *dhfr* N51I) and buffers obtained from the biopolymer factory, Soflinger Str. 100 D-89077 Ulm, Germany. The digested products were visualised by electrophoresis on 2% agarose gels and visualised by ultraviolet trans illumination.

Structured questionnaires were used to harvest demographic data (age, sex etc) from the pupils who participated in this study.

Data were finally analysed using the Statistical Package for Social Sciences (SPSS) version 21.

Ethical Considerations

Ethical clearance was sought and obtained from the Niger State Ministry of Health Ethical Review Committee (Ref. MOH/NS/EC/777/88/32). A letter of introduction which spelt out the purpose of the research work and benefits to be derived was taken to the Local Government Education Board, the Education Secretary, Area supervisors and Head Teachers of the various schools.

Results

Prevalence of molecular markers of *plasmodium falciparum* resistance to chloroquine and sulphadoxine/pyrimethamine

One hundred (100) selected *Plasmodium falciparum* isolates were typed with markers of Chloroquine (pfcrt and pfmdr 1) and Sulphadoxine/Pyrimethaxine (pfdhfr and pfdhps) resistance at five codons namely **K76T**, **N86Y**, **S108N**, **N51I** and **A437G**. Whereas the prevalence of Wild (sensitive) type alleles **N86** of pfmdr1 (59.0%) was higher than the mutant alleles **Y86** (41.0%), prevalence of mutant alleles **T76** (40.0%) of pfcrt was considerably higher than wild type alleles **K76**. At codons 51 and 108, the prevalence of mutant alleles **I51** (55.0%) and **N108** (51.0%) was higher than wild type alleles at the two codons. However, the differences were not statistically significant. At codon 437, the Wild type alleles **A437** (53%), was more represented and prevalent than the mutant alleles **G437** (47%). Twenty-nine percent and 16% of the isolates recorded double mutation and triple mutation at codons (**N108/ I51**) and (**N108/ I51/ G437**) respectively (Table 1).

Prevalence of molecular markers associated with *plasmodium falciparum* resistance to chloroquine and sulphadoxine-pyrimethamine in study population according to age

The ages of 100 participants for drug resistance typing ranges from 1year to above 30 and were grouped into four: 1-10years, 11-20, 21-30 and greater than 30. The age groups 1-10 and 11-20 were more represented being 62% and 24% respectively. The least representation was observed in the age groups 21-30 and > 30 being 9% and 5% respectively.

Of the five codons examined for the presence of mutant alleles, T76 and I51 of pfcr1 and pfdhfr had the highest overall prevalence of mutant alleles, followed by N108 and G437 of pfdhfr. The least overall prevalence of mutant alleles was observed among Y86 of pfmdr1. The prevalence of mutant alleles by age showed an increasing rate of mutation with age in most of the codons but analysis of data by age categories did not discover any statistical difference ($p < 0.05$). (Table 2).

Prevalence of drug resistance marker by sex

Of the 100 isolates that were successfully typed by drug resistance markers, 44 were males while 55 were females. Thus females were more represented. While higher prevalences of mutant alleles was observed in both sexes at codons 76 and 51 of pfcr1 and dhfr respectively, lower prevalence of mutant alleles was observed in both sexes at codon 86 of pfmdr1. At codons 437 and 108 of dhps and dhfr respectively, males had higher prevalence of mutant alleles than females ($p > 0.05$). However, there is no significant difference in the distribution of mutant alleles in both sexes (Table 3).

Prevalence of drug resistant markers in relation to the local government areas of the participants

Of the three Local Government Areas where participants who consented to the study were drawn for participation, Borgu LGA had the highest representation, followed by Magama while Mashegu had the least representation. Both mutant and wild type alleles were discovered at all codons examined in all the L.G.A.S. In Mashegu, except for codon 86 of pfmdr1 in which the Wild type alleles was more prevalent (53.0%), analysis of other codons showed higher prevalence ($p < 0.05$) of mutant alleles whereas at Magama, higher prevalence of Wild type genotypes was observed at all codons except for codon 76 of pfcr1 in which mutant alleles had higher prevalence. However, the differences were not statistically significant ($p < 0.05$). At Borgu, prevalence of Wild type alleles was higher at codons 437 and 86 of dhps and mdr1 respectively whereas equal prevalences of both Mutant and Wild type alleles was observed at codon 108 of dhfr but at codons 51 and 86 of dhfr and pfcr1 respectively, higher prevalence of mutant alleles was observed (Table 4).

Table 1: Prevalence of drug resistant markers among confirmed *Plasmodium falciparum* infected Population

Drug Resistance Markers	% of Mutant Alleles	% of Wild-type Alleles
Pfcr (K76T)	60	40
Pfmdr 1 (N86Y)	41	59
Pfdhfr (S108N)	51	49
Pfdhfr (N511)	55	45
PfdhfrN108/I51	29	71
dhps(A437G)	47	53
dhfr/dhps N/108/I51/G437	16	84

Table 2: Prevalence of Molecular Markers Associated with *P. falciparum* Resistance to Chloroquine and Sulphadoxine-Pyrimethamine by Age

Age	% Examined	No (%) of Mutant Alleles Examined				
Group	(100)	T76	Y86	N108	I51	G437
1-10	62	36(58.1)	25(40.3)	29(46.8)	34(54.8)	30(48.4)
11-20	24	15(62.5)	10(41.7)	13(54.2)	13(54.2)	9(37.5)
21-30	9	6(66.7)	4(44.4)	5(55.6)	5(55.6)	4(44.4)
>30	5	3(60.0)	2(40.0)	4(80.0)	3(60.0)	4(80.0)
Total	100	60(60.0)	41(41.0)	51(51.0)	55(55.0)	47(47.0)

Table 3: Prevalence of Drug-Resistance Markers by Sex

Drug Resistance Markers	Males (44)	FEMALES (56)
	No.(%) of Mutant	No.(%) of Mutant
pfcr (T76)	26(59.1)	34(60.7)
pfmdr1(Y86)	15(34.1)	26(46.4)
pfdhfr(N108)	28(63.6)	23(41.1)
pfdhfr(I51)	24(54.5)	31(55.4)
pfdhfrN108/I51	13(29.5)	16(28.6)
pfdhps(G437)	23(52.3)	24(42.9)
dhfr/dhps N108/I51/G437	7(15.9)	9(16.1)

Table 4: Prevalence of Drug-Resistant Markers by LGA

Number examined by L.G.As	Pfcr 76		Pfmdr1 86		Pfdhfr 108		pfdhrt 51		Dhps437	
	Mutant (%)	Wild (%)	Mutant (%)	Wild (%)	Mutant (%)	Wild (%)	Mutant (%)	Wild (%)	Mutant (%)	Wild (%)
<u>Mashegu</u> (13)	10 (76.9)	3 (23.1)	6 (46.2)	7 (53.8)	8 (61.5)	5 (38.5)	9 (69.2)	4 (30.8)	8 (61.5)	5 (38.5)
<u>Magama</u> (29)	16 (55.2)	13 (44.8)	13 (44.8)	16 (55.2)	14 (48.3)	15 (51.7)	14 (48.3)	15 (51.7)	13 (44.8)	16 (55.2)
<u>Borgu</u> (58)	34 (58.6)	24 (41.4)	22 (37.9)	36 (62.1)	29 (50.0)	29 (50.0)	32 (55.2)	26 (44.8)	26 (44.8)	32 (55.2)
Total	60 (60.0)	40 (40.0)	41 (41.0)	59 (59.0)	51 (51.0)	49 (49.0)	55 (55.0)	45 (45.0)	47 (47.0)	53 (53.0)

Discussion

Resistance to Chloroquine

One of the factors to be considered in the prophylaxis, treatment and control of *P. falciparum* malaria is the resistance of parasite strains that may arise against every drug available with time. Chloroquine-resistant *P. falciparum* malaria was a major health problem, particularly in Sub-Saharan Africa that led to change in treatment policy (FMH, 2005). According to Atroosh *et al.* (2012), chloroquine resistance has been associated *in vitro* with point mutations in two genes, *pfcr* and *pfmdrl*, which encodes the *P. falciparum* digestive-vacuole transmembrane proteins and *p*-glycoprotein-mediated multidrug resistance (*Pgh1*), respectively. Identification of *pfcr* as the central determinant of chloroquine-resistant *P. falciparum* malaria provides a molecular marker that can be used for surveillance of resistance to evaluate drug treatment and prophylaxis policies. Analysis of well-characterized molecular markers of *P. falciparum* resistance to the 4-amino-quinolines and the antifolates revealed a high prevalence of resistant genotypes.

The T76 mutation which is associated with chloroquine resistant i.e the substitution of threonine (T76) for lysine (K76) at position 76 (K76T) of the amino acid sequence in *pfcr* which encodes a transporter protein of the *P. falciparum* digestive vacuole (ref) was found in 60.0% of samples with *P. falciparum* malaria infection and Y86 mutation (associated with resistance to amodiaquine, mefloquine, halofantrine and lumefantrine) that is the substitution of tyrosine (Y86) for asparagine (N86) at position 86 (N86Y) was found in 41.0%.

The high prevalence of resistance to chloroquine observed in this study indicates a persistence of resistance to chloroquine 10 years after the change of malaria treatment policy in Nigeria (FMH, 2005). The result is consistent with the findings from other parts of Nigeria and most regions of high drug resistance (; Micheal *et al.*, 2011; Ojurongbe *et al.*, 2011; Olasehinde *et al.*, 2014). This result is however in sharp contrast to findings from Malawi where plasmodium became susceptible to chloroquine after cessation of usage (ref). Also in Tanzania, chloroquine regained its sensitivity after two and half years of withdrawal of drug pressure (ref).

Chloroquine (CQ) and Sulphadoxine (SP) were replaced with artemisinin in 2005 due to widespread resistance and high level clinical failure across the country. It is expected that the sensitivity to CQ will be restored about 10 years after withdrawal as it happened in Malawi and Tanzania. Persistence of CQ resistance witnessed in this study might not be unconnected with poor drug policy in the country which allows the drug to be freely available for use outside government hospitals. Cross-resistance between CQ and amodiaquine (AQ) may also be a contributory factor due to the similarities in their modes of action. Amodiaquine is currently a partner drug with artemisinin in the treatment of malaria in Nigeria. Although this drug remains

effective in areas of substantial CQ resistance, the two drugs are chemically related and several clinical and *in vitro* reports have shown cross resistance between CQ and AQ or active metabolite of AQ (Micheal *et al.*, 2011).

Adagu and Warshurst (1999) observed that *pfcr*t K76 and the *pfmdr*1Y86 alleles are closely associated in chloroquine resistant strains. A similar observation was found in this study as all isolates carrying *pfmdr*1 mutated allele were also positive for *pfcr*t mutated allele. However, studies comparing the associations of the *pfmdr*1 variant and the *pfcr*tK76 variant have also shown that the impact of the *pfcr*t gene was stronger than that of the *pfmdr* gene (Ojurongbe *et al.*, 2007). It has been suggested that the degree of chloroquine resistance is further modulated by factors linked to genes other than *pfcr*t or *pfmdr* (Chen *et al.*, 2001). Consistent with other studies, one can assume that the prevalence of *pfcr*tT76 is a function of the actual chloroquine level and influence of the acquired immunity and natural resistance factors of the host. Furthermore, one can also suggest that chloroquine intake contributes essentially to the selection of the *pfcr*tT76 allele.

Regarding *pfmdr*1, the present study showed a predominance of wild type of *pfmdr*1 N86 (59%). The role of *pfmdr*1 gene mutations in anti-malarial drug resistance is still controversial. Previous *in vivo* studies reported a strong association between *pfmdr*1 86 in the chloroquine resistant infections (Von *et al.*, 1997). Similarly, a previous study by Zakeri *et al.* (2008) reported a strong association between *pfcr*tK76T but not *pfmdr*1 N86Y mutation *in vivo* chloroquine resistance. In contrast, a recent study reported an association with *pfcr*t gene (Andriantsoanirina *et al.*, 2010). Moreover, an *in vitro* study showed that *pfmdr*1 mutations in *P.falciparum* can confer resistance to high levels of chloroquine and that these *pfmdr*1 mutations have an important role in the resistance of *P.falciparum* to mefloquine and quinine (Reed *et al.*, 2000).

The high prevalence of wild alleles of *pfmdr*1 N86 could be considered as an indicator for low susceptibility of *P.falciparum* isolates to mefloquine, amodiaquine and quinine. On the other hand, previous studies have found increased sensitivity to the anti-malarials mefloquine and artemisinin in *P. falciparum* isolates with mutations in the *pfmdr*1 gene (Sidhu *et al.*, 2005). However, a previous *in vivo* study found that the treatment failure with mefloquine is significantly associated with increased *pfmdr*1 (Price *et al.*, 2004).

This current study did not discover any positive association in the prevalence of mutant alleles in relation to either sex or age of participants, although lower age had lower prevalence of mutant alleles T76 (58.1%) and Y86 (40.3%). However, the differences were not statistically significant, similar to the findings of Atroosh *et al.* (2012).

Resistance to sulfadoxine and pyrimethamine

Resistance to sulfadoxine and pyrimethamine was observed in this study among the isolates analysed for resistance markers, *pf dhfr*S108N/N51I and *pf dhps*A437G genes coding for pyrimethamine and sulfadoxine respectively. It has been earlier observed that resistance to pyrimethamine is primarily conferred by a non-synonymous point mutation at codon 108 and is consecutively enhanced by mutations at codons 51 and 59 of the *P.falciparum* *pf dhfr* gene located on chromosome 4 while point mutations at codon 437 and 540 of the *pf dhps* gene located on chromosome 8 of *P.falciparum* are considered responsible for sulfadoxine resistance (Sharma *et al.*2015).

Selection for sulfadoxine/pyrimethamine(SP) resistance markers in all the study areas remained high even after the replacement of SP as second-line treatment of uncomplicated malaria in 2005. A similar high prevalence of mutant *dhfr*and *dhps* alleles was recorded by Ojurongbe *et al.* (2011). in a study conducted at Osogbo, Oyo State, Nigeria. The result is also comparable to that of Agomo *et al.*(2016) and Matondo *et al.*(2014).

High prevalence of *dhfr* and *dhps* mutant alleles recorded in this study especially with the presence of double and triple mutant alleles is an indication that there is widespread sulfadoxine/pyrimethamine resistance and this is likely due to continued use of sulfadoxine/pyrimethamine for intermittent preventive therapy in pregnancy (IPTp). Sulfadoxine/pyrimethamine is readily available in both public and the private sector making its restriction to only IPTp impossible. In the current situation it is unlikely that self-medication with sulfadoxine/pyrimethamine can be prevented especially due to its low cost compared to artemisinin-based combination therapy (ACT), which may also explain the reason for the observed high prevalence of SP resistance markers despite its replacement with ACT. Use of artesunate/sulfadoxine/pyrimethamine combination is also another selection factor for sulfadoxine/pyrimethamine resistance markers. The continued use of pyrimethamine has been based partly on the belief that the drug retains activity against the liver stages of *P.falciparum* (Nahlen *et al*, 1989) . Several studies have also shown that although implementation of SP-IPTp does not prevent malaria infection during pregnancy, especially in the presence of high prevalence of SP-resistance markers (Gutman *et al.*, 2013), there is a significant protection against severe outcomes of pregnancy in malaria, such as low birth weight, maternal and fetal outcomes (Eisle *et al.*,2012) . This is the reason behind WHO's continued recommendation of SP for IPTp (WHO-MPAC 2013).

It is concluded that the high prevalence of molecular marker of chloroquine resistance (*pf crt*76) is suggestive of persistent drug pressure and continuing non efficacy of chloroquine for malaria treatment in Nigeria. And that the high prevalence of *dhfr* and *dhps* gene mutations recorded in this study is an indication that an alternative drug would be necessary in the near future for successful

implementation of IPTp in Nigeria. Therefore we recommend an urgent re-evaluation of the malaria treatment policy in Nigeria, and ensure effective regulation of the manufacture, importation, sale and use of chloroquine if the purpose behind its withdrawal would be realized. Finally, Regular assessment of the effectiveness of sulfadoxine and pyrimethamine among children and pregnant women is desirable.

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