Journal of Advances in Biology & Biotechnology

20(3): 1-8, 2018; Article no.JABB.47648
ISSN: 2394-1081

Prevalence of Mutant Alleles Responsible for Chloroquine Resistance among *Plasmodium falciparum* Isolates in North Central, Nigeria

H. A. Edogun[1*], G. O. Daramola[1], A. O. Ojerinde[2], C. O. Esan[3], A. T. Adegbuyi[4], F. Adewumi[5], A. Egbebi[6], E. F. Akerele[1] and B. R. Oyedeji[6]

[1]Department of Medical Microbiology and Parasitology, Ekiti State University Teaching Hospital, Ado-Ekiti, Ekiti State, Nigeria.
[2]University Health Centre, Federal University, Oye-Ekiti, Ekiti State, Nigeria.
[3]University Health Centre, Ekiti State University, Ado-Ekiti, Ekiti State, Nigeria.
[4]Department of Pharmacology, Ekiti State University, Ado-Ekiti, Ekiti State, Nigeria.
[5]Department of Medical Microbiology and Parasitology, Ekiti State University, Ado-Ekiti, Ekiti State, Nigeria.
[6]Department of Medical Laboratory Science, Afe Babalola University, Ado-Ekiti, Ekiti State, Nigeria.

Authors' contributions

This work was carried out in collaboration among all authors. Author HAE designed the study and carried out the statistical analysis. Author GOD assisted with the manuscript. Authors AOO, COE, ATA, FA, AE, EFA and BRO gave support. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JABB/2018/v20i330078
Editor(s):
(1) Dr. Michael BamitaleOsho, Department of Biological Sciences, McPherson University, Nigeria, and Department of Microbiology, Faculty of Sciences, OlabisiOnabanjo University, Ago-Iwoye, Nigeria.
Reviewers:
(1) Mra Aye, Melaka Manipal Medical College, Melaka, Malaysia.
(2) Joshua Raji, Florida International University, USA.
Complete Peer review History: http://www.sdiarticle3.com/review-history/47648

Original Research Article

Received 22 November 2018
Accepted 27 February 2019
Published 14 March 2019

ABSTRACT

Background: Although chloroquine (CQ) has been officially replaced with artemisinin combination therapy (ACT) as first line drug for the treatment of malaria in Nigeria since 2005, a lot of people still believe that chloroquine is more effective chiefly because of the decline in the sensitivity of *Plasmodium falciparum* to ACT. Thus resulting into unofficial use of CQ for self medication. This study was conducted in order to survey the current status of chloroquine resistant strains of *pfcrt* and *pfmdr1* in view of possible re-introduction of chloroquine for malaria treatment.

**Corresponding author: E-mail: abik.edogun@gmail.com;*

Methods: DNA was extracted from one hundred (100) microscopically confirmed *Plasmodium falciparum* positive blood samples spotted on 3 mm Whatman filter paper. The detection of mutations in *Plasmodium falciparum* chloroquine resistance transporter (*Pfcrt*) and *Plasmodium falciparum* multidrug resistance (*Pfmdr1*) genes was performed by nested polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP).
Results: Results showed the presence of mutant alleles of *Pfcrt* and *Pfmdr1* in 60% and 41% of the samples respectively. However, there was no significant correlation in the prevalence of mutant alleles (T76/Y86) in relation to gender (p = 0.59/ 0.08) and age (p=0.59/0.93) of participants respectively.
Conclusion: The observed high prevalence of chloroquine resistance despite thirteen years of withdrawal calls for serious concern.

Keywords: Chloroquine; malaria; Plasmodium falciparum; mutation; mutant allele.

1. INTRODUCTION

Malaria poses a significant challenge to the health and well being of populations living in malaria-endemic regions. Although the number of cases and deaths due to malaria has declined globally, the disease is still responsible for about 200 million cases leading to 500,000 deaths annually [1]. The burden of malaria is heaviest on African nations (and particularly Nigeria) where 90% of global deaths due to it occur [2]. Efforts to effectively control malaria have in the recent decades been hampered by emergence of resistant strains of *Plasmodium*. The development and spread of resistance to *Plasmodium falciparum*, the most virulent *species* of malaria parasite is one of the greatest challenges to malaria control today [3].

CQ had historically played a key role in the management of the disease, being the mainstay of malaria therapy worldwide from 1940s up to the 1990s [4]. However, extensive use of CQ as a monotherapy for prophylaxis and chemotherapy led to the emergence of resistant strains of *Plasmodium falciparum* across many malaria-endemic countries prompting policy change which eventually brought about the withdrawal of CQ in favour of artemisinin combination therapy (ACT) in 2005 in Nigeria accordance to WHO recommendation. Nonetheless, CQ has remained available on the counter and is being dispensed without prescription [5].

Essential qualities of CQ which made it a drug of choice in the past decades include its low cost, low toxicity and high efficacy in clinical cases. Chloroquine acts by targeting the parasite haematin detoxification pathway in the digestive vacuole in a two-pronged attacks where it adsorbs onto growing hemozoin polymers and also binds to toxic haematin molecules generated as the parasite digests host haemoglobin resulting in accumulation of toxin haematin in the digestive vacuole [6].

Resistance to CQ is primarily mediated by the genetic replacement at codon 76 (K76T) of lysine with threonine in the *P. falciparum c*hloroquine resistance transporter gene (*pfcrt*) which increases efflux of CQ from the digestive vacuole of the parasite thereby rendering the drug ineffective [7]. Polymorphisms in the *Plasmodium falciparum* multidrug resistance gene-1 (*pfmdr*1) which encodes the P-glycoprotein homolog, modulates chloroquine resistance in mutant *pfcrt*-harbouring parasites in vitro and impact on the sensitivity multiple antimalarial drugs. Changes in pfmdr1 sequence alter transport of multiple drugs in and out of the parasite food vacuole with individual polymorphisms leading to opposite effects on different drugs. Mutations at pfmdr1 have been linked to decreased sensitivity to chloroquine and amodiaquine [8]. Drug efficacy studies are critical in guiding drug policy as monitoring and evaluation are also essential. CQ has been replaced with ACT since 2005 in Nigeria. This study was therefore conducted in order to assess the sensitivity of CQ given the time interval since its withdrawal, and also in the light of emerging resistance to the current drug of choice.

2. MATERIALS AND METHODS

2.1 Study Design

This is a cross-sectional descriptive study and a subsection of a larger study conducted between 2014 and 2016 aimed at characterizing the genetic diversity of *Plasmodium falciparum* in Niger State, North Central, Nigeria.

2.2 Study Area

The study was conducted at Swashi Clinic and Maternity, Niger State, Nigeria.

2.3 Ethical Clearance

Ethical clearance was obtained from ethical review committee of University of Ilorin Teaching Hospital while informed consent of all participants was obtained before sample collection.

2.4 Sample Collection

Blood samples of all patients aged 1 to >30 years with fever or history of fever 48 hours prior to presentation were collected on microscope slides and on 3 MM Whatman filter paper. Patients with microscopically confirmed *falciparum* parasitaemia were recruited into the study.

2.5 Laboratory Analysis

2.5.1 Microscopy

Thick and thin blood films on were stained with Giemsa stain and examined under compound microscope for the asexual stage of *P. falciparum*. Parasite density was determined by counting the number of asexual parasite against 200 leucocytes taking the total leucocytes count to be 8,000/ul of blood.

2.6 DNA Extraction

DNA was extracted from microscopically confirmed *P. falciparum* blood samples spotted on filter paper by using Chelex Extraction method earlier described [9].

PCR for detection of *Pfcrt* and *pfmdr*1 genes.

The detection of mutations responsible for chloroquine resistance was performed by amplifying sequences marking the *Plasmodium falciparum* chloroquine resistance transporter *(pfcrt)* and *Plasmodium falciparum* multidrug resistant 1 (*pfmdr*) genes using nested PCR followed by restriction fragment length polymorphism (RFLP) according to previously described procedures [10].

Primers used for *pfcrt* (K76T) and *pfmdr*1 (N86Y) primary amplifications included *Crtp*1, *Crtp*2, *Mdr*1 and *Mdr*2 and their sequences are as shown in Tables 1 and 2 respectively. The primary PCR components in a final volume of 15µl was buffer 10x, 25 mM $Mgcl_{2,}$ 50 mM deoxynucleotide triphosphate (dNTPs), 10 µm of each of the primer (Crtp1, Crtp2, Mdr1 and Mdr2), 5u/µl mM deoxynucleotide triphosphate (dNTPs), 10 µm of Taq polymerase and 2 µl of DNA samples. The cycling protocol for *pfcrt* was as follows: initial denaturation at 94°C for 3 minutes followed by 45 cycles of 94°C for 30 seconds, 56°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 PCR and RFLP for Pfcrt and Mdr1 mutation-specific detection Secondary PCR was conducted by using the forward primer Crtd1 and the reverse primer Crtd2 (Table 1) 2µl of 10x dilution of primary PCR was used in a follow-up, nested, allele specific PCR amplifications to detect the codon for pfcrt 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 amplification, 20 µl 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 transillumination.

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 (Table 2) after which restriction with *ApoI* 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

Table 1. PCR primer sequences for amplification of Codon 76 of *Pfcrt*

Primer Name	Sequence 5' - 3'
Pfcrt-P1 (Forward)	CCG TTA ATA ATA AAT ACA CGC AG
Pfcrt-P2 (Reverse)	CGG ATG TTA CAA AAC TAT AGT TAC C
Pfcrt-D1 nested1 (Forward)	TGT GCT CAT GTG TTT AAA CTT
Pfcrt-D2 nested 2 (Reverse)	CAA AAC TAT AGT TAC CAA TTT TG

Table 2. PCR primer sequences for amplification of Codon 86 of Pfmdr

Primer Name	Sequence 5' - 3'
*Mdr*1 (Forward)	GCG CGC GTT GAA CAA AA A GAG TAC CGC GTG
*Mdr*2 (Reverse)	GGG CCC TCG TAC CAA TTC CTG AAC T CAC
*Mdr*3 (Forward)	TTT ACC GTT TAA ATG TTT ACC TGC
*Mdr*4 (Reverse)	CCA TCT TGA TAA AAA ACA CTT CTT

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.

2.7 Data Analysis

Data were statistically analysed by using Chi-square and Fisher's exact test. Statistical significance was set at $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 Prevalence of molecular markers *P. falciparum* resistance to chloroquine

100 isolates of *Plasmodium falciparum* were typed with markers of chloroquine resistance (*pfcrt* and *pfmdr* 1) at codons K76T and N86Y. Whereas the prevalence of

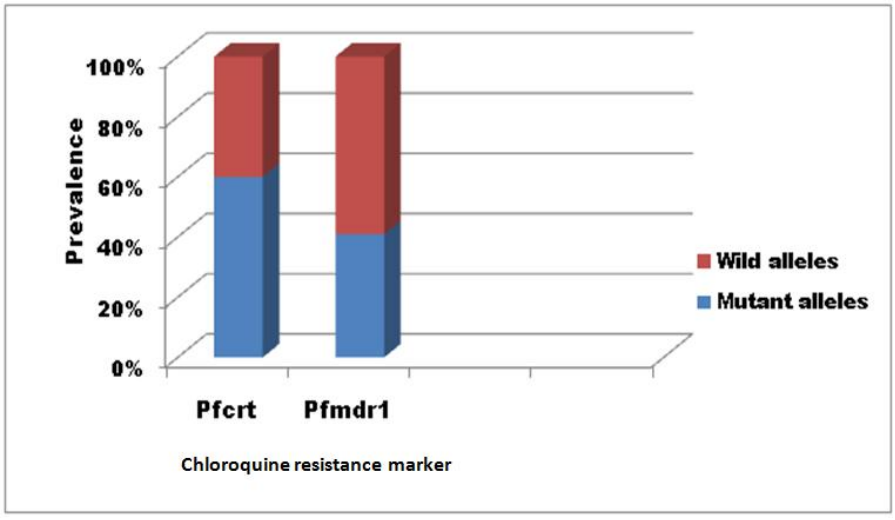

Fig. 1. Prevalence of molecular markers *P. falciparum* resistance to Chloroquine

wild (sensitive) alleles N86 of *pfmdr*1 (59.0%) was higher than the mutant alleles Y86 (41.0%), the prevalence of mutant alleles T76 (60.0%) of *pfcrt* was considerably higher than that of the wild type alleles K76.

3.1.2 Prevalence of drug-resistance marker by gender

Out of a total of 100 isolates that were successfully typed by drug resistance markers, 44 were males while 55 were females as shown in Table 3. Thus females were more represented and also recorded higher higher prevalences of mutant alleles at the two codons, however, this was not statistically significant (p>0.05).

3.1.3 Prevalence of molecular markers associated with *P. falciparum* resistance to Chloroquine by age

The age of the 100 participants for drug resistance typing ranges from 1 year to above 30 and were grouped into four: 1-10 years, 11-20, 21-30 and greater than 30. The age groups 1-10 and 11-20 were more represented being 62% and 24% respectively as shown in Table 4. The least representation was observed in the age groups 21-30 and > 30 being 9% and 5% respectively.

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).

3.2 Discussion

Chloroquine resistance has been associated *in vitro* with point mutations in two genes, *pfcrt* and *pfmdr1*, which encode the *P. falciparum* digestive-vacuole transmembrane proteins and P-glycoprotein-mediated multidrug resistance (*Pgh*1), respectively. Identification of *Pfcrt* as the central determinant of chloroquine-resistant *P. falciparum* malaria provides a molecular marker that can be used for surveillance of resistance and to evaluate drug treatment and prophylaxis policies.

Analysis of well-characterized molecular markers of *P. falciparum* resistance to the 4-amino-quinolines 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 *pfcrt* which encodes a transporter protein of the *P. falciparum* digestive vacuole was found in 60.0% of samples with *falciparum* malaria infection while and Y86 mutation (associate with resistance to amodiaquine, mefloquine, halofantrine and lumefantrine) that is the substitution of tyrosine (Y86) for asparagines (N86) at position 86 (N86Y) was found in 41.0%.

The high prevalence of resistance observed in this study indicates a pertinacity of resistance to chloroquine for more than a decade after the change of malaria treatment policy in Nigeria. This result is consistent with the findings from

Table 3. Prevalence of drug-resistance markers stratified by gender

Drug Resistance Marker	Males (44)	Females (56)	p-Value
T76	26 (59.1%)	34 (60.7%)	0.77
Y86	15 (34.1%)	26 (46.4%)	0.08

Table 4. Prevalence of molecular markers associated with *P. falciparum* resistance to chloroquine by age

		No(%) of mutant alleles	
Age Group	% Examined	T76	Y86
1-10	62	36 (58.1%)	25 (40.3%)
11-20	24	15 (62.5%)	10 (41.7%)
21-30	09	06 (66.7%)	04 (44.4%)
>30	05	03 (60.0%)	02 (40.0%)
Total	100	60 (60.0%)	41 (41.0%)
p-Value		0.582332	0.4531

other parts of Nigeria and most regions of high drug resistance [11-14]. The result is however in sharp contrast to findings from Malawi where *plasmodium* became susceptible to chloroquine after cessation of usage [15]. Also in Tanzania, chloroquine regained its sensitivity after two and half years of withdrawal of drug pressure [16].

CQ was abandoned for artemisinin combination therapy in 2005 due to widespread resistance and highly significant clinical failure across the country. It is expected that the susceptibility of 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 control policy in the country which permits the drug to be freely available for use outside government hospitals. Cross-resistance between CQ and Amodiaquine might also be a contributory factor due to the similarities in their modes of action. Presently, Amodiaquine is 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 [13].

The present study showed a predominance of wild type of *pfmdr1* N86 (59%), this could be considered as an indicator for low susceptibility of *P. falciparum* isolates to mefloquine, amodiaquine and quinine [17,18]. Mutations in *Pfmdr* 1 has also been associated with with decreased susceptibility to artemether and lumefantrine drugs separately [19-22]. However, mutations at *pfmdr*1 gene alone do not seem to be sufficient to explain *in vitro* resistance to antimalarial drugs.

This study did not discover any positive association in the prevalence of mutant alleles in relation to sex and age of the participants, although lower age had lower prevalence of mutant alleles T76 (58.1%) and Y86 (40.3%) this may not be unconn ected with acquired immunity of the adults. However, the differences were not statistically significant. This is consistent with findings of Atroosh et al. [23].

4. CONCLUSION

The high prevalence of molecular marker of chloroquine resistance recorded in this study is an indication of persistent drug pressure and steady inefficacy of CQ for malaria treatment in Nigeria. Thus, there is an urgent need to re-evaluate the malaria treatment policy in Nigeria, and ensure effective legislation against the manufacture, importation, sales and use of chloroquine if the purpose behind its withdrawal would be realized.

CONSENT

Written consent of all participants was obtained before sample collection.

ETHICAL APPROVAL

Ethical approval was obtained from ethical review committee of University of Ilorin Teaching Hospital.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. World Health Organisation (WHO). Malaria key facts; 2018.
Avalable:http://www.who.int/news-room/fact-sheets/detail/malaria
2. Onwujeke O. Why individual Nigerians carry the heaviest malaria cost burden in Africa; 2016.
Available:http://theconversation.com/why-individual-nigerians-carry-the-heaviest malaria-cost-burden-in-africa-57786
3. WHO. Malaria-responding to antimalarial drug resistance; 2018.
Avalable:http://www.who.int/malaria/areas/drug_resistance/overview/en/
4. Lambert HP. Malaria: Past and present history of treatment and prophylaxis; 2014.
Available:www.nobelprize.orgeducationalmedicine-malaria/readmore/treatment.html
5. Efunshile M, Runsewe-Abiodun T, Ghebremedhin B, Konig W, Konog B. Prevalence of molecular marker of chloroquine resistance (pfcrt 76) in Nigeria 5 years after withdrawal of drug as first-line antimalarial: A cross sectional study. S A Journal of Child Health. 2011;5(2).
6. Pacifici GM. Clinical pharmacology of the antimalarial chloroquine in children and their mothers. Int J. Pediatr. 2018;6(6): 7733-58.
DOI: 10.22038/IJP.2018.30912.2727

7. Awasthi G, Das A. Genetics of chloroquine-resistant malaria: A haplotypic view. Mem Inst Oswaldo Cruz. 2013;108(8):947-961.
8. Olasehinde GI, Ojurongbe OO, Fagade EO, Ruchi S, Egwari LO, Ajayi AA, Adeyeba OA. Detection of molecular markers of antimalarial drug resistance in *Plasmodium falciparum* from South-Western Nigeria. Covenant Journal of Physical and Life; 2014.
9. Sandor B, Andreas M, Pedro G, Anna Farnert. Short report: Rapid DNA extraction from archive blood spots on filter paper for genotyping of *Plasmodium falciparum*. American Journal of Tropical Medicine and Hygiene. 2005;72S(3):249-251.
10. W.H.O. Methods and techniques for clinical trials on antimalarial drug efficacy: Genotyping to identify parasite populations; 2008.
    Available:www.who.int./publications/2008
    (Accessed on 14/3/2014)
11. Olasehinde GI, Ojurongbe OO, Fagade EO, Ruchi S, Egwari LO, Ajayi AA, Adeyeba OA. Detection of molecular markers of antimalarial drug resistance in *Plasmodium falciparum* from South-Western Nigeria. Covenant Journal of Physical and Life Sciences (CJPL). 2014;1(2):1-15.
12. Olukosi YA, Oyebola MK, Ajibaye O, Orok BA, Aina OO, Agomo CO, Iwalokun BA, Akindele SK, Enya NV, Okoh HI. Persistence of markers of chloroquine resistance among *P. falciparum* isolates recovered from two Nigerian communities. Malaria World Journal. 2014;5(3):2214-4374.
13. Michael E, Tamramat RA, Beniam G, Wolgang K, Brigitte K. Prevalence of molecular marker of chloroquine resistance (*pfcrt* 76) in Nigeria 5 years after withdrawal of the drug as first-line antimalarial: A cross-sectional study. South-African Journal of Child Health. 2011;5(2):39-42.
14. Ojurongbe O, Fagbenro-Beyioku AF, Adeyeba OA, Kun JF. Allelic diversity of merozoite surface protein 2 gene of *Plasmodium falciparum* among children in Osogbo, Nigeria. West Indian Medical Journal. 2011;60(1):19-23.
15. Laufer MK, Takala-Harrison S, Dzinjalamala FK, Stine OC, Taylor TE, Plowe CV. Return of chloroquine-susceptible *falciparum* malaria in Malawi was a reexpansion of diverse susceptible parasites. Journal of Infectious Diseases. 2010;202(5):801-808.
    DOI: 10.1086/655659
16. Temu EA, Kimani I, Tuno N, Kawada H. Monitoring chloroquine resistance using *Plasmodium falciparum* parasites isolated from wild mosquitoes in Tanzania. American Journal of Tropical Medicine and Hygiene. 2006;75:1182-1187.
17. Rungsihirunrat K, Chaijareonkul W, Seugorn A, Na-Bangchang K, Thaithong S. Association between chloroquine resistance phenotypes and point mutations in *pfcrt* and *pfmdr*1 in *Plasmodium falciparum* isolates from Thailand. Acta Tropical. 2009;109:37–40.
18. Baliraine FN, Nsobya SL, Achan J, Tibenderana JK, Talisun AO, Greenhouse B, Rosenthal PJ. Limited ability of *Plasmodium falciparum pfcrt, pfmdr*1, and *pfnhe*1 polymorphisms to predict quinine *in vitro* sensitivity or clinical effectiveness in Uganda. Antimicrobial Agents and Chemotherapy. 2011;55:615–622.
19. Lekana-Douki JB, Dinzouna SD, Zatra R, Zang SE, Ekomy H, Bisvigou U, Toure-Ndouo FS. Increased prevalence of the *Plasmodium falciparum pfmdr*1 86 N genotype among field isolates from Franceville, Gabon after replacement of chloroquine by artemether-lumefantrine. Infect. Genet. Evol. 2011;11:512-517.
20. Mwai L, Kiara SM, Abdiranhman A, Pole L, Rippert A, Diriye A, Bull-Marsh PK, Borrmann S, Nzila A. *In-vitro* activities of piperaquine, lumenfantrine dihydroartemisinin in Kenyan *Plasmodium falciparum* isolates and polymorphisms in *pfcrt* and *pfmdr*1. Antimicrobial Agents Chemotherapy. 2009;53:5069-5073.
21. Ngo T, Duraisigh M, Reed M, Hipgrave D, Biggs B, Cowman AF. Analysis of *pfcrt, pfmdr1, dhfr* and *dhps* mutations and drug sensitities in *Plasmodium falciparum* isolates from patients in Vietnam before and after treatment with artemisinin. Am. J. Trop. Med. Hyg. 2003;68:350-356.
22. Nathalie W, Becaye F, Aurelie P, Mansour F, Eric B, Cheikhou C, Aminata N, Bakary D, Khadidiatou BF, Pape SM, Yaya D,

Raymond B, Boubacar W, Bruno P. Antimicrobial Agents Chemotherapy. 2014;58(12):7032-7040.

23. Atroosh WM, Al-Mekhlafi M, Mohammed AM, Johari S. The detection of *pfcrt* and *pfmdr*1 point mutations as molecular markers of chloroquine resistance in Pahang, Malaysia. Malaria Journal. 2012;11:251.
DOI: 10.1186/1475-2875-11-257

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sdiarticle3.com/review-history/47648