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Molecular surveillance of drug-resistant *Plasmodium falciparum* in two distinct geographical areas of Nigeria

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Analyse von Therapie-resistenten Malariaerregern in zwei Regionen Nigerias

Zusammenfassung. Die Entwicklung von therapie-resistenter *Plasmodium falciparum*-Malaria ist seit langem als Haupthindernis zur Bekämpfung von Mortalität und Morbidität erkannt. Wir haben daher die Verbreitung von Genveränderungen, die mit der Resistenz gegen Chloroquine und Pyrimethamin assoziiert sind, in *P. falciparum*-Isolaten aus zwei geographisch distinkten Gegenden in Nigeria untersucht. Mit Hilfe von RT-PCR und DNA-Sequenzierung wurde die Prävalenz dieser Mutationen bestimmt. Die Prävalenz der *pfcrt* T76-Mutation in den beiden Gegenden war 92.3 % gegen 86 % in Osogbo verglichen mit 93 % in Lafia ($P = 0.4453$). Sequenzanalyse des *pfcrt* Haplotyps (Aminosäuren 72–76) ergab CVIET als einzigen resistenten Haplotyp an beiden Orten. Die Häufigkeit von *pfmdr1*-Polymorphismen war höher in Lafia (39 %) als in Osogbo (35 %); die kombinierte Prävalenz in beiden Orten war 45.5 % ($P = 0.6604$). Die Prävalenz der *pfdhfr*-Triplemutante war hoch in beiden Gegenden: in Osogbo 84 % gegenüber 91 % in Lafia für I51, sowie 88 % gegen 87 % und 96 % gegen 96 % für die R59 and N108 Mutationen. Die kombinierte Prävalenz von *pfcrt*- und *pfmdr1*–Mutationen in Osogbo und Lafia war 44.2 % mit einem Risiko von 0.4164; die kombinierte Prävalenz aller Genveränderungen in *pfcrt*, *pfmdr1* and *pfdhfr* war 40.4 % mit einem Risiko von 1.081. Diese Ergebnisse zeigen die weite Verbreitung der Resistenz gegen Chloroquin und Pyrimethamin in beiden untersuchten Regionen.

Summary. Drug resistance against *P. falciparum* has been recognized as the crucial obstacle to curbing mortality and morbidity from malaria. We therefore determined the baseline distribution of *pfcrt* and *pfmdr1* genes associated with resistance to chloroquine and *pfdhfr* gene associated with resistance to pyrimethamine in *P. falciparum* isolates collected from two geographically distinct areas of Nigeria. We use RT-PCR assays and sequencing to determine the prevalence of these mutations. The combined prevalence of *pfcrt* T76 mutation in the two sites was 92.3% with 86% from Osogbo compared to 93% from Lafia. Sequencing analysis of the (*Pfcrt*) K76T haplotype (amino acids 72–76) revealed CVIET as the only resistance haplotype present in the two areas. The frequency of *pfmdr1* polymorphisms was higher in Lafia (39%) compared to that in Osogbo (35%) and the combined prevalence from the two sites was 45.5%. The prevalence of the *pfdhfr* triple mutant alleles was high in both locations. The Osogbo *vs* Lafia prevalence for *pfdhfr* mutations was 84% *vs* 91%, 88% *vs* 87% and 96% *vs* 96% for I51, R59 and N108, respectively. None of the samples from the two locations had the T108 mutation. The combined prevalence of *pfcrt* and *pfmdr1* in Osogbo and Lafia was 44.2% with a risk ratio of 0.4164 while the combined prevalence of *pfcrt, pfmdr1* and *pfdhfr* was 40.4% with a risk ratio of 1.081. These results strongly suggest the widespread distribution of CQ and pyrimethamine resistance without any marked distinction between the two locations.

Key words: Malaria, drug resistance, real time PCR, Nigeria.

Introduction

Plasmodium falciparum resistance to antimalarials has progressed at a steady rate during the last decades and malaria has continued to be the leading cause of morbidity and mortality, especially in children less than five years old in Sub-Saharan Africa [1]. In Nigeria due to the loss of efficacy of chloroquine (CQ), sulfadoxine-pyrimethamine (SP) was gradually introduced. However, like in most ma-

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laria endemic countries, artemisinin combination therapy (ACT) has been adopted as the first-line treatment against malaria [2]. The identification and monitoring of genes and mutations responsible for resistance to antimalarias are essential for the effective control of malaria.

Increased understanding of the molecular mechanisms of parasite resistance to drugs has led to a proliferation of field studies investigating the role of molecular markers in detecting drug resistance [3]. Polymorphisms in the *P. falciparum* chloroquine resistance transporter (*pfcrt*) confer resistance to chloroquine [4, 5], and mutations in the P-glycoprotein homologue (Pgh1) encoded by *P. falciparum* multi drug resistance 1 (*pfmdr1*) modulate this resistance [6]. In general, CQ-resistant isolates possess *pfcrt* alleles with multiple mutations corresponding to the amino acid haplotype CVIET (residues 72–76) or a SVMNT haplotype [7–9]. CQ-sensitive strains are characterized by the CVMNK specific set of *pfcrt* mutations [10]. Polymorphisms in *pfmdr1* and amplifications of this gene also affect susceptibility to structurally unrelated antimalarial drugs, including mefloquine, artesunate, lumefantrine and quinine [11, 12]. Polymorphisms in *P. falciparum* dihydrofolate reductase (*pfdhfr*) cause resistance to the antifolate drugs including pyrimethamine and other *pfdhfr* inhibitors, and polymorphisms in dihydropteroate synthase (*pfdhps*) cause resistance to sulphadoxine and its derivatives [13, 14].

The limitations of *in vivo* and *in vitro* methods for studying drug-resistant malaria and elucidating molecular mechanisms of resistance to some antimalarial drugs have stimulated the widespread use of molecular markers for resistance [15] and RT-PCR fluorescent dye-based genotyping technologies are now emerging as high-throughput genotyping platforms [16]. In this study, the analysis of the genetic mutations of *pfcrt*, *pfmdr1* and *pfdhfr* genes in clinical isolates from two geographical locations in Nigeria is described using RT-PCR Fret hybridization and dual labeled probes. The aim of this study was to provide a picture of the diversity of these resistant genes in Nigeria.

Methods

Study Site and recruitment of patients

Samples were collected between 2006 and 2007 in Ladoke Akintola University Teaching hospital, Osogbo Osun State and Lafia general hospital, Nasarawa State both in Nigeria. Osogbo is located in the western part of Nigeria and it is the state capital of Osun state Nigeria while Lafia is located in north-central Nigeria about 800 km from Osogbo. Both cities represent typical urban settings in Nigeria. In Osogbo malaria is present throughout the year with a marked increase during the raining season (i.e. April – September) [17]. In Lafia, malaria transmission has formerly been described as stable and uniformly intense through most of the year [18]. Informed consent was obtained from each patient or the parental guide. The study was approved by the ethical review committees of Ladoke Akintola University Teaching Hospital and Nasarawa State Ministry of Health.

Children with clinically diagnosed and laboratory confirmed uncomplicated *P. falciparum* malaria (fever ≥ 38.0°C, parasitaemia ≥ 2000/µl) were recruited for the study. From each patient a finger-prick blood sample was obtained for thick and thin blood films which were stained with 10% Giemsa. Two drops of blood were also blotted onto 3 MM Whatman filter paper. The Giemsa-stained blood films were examined microscopically for malarial parasites (per 200 white blood cells) and densities were assessed based on an assumed mean WBC count of 8000/µl. Genomic DNA of parasite was extracted from the filter paper sample using QIAamp DNA blood kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions.

Genotyping of pfcrt, pfmdr1 *and* pfdhfr *mutations*

The *pfcrt* 76 and *pfmdr1* 86 alleles were rapidly determined using hybridization probe method based on RT-PCR as previously described [16]. For the *pfdhfr* gene, the mutations at positions 51 and 59 were detected simultaneously in a quadruplex assay (N51I and C59R), while mutations at position 108 were detected in a triplex assay (N108, S108 and T108). A pair of Locked Nucleic Acid (LNA) probes labeled with a florescent dye at the 5′ position and BBQ quencher at the 3′ position was designed to be complimentary to either the wild or the mutant allele at each position. The probes and primers were designed and synthesized by TIB MOLBIOL DNA synthesis service, (Berlin, Germany) (accession number AF248537). For the positions 51 and 59, the forward and the reverse primer sequences were TGAGGTTTTTAATAAC TACACATTTAGAGGTCT and TATCATTTACATTATCCACAGTTTCTTTGTT, respectively. The probe for the wild-type allele at position 51 (FAM-AAATGTAATTCCCTAGATATG–BBQ) was labeled with FAM fluorescent dye while the probe for the mutant (JOE-AAATGTATTTCCCTAGATATG–BBQ) was labeled with JOE fluorescent dye. For the wild-type allele at position 59, the probe (ROX-ATATTTTTGTGCAGTTAC–BBQ) was labeled with ROX fluorescent dye while the mutant probe (Cy5-ATATTTTCGTGCAGTTAC–BBQ) was labeled with Cy5 fluorescent dye. For position 108, the forward and the reserve primer sequences were TGGATAATGTAAATGATATGCCTAATTCTAA and AATCTTCTTTTTTTAAGG TTCTAGACA ATATAACA, respectively. Two mutant LNA probes labeled with Cy5 (N108) and FAM (T108) (Cy5-AGAACAAACTGGGAAAG–BBQ and FAM-AGAACAACCTGGGAAA-BBQ) were designed to be complimentary for the two possible mutations while the wild-type allele (S108) (ROX-AGAACAACTGGGAAA–BBQ) was labeled with ROX fluorescent dye.

RT-PCR Amplification conditions

The amplification conditions for *pfcrt* and *pfmdr1* has been previously described [16]. For *pfdhfr* gene, RT-PCR amplification of positions 51 and 59 was carried out in duplicate in 50 µl final volume while for position 108 the final volume was 30 µl. The PCR mix contains 18 µl Multiplex PCR Master Mix (Qiagen, Hilden, Germany), 0.4 µM forward and reverse primers, both mutant and wild-type probes which were used at a final concentration of 0.2 µM and 5 µl of DNA. The PCR program started with an initial denaturing step at 95°C for 15 min and cycled 40 times, with each cycle consisting of 94°C for 20s and 60°C for 45s. Amplifications were performed with Rotor Gene 3000 (Corbbett, Sydney, Australia) and the probes and primers were designed and synthesized by TIB MOLBIOL DNA synthesis service (Berlin, Germany).

Determination of the pfcrt *haplotypes*

The *pfcrt* haplotype was determined by sequencing the PCR fragments that was amplified using the set of primers and conditions used for the RT-PCR. The resulting PCR products were subjected to gel electrophoresis on agarose gels and stained with SYBR green. All amplicons with the correct PCR band were purified using PeqGOLD Cycle pure kit (Classic-Line) (peQLab

Biotechnologie GmbH, Erlangen, Germany) according to manufacturer's instructions. The purified amplicons was re-amplified, purified with Sephadex TM G-50 and the DNA sequence was determined using an ABI PRISIM 3100 Genetic Analyser (Applied Biosystem). Sequence results were analyzed using the freely available BioEdit Sequence Alignment Editor software.

Statistical analysis

The risk ratio was used to compare the probability of the occurrence of mutant alleles in Osogbo and Lafia. Analysis was done with Graph-pad instat of Graphpad software Incorporation San Digeo, USA.

Results

A total of 156 (81 from Osogbo + 75 from Lafia) blood samples from *P. falciparum*-infected individuals collected from two geographical areas of Nigeria were examined for the presence of *pfcrt*, *pfmdr1* and *pfdhfr* mutations associated with malarial drug resistance. The patient's characteristics are shown in Table 1. The prevalence of *pfcrt* T76 and *pfmdr1* Y86 in the two locations was 92.3% and 45.5%, respectively. For *pfdhfr* gene, mutation at position 108 (N108) was the highest with 98.7% followed by positions 59 (R59) and 51 (I51) with 90.4% and 89.7%, respectively. The breakdown of the mutations according to the site showed that Lafia had a higher prevalence of both *pfcrt* T76 (93%) and *pfmdr1* Y86 (39%) mutations compared to Osogbo (*pfcrt* T76 (86%), *pfmdr1* Y86 (26%)) (Table 2). For *pfdhfr* mutation at position 108, both Osogbo and Lafia recorded a very high prevalence of 96% each. The prevalence of all the mutant alleles was comparably high in both locations.

Table 1. Characteristics of the patients

	Osogbo	Lafia
No. of patients	81	75
Mean Age (range) ± SD	42.1 (6–144) ± 34.27	32.6 (6-96) ± 17.64
Sex M:F	49:32	26:49
Temp oC ± SD	38.2 ± 3.075	37.5 ± 0.993
Median Parasite density (IQR)	9520 (13000)	12060 (49409)

Table 2. Prevalence of *pfcrt*, *pfmdr1* and *pfdhfr* genotypes in Osogbo and Lafia

Gene	Osogbo		Lafia		Osogbo + Lafia
	N = 81		*N* = 75		*N* = 156
	Mutant	Mixed	Mutant	Mixed	Mutant + Mixed
Pfcrt K76T	70 (86%)	3 (4%)	70 (93%)	1 (1%)	144 (92.3%)
pfmdr1 N86Y	28 (35%)	7 (9%)	29 (39%)	7 (9%)	71 (45.5%)
DHFR N51I	68 (84%)	2 (3%)	68 (91%)	2 (3%)	140 (89.7%)
DHFR C59R	71 (88%)	2 (3%)	65 (87%)	3 (4%)	141 (90.4%)
DHFR S108N	78 (96%)	2 (3%)	72 (96%)	2 (3%)	154 (98.7%)

The concurrent prevalence of *pfcrt* T76 + *pfmdr1* Y86 in Osogbo and Lafia was 44.2%. For *pfdhfr* mutations, R59 + N108 had the highest of 90.3% while the triple combination of the *pfdhfr* mutations was 86.5%. The concurrent prevalence of all the mutant alleles (*pfcrt* + *pfmdr1* + *pfdhfr*) in the two locations was 40.4% with a risk ratio of 1.081 (Table 3). The *pfcrt* CVIET haplotype pattern accounted for the vast majority of *P. falciparum* infections with 89% in Osogbo and 88% in Lafia while CVMNK wild-type haplotype pattern accounted for 7% and 4% in Osogbo and Lafia, respectively. No SVMNT haplotype was detected in the 2 locations.

Discussion

The data presented here together with our previous study on the association of *pfcrt* and *pfmdr1* on *in vivo* CQR in Osogbo Nigeria [16] might be used as a baseline for the

Table 3. Prevalence of *pfcrt*, *pfmdr1* and *pfdhfr* in isolates from Osogbo and Lafia Nigeria together and each separated

Genes combination	Osogbo + Lafia			Osogbo			Lafia			Risk Ratio
	N = 156			*N* = 81			*N* = 75			
alleles	MU (%)	WI (%)	MI (%)	MU (%)	WI (%)	MI (%)	MU (%)	WI (%)	MI (%)	
pfcrt T76 + pfmdr1 Y86	69 (44.2)	5 (3.2)	82 (52.5)	30 (37)	5 (6.2)	46 (56.8)	26 (34.6)	2 (2.7)	47 (62.7)	0.4164
pfdhfr I51 + R59	136 (87.2)	11 (70.5)	9 (5.8)	66 (81)	6 (7.4)	9 (11.1)	63 (84%)	5 (6.7)	7 (9.3)	0.8929
pfdhfr I51 + N108	140 (89.7)	1 (0.6)	15 (9.6)	71 (87.6)	1 (1.2)	9 (11.1)	66 (88%)	1 (1.3)	8 (10.7)	1.081
pfdhfr R59 + N108	141 (90.3)	2 (1.3)	13 (8.3)	68 (84)	1 (1.2)	12 (14.8)	63 (84%)	1 (1.3)	11 (14.7)	1.081
pfdhfr I51 + R59 + N108	135 (86.5)	2 (1.3)	19 (12.2)	66 (82)	1 (1.2)	14 (17.2)	60 (80)	1 (1.3)	14 (18.7)	1.081
Pfcrt T76 + *Pfmdr1* Y86 + *pfdhfr* I51 + R59 + N108	63 (40.4)	0 (0)	93 (59.6)	27 (33.3)	0 (0)	54 (66.6)	21 (28)	0 (0)	54 (72)	1.081

Alleles: Mu = Mutant; WI = Wild type; MI = Mixed
The risk ratio is the prevalence ratio of the combinations between Osogbo and Lafia.

surveillance of resistance markers after introduction of ACT. Arthemeter + Lumefantrin (Coartem) has been adopted as the first-line malarial treatment in Nigeria due to resistance to chloroquine and SP monotherapy [2]. Other ACT combinations like Artemisinin + Amodiaquine or SP are being widely used because they are cheaper compared to Coartem.

Pfmdr1 showed considerable polymorphism in these Nigerian isolates. Single nucleotide polymorphisms (SNPs) in the *pfmdr1* have been associated with altered *in vitro* and *in vivo* parasite response to arylaminoalcohols [19], including lumefantrine [20]. It has been previously reported that *in vivo* exposure selects for *pfmdr1* 86N in new inoculations (reinfections) post-treatment [21]. Due to this finding, it has been suggested that the selection of 86N may represent a marker of tolerance to lumefantrine [22] and *pfmdr1* copy numbers seem to influence susceptibility to lumefantrine [23], mefloquine and artemisinin [24, 25]. The precise role of *pfmdr1* polymorphisms (and copy numbers) is still relatively vague, pointing to the need for further intensive investigation. In this study, such an association could not be reported due to the limitations of the study design but continuing surveillance would be interesting concerning *pfmdr1* polymorphisms and its association to ACTs.

The occurrence of high triple *pfdhfr* mutations (51, 59 and 108) in our study site is similar to findings from other sites in Nigeria and other African countries where antifolate drugs have been used intensively owing to high level of resistance to CQ [26–28]. The prevalence of the triple-mutant alleles in our study area makes it clear that SP-driven selection for mutant alleles of *pfdhfr* is certainly in progress, although the predictive value of these markers for SP treatment failure has not been established in the study areas.

High frequencies of the *pfcrt* mutation were observed in the two locations surveyed and high frequencies have been described in other African countries such as Cameroon [29], Gabon [30], Democratic Republic of Congo [31] and Nigeria [16, 32] where CQ has been widely used. The *pfcrt* haplotypes (amino acids 72–76) CVIET known to be associated with CQ resistance is the most predominant haplotype observed followed by the susceptible type CVMNK. One important result of this study is that the South American and PNG *pfcrt*-haplotype SVMNT recently seen in some African countries were not found in our populations. There are also interesting reports of changing patterns in haplotype prevalence when followed for years in geographical areas [33, 34]. It will be interesting to see what the prevalence pattern in this study area will look like in the next 2 or 3 years. Continuous surveillance of drug resistance is therefore necessary in these areas for timely adjustment and enforcement of local drug policies for more effective malarial control.

The result of this study clearly shows a widespread distribution of *P. falciparum* drug-resistant alleles in Nigeria. Regional drug pressure, as well as use of SP and CQ probably partly explains the high prevalence of the mutant alleles observed in this study. At present the current National Malaria Treatment Guideline and Policy recommends SP as first-line agent for intermittent preventive treatment (IPTp) and quinine for treatment of clinical malaria in all trimesters of pregnancy and Artemisinin-based combination therapy (ACT) is considered safe second-line agents in the second and third trimesters [35]. SP drug pressure in Nigeria may also have been further enhanced by the use of other antifolate compounds, such as the combination antibiotic cotrimoxazole, which have cross-resistance with SP. Cotrimoxazole is commonly prescribed as prophylaxis against opportunistic infections to HIV patients [36], and may have contributed to high triple dhfr mutation in malarial parasites in Nigeria. While chloroquine is no longer part of the treatment guideline for malaria in Nigeria, the drug is still readily available and it's still been purchased on a large scale in the private market.

In conclusion, the overall finding of this study showed that mutant alleles of *pfcrt*, *pfmdr1* and *pfdhfr* are widespread in these different regions of the country. These molecular makers and the use of RT-PCR methods are valuable for describing the epidemiology of drug-resistant *P. falciparum* which we hope will help the policy makers in the choice of drug combination in these areas and in the country as a whole. Also the two RT-PCR methods used in this study were demonstrated to be rapid, sensitive, and specific for the detection of the genetic markers.

Author's contribution

OO performed the sample collection and the molecular analysis, and drafted the manuscript. SIO performed sample collection. WAO participated in the design and supervision of sample collection. AFF-B supervised the design of the study. JFJK: supervised the molecular work and the interpretation of the data and helped to draft the manuscript. All authors read and agreed to the content of the manuscript.

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Conflicts of interest
None declared.

Ethical approval
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Reference

1. WHO (2005) World Malaria Report. Geneva.
2. FMOH (2005) Federal Republic of Nigeria National antimalarial treatment policy. Abuja: Federal Ministry of Health.

3. Talisuna AO, Bloland P, D'Alessandro U. History, dynamics, and public health importance of malaria parasite resistance. Clin Microbiol Rev 2004;17:235–54.
4. Fidock DA, Nomura T, Talley AK, Cooper RA, Dzekunov SM, et al. Mutations in the P. falciparum digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance. Mol Cell 2000;6:861–71.
5. Sidhu AB, Verdier-Pinard D, Fidock DA. Chloroquine resistance in Plasmodium falciparum malaria parasites conferred by pfcrt mutations. Science 2002;298:210–3.
6. Reed MB, Saliba KJ, Caruana SR, Kirk K, Cowman AF. Pgh1 modulates sensitivity and resistance to multiple antimalarials in Plasmodium falciparum. Nature 2000;403:906–9.
7. Chen N, Kyle DE, Pasay C, Fowler EV, Baker J, et al. pfcrt Allelic types with two novel amino acid mutations in chloroquine-resistant Plasmodium falciparum isolates from the Philippines. Antimicrob Agents Chemother 2003;47:3500–05.
8. Durrand V, Berry A, Sem R, Glaziou P, Beaudou J, et al. Variations in the sequence and expression of the Plasmodium falciparum chloroquine resistance transporter (Pfcrt) and their relationship to chloroquine resistance in vitro. Mol Biochem Parasitol 2004;136:273–85.
9. Mehlotra RK, Fujioka H, Roepe PD, Janneh O, Ursos LM, et al. Evolution of a unique Plasmodium falciparum chloroquine-resistance phenotype in association with pfcrt polymorphism in Papua New Guinea and South America. Proc Natl Acad Sci USA 2001;98:12689–94.
10. Keen J, Farcas GA, Zhong K, Yohanna S, Dunne MW, et al. Real-time PCR assay for rapid detection and analysis of PfCRT haplotypes of chloroquine-resistant Plasmodium falciparum isolates from India. J Clin Microbiol 2007;45:2889–93.
11. Price RN, Cassar C, Brockman A, Duraisingh M, van Vugt M, et al. The pfmdr1 gene is associated with a multidrug-resistant phenotype in Plasmodium falciparum from the western border of Thailand. Antimicrob Agents Chemother 1999;43:2943–49.
12. Duraisingh MT, Jones P, Sambou I, von Seidlein L, Pinder M, et al. The tyrosine-86 allele of the pfmdr1 gene of Plasmodium falciparum is associated with increased sensitivity to the anti-malarials mefloquine and artemisinin. Mol Biochem Parasitol 2000;108:13–23.
13. Plowe CV, Roper C, Barnwell JW, Happi CT, Joshi HH, et al. World Antimalarial Resistance Network (WARN) III: molecular markers for drug resistant malaria. Malar J 2007;6:121–30
14. Brooks DR, Wang P, Read M, Watkins WM, Sims PF, et al. Sequence variation of the hydroxymethyldihydropterin pyrophosphokinase: dihydropteroate synthase gene in lines of the human malaria parasite, Plasmodium falciparum, with differing resistance to sulfadoxine. Eur J Biochem 1994;224:397–405.
15. Basco LK. Molecular epidemiology of malaria in Cameroon. XII. In vitro drug assays and molecular surveillance of chloroquine and proguanil resistance. Am J Trop Med Hyg 2002;67:383–7.
16. Ojurongbe O, Ogungbamigbe TO, Fagbenro-Beyioku AF, Fendel R, Kremsner PG, et al. Rapid detection of Pfcrt and Pfmdr1 mutations in Plasmodium falciparum isolates by FRET and in vivo response to chloroquine among children from Osogbo, Nigeria. Malar J 2007;6:41–8.
17. Ogungbamigbe TO, Ojurongbe O, Ogunro PS, Okanlawon BM, Kolawole SO Chloroquine resistant Plasmodium falciparum malaria in Osogbo Nigeria: efficacy of amodiaquine + sulfadoxine-pyrimethamine and chloroquine + chlorpheniramine for treatment. Mem Inst Oswaldo Cruz 2008;103:79–84.
18. Bruce-Chwatt LJ. Malaria in Nigeria. Bull World Health Organ 1951;4:301–27.
19. Duraisingh MT, Cowman AF. Contribution of the pfmdr1 gene to antimalarial drug-resistance. Acta Trop 2005;94:181–90.
20. Duraisingh MT, Roper C, Walliker D, Warhurst DC. Increased sensitivity to the antimalarials mefloquine and artemisinin is conferred by mutations in the pfmdr1 gene of Plasmodium falciparum. Mol Microbiol 2000;36:955–61.
21. Sisowath C, Stromberg J, Martensson A, Msellem M, Obondo C, et al. In vivo selection of Plasmodium falciparum pfmdr1 86N coding alleles by artemether-lumefantrine (Coartem). J Infect Dis 2005;191:1014–7.
22. Hastings I, Ward S. Coartem (artemether-lumefantrine) in Africa: the beginning of the end? J Infect Dis 2005;192:1290–3.
23. Price RN, Uhlemann AC, van Vugt M, Brockman A, Hutagalung R, et al. Molecular and pharmacological determinants of the therapeutic response to artemether-lumefantrine in multidrug-resistant Plasmodium falciparum malaria. Clin Infect Dis 2006;42:1570–7.
24. Uhlemann AC, McGready R, Ashley EA, Brockman A, Singhasivanon P, et al. Intrahost selection of Plasmodium falciparum pfmdr1 alleles after antimalarial treatment on the northwestern border of Thailand. J Infect Dis 2007;195:134–41.
25. Alker A, Lim P, Sem R, Shah N, Yi P, et al. Pfmdr1 and in vivo resistance to artesunate-mefloquine in falciparum malaria on the Cambodian-Thai border. Am J Trop Med Hyg 2007;76:641–7.
26. Falade CO, Salako LA, Sowunmi A, Oduola AM, Larcier P. Comparative efficacy of halofantrine, chloroquine and sulfadoxine-pyrimethamine for treatment of acute uncomplicated falciparum malaria in Nigerian children. Trans R Soc Trop Med Hyg 1997;91:58–62.
27. Happi CT, Gbotosho GO, Folarin OA, Akinboye DO, Yusuf BO, et al. Polymorphisms in Plasmodium falciparum dhfr and dhps genes and age related in vivo sulfadoxine-pyrimethamine resistance in malaria-infected patients from Nigeria. Acta Trop 2005;95:183–93.
28. Bates SJ, Winstanley PA, Watkins WM, Alloueche A, Bwika J, et al. Rare, highly pyrimethamine-resistant alleles of the Plasmodium falciparum dihydrofolate reductase gene from 5 African sites. J Infect Dis 2004;190:1783–92.
29. Basco LK. Molecular epidemiology of malaria in Cameroon. XIII. Analysis of pfcrt mutations and in vitro chloroquine resistance. Am J Trop Med Hyg 2002;67:388–91.
30. Binder RK, Borrmann S, Adegnika AA, Missinou MA, Kremsner PG, et al. Polymorphisms in the parasite genes for pfcrt and pfmdr-1 as molecular markers for chloroquine resistance in Plasmodium falciparum in Lambarene, Gabon. Parasitol Res 2002;88:475–6.
31. Wilson PE, Kazadi W, Kamwendo DD, Mwapasa V, Purfield A, et al. Prevalence of pfcrt mutations in Congolese and Malawian Plasmodium falciparum isolates as determined by a new Taqman assay. Acta Trop 2005;93:97–106.
32. Happi CT, Gbotosho GO, Folarin OA, Bolaji OM, Sowunmi A, et al. Association between mutations in Plasmodium falciparum chloroquine resistance transporter and P. falciparum multidrug resistance 1 genes and in vivo amodiaquine resistance in P. falciparum malaria-infected children in Nigeria. Am J Trop Med Hyg 2006;75:155–61.
33. Alifrangis M, Dalgaard MB, Lusingu JP, Vestergaard LS, Staalsoe T, et al. Occurrence of the Southeast Asian/South American SVMNT haplotype of the chloroquine-resistance transporter gene in Plasmodium falciparum in Tanzania. J Infect Dis 2006;193:1738–41.
34. Mittra P, Vinayak S, Chandawat H, Das MK, Singh N, et al. Progressive increase in point mutations associated with chloroquine resistance in Plasmodium falciparum isolates from India. J Infect Dis 2006;193:1304–12.
35. FMOH (2005) National guidelines and strategies for malaria prevention and control during pregnancy. A publication of the Federal Ministry of Health, Nigeria; Malaria Control Programme Abuja: FMOH.
36. White NJ. Antimalarial drug resistance. J Clin Invest 2004;113:1084–92.