Molecular surveillance of chloroquine drug resistance markers (*Pfcr*t and *Pfmdr*1) among imported *Plasmodium falciparum* malaria in Qatar

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ABSTRACT

Imported malaria has been a great challenge for public health in Qatar due to influx of large number of migrant workers. Antimalarial drug resistance has emerged as one of the greatest challenges facing malaria control today. Monitoring parasite haplotypes that predict susceptibility to major antimalarial can guide treatment policies. This study aimed to determine molecular drug resistance pattern in imported malaria cases in Qatar. Blood samples from the uncomplicated *P. falciparum* malaria patients were collected at Hamad General Hospital, HMC, Doha, Qatar. The samples were further confirmed by nested-polymerase chain reaction (PCR) for *P. falciparum*. Molecular markers of chloroquine (*Pfcr*t and *Pfmdr*1) were analyzed by using nested PCR- RFLP method to determine the key point mutations associated with chloroquine (CQ) drug resistance. A total 118 blood samples were positive for *P. falciparum*. Overall, by RFLP, 72% harboured wild type allele (N86) of *Pfmdr*1 gene. The prevalence of *Pfcr*t mutant (T76), WT (K76) and mixed alleles (K76T) was 63.6% ($n = 75$), 22.9% ($n = 27$) and 13.5% ($n = 16$), respectively. Mean parasitaemia level was higher among the wild type alleles of *Pfcr*t gene as compared to the mixed/mutant alleles whereas mixed alleles of *Pfmdr*1 gene having high parasitaemia. Molecular surveillance strategy based on imported malaria cases can be used to detect and track CQ drug-resistant malaria. The data presented here might be helpful for enrichment of molecular surveillance of antimalarial resistance and will be useful for developing and updating antimalarial guidance for non-immune imported cases in Qatar.

KEYWORDS

Plasmodium falciparum; chloroquine; molecular drug resistance; imported malaria; Qatar

Introduction

Antimalarial drug resistance has emerged as one of the greatest challenges facing malaria control today. It has been implicated in the spread of malaria to new areas and in re-emergence of malaria in areas where malaria had previously been eradicated.¹ In the last decades, due to its effectiveness and reasonable cost, chloroquine has represented the best and more widely used drug in the treatment of uncomplicated malaria. Unfortunately, within a decade of its introduction, *Plasmodium falciparum* parasites were observed chloroquine (CQ) resistant in malaria-endemic countries [1–3] and now it is a hurdle for malaria control [4,5]. Currently, artemisinin combination therapies (ACTs) has shown high efficacy and recommended in all malaria-endemic countries [1]. The current antimalarial treatment policy in Qatar has also been changed from CQ to ACTs. However,

administration of CQ and quinine plus doxycycline (adults)/or clindamycin (children) is recommended for uncomplicated and complicated/severe *P. falciparum* malaria, respectively. In addition, mixed infections are usually treated as *P. falciparum* malaria.

Based on reports from the different geographical areas of the world, CQ resistance in *P. falciparum* is strongly associated with genetic polymorphisms in *P. falciparum* CQ resistance-transporter (*Pfcr*t) T76 mutation and multidrug resistance 1 (*Pfmdr*1) Y86 mutation, which remain the most important single nucleotide polymorphisms (SNPs) [6].

Indigenous malaria transmission has been eliminated from Qatar but, the risk of imported malaria still exists due to the large number of immigrant workers and expatriates originating from the Indian subcontinent and Africa [7,8] Additionally, receptivity of the country

to a reintroduction of malaria exists because of the presence of two potential vectors of malaria, *Anopheles stephensi* and *An. multicolor* [9]. The proportion of all imported malaria, mostly due to *P. falciparum*, in travelers and relatives in Qatar has increased in recent years [8,10,11]. This increase in cases could pose a serious public health problem in Qatar as: the populations is 'naïve' and if infection is transmitted to this native population serious life-threatening infection can occur; spread to native population could trigger epidemics. In addition, human migration can also interact with natural features to establish drug resistance in non-endemic areas [12].

Monitoring drug resistance is essential for early detection and subsequent prevention of the spread of drug resistance by timely changes of treatment policy and control programs. Therefore, the present study was conducted to determine the mutation of CQ (*Pfcr*-codon76, *Pfmdr1*-codon86) drug resistance genes in imported *P. falciparum* malaria cases in Qatar. The data from this study could enrich the molecular surveillance of antimalarial resistance and will be useful for developing national strategy for non-immune imported cases in Qatar.

Materials and methods

Study area, sample collection and ethical statement

The study was conducted in Doha, Qatar (geographical coordinates 25.3548° N and 51.1839° E). During January 2013–September 2016, after obtaining a written informed consent, symptomatic patients were screened for malaria parasites and included in this study. The present study was approved (Protocol No. 14-00097) by the Institutional Review Board of HMC and Weill Cornell Medicine-Qatar.

Intravenous blood samples, in sterile vacutainer, were collected from the patients attending the Hamad General Hospital, HMC, Qatar. The inclusion criteria included signs and symptoms of acute uncomplicated malaria (according to WHO), patients with *P. falciparum* asexual parasite without severe anemia [1]. Pregnant women, patients with signs and symptoms of severe

and complicated malaria (as defined by WHO) [1], and patients with severe anemia or if they have second major illness, were excluded from this study. All the samples were first screened for malaria parasites by microscopy using Giemsa staining and confirmed by nested polymerase chain reaction (PCR). All subjects were interviewed using a structured questionnaire to collect socio-demographics and risk factors such as travel history, blood transfusions. Other data from medical records include age, nationality, clinical history, parasitaemia and hemoglobin were collected from each subject. Patients were given treatment as per the Ministry of Public Health Qatar.

Preparation of DNA extraction and diagnosis by PCR

Genomic DNA was extracted by QIAamp DNA blood mini kit from whole blood according to the manufacturer's instructions (Qiagen, CA, USA), and stored at –80 °C for further assay. Genus and species specific nested PCR was performed using 18S rRNA as the target gene to detect *P. falciparum* infection [13].

Detection of CQ *Pfcr* and *Pfmdr1* polymorphisms by nested PCR-RFLP genotyping

The polymorphic region of selected target genes (*Pfcr*-codon76 and *Pfmdr1*-codon86) were carried out as described earlier [6]. Briefly, genomic DNA was amplified by nested PCR with primers flanking codon 86 of *pfmdr1* and codon 76 of *pfcr*, which was followed by Restriction Fragment Length Polymorphism (RFLP) (Table 1) [14]. All the samples were analyzed in duplicates. A negative control (nuclease free water) and DNA of resistant (RKL9) and sensitive (3D7 and MRC2) strains of *P. falciparum*, kind gift from Dr. R Sehgal, Chandigarh, were included as positive controls in each amplification reaction.

Statistical analysis

Statistical analyses were performed using IBM SPSS version 22.0. The distribution of *Pfmdr1* and *Pfcr* genotypes

Table 1. The demographic and clinical profile of patients' population in this study according to nationality in Qatar.

Nationality (No.)	Age		Gender		Parasitemia Median (95% CI)	Hemoglobin (g/dl; mean $\pm$ SD)
	<18	>18	Male	Female		
East Africa [§] * (71)	2	69	58	13	0.8 (0.3–2.8)	13.3 $\pm$ 2.3
Indian subcontinent [‡] (24)	2	22	22	2	0.7 (0.4–1.4)	12.2 $\pm$ 1.5
West and Central Africa ^{***} (21)	–	21	20	1	1.7 (0.2–2.9)	12.8 $\pm$ 2.6
GCC [@] (2)	–	2	2	–	0.3 (0.1–0.5)	10.9 $\pm$ 2.8
Total	4	114	102	16	0.8 (0.3–2.7)	13.0 $\pm$ 2.3

*Two patients (one of each from Spain and Romania) had a travel history to Tanzania; **Two patients (one of each from USA and Syria) had a travel history to Nigeria and Cameroon, respectively; [§]East Africa: Sudan ($n = 36$), Kenya ($n = 16$), Eritrea ($n = 10$), Ethiopia ($n = 3$), Rwanda ($n = 2$), Djibouti ($n = 1$) and Tanzania ($n = 1$).

[‡]Indian subcontinent: India (18), Pakistan ($n = 4$), Nepal ($n = 1$) and Sri Lanka ($n = 1$).

^{***}West & Central African countries: Nigeria ($n = 11$), Ghana ($n = 3$), Cameroon ($n = 2$), Democratic Republic of Congo ($n = 1$), Republic of Côte d'Ivoire ($n = 1$) and Chad ($n = 1$).

[@]Gulf Cooperation Council (GCC): one of each patient from Qatar and Saudi Arabia had a travel history to Oman.

among different nationalities was summarized using frequency distribution. Frequencies of mutations and genotypes among groups were compared using the *t*-test. The *P* values < 0.05 were considered statistically significant. The association of genotypes (wild, mutant and mixed) with parasitemia was presented in Table 3, and median parasitemia between two genotypes were compared using Mann Whitney test (*Z*) of wild, mutants and mixed alleles according to mean parasite density was assessed using the *t*-test (Table 3).

Results

Demographic profile

During the study period, a total of 583 patients with malaria symptoms were screened, 76.8% (*n* = 448) were found to be positive for a malaria parasite *P. falciparum* (*n* = 118), *P. vivax* (*n* = 318) or a mixed *P. falciparum* and *P. vivax* (*n* = 12). Of 118 microscopically and PCR positive mono-infected *P. falciparum* patients, who fulfilled the enrolment criteria were included in the study. All 118 (102 males, 16 females) uncomplicated *P. falciparum* malaria cases were analyzed for the study. Clinical and parasitological characteristics of the study subjects are depicted in Table 1. Median age of study participants was 33.0 years (95% CI 30.0–35.0) based on 1000 bootstrap samples. The majority (60.2%, *n* = 71) of patients were from East Africa followed by Indian Sub-continent (20.3%, *n* = 24), West & Central Africa (17.8%, *n* = 21) and GCC (1.7%, *n* = 2) (Table 1).

Pfcr and Pfmdr1 genotypes

All samples analyzed by PCR-RFLP were amplified successfully for *Pfcr* & *Pfmdr1* genes. Out of 118 samples genotyped at codon 86 of the *Pfmdr1* gene, 72% (*n* = 85) carried the wild type (WT) allele (N86) while 19.5% (*n* = 23) were mutants (Y86) and only 8.5% (*n* = 10) had mixed alleles (N86Y). However, the prevalence of *Pfcr*

mutant (T76), WT (K76) and mixed alleles was 63.6% (*n* = 75), 22.9% (*n* = 27) and 13.5% (*n* = 16), respectively (Table 2).

Further analysis among different nationality revealed that WT allele of *Pfmdr1* were higher than mutant/mixed alleles (*Z* = 6.9134, *p* < 0.001), while in *pfcrt* gene more than 75% patients had mixed/mutant alleles than WT allele (*Z* = 8.2956, *p* < 0.001). Overall, only 15.2% (*n* = 18) patients had WT allele for the both genes. Additionally, 8.5% (*n* = 10) had mixed alleles for *Pfmdr1* gene and 13.5% (*n* = 16) *Pfcr* gene and most of the samples were from patients who originally come from Africa (East, West & Central Africa) (Data not shown). Mean parasitaemia level was higher among the wild type alleles of *Pfcr* gene as compared to the mixed/mutant alleles, whereas mixed alleles of *Pfmdr1* gene having high level of parasitaemia (Table 3).

It is noteworthy that there was no statistically significant association between the distribution of *Pfcr* and *Pfmdr1* genotypes with age, gender, hemoglobin level and mean parasitaemia.

Discussion

The present study was conducted during the 2013–2016 to determine the chloroquine resistance related genotype *Pfcr* at amino acid 76 and *Pfmdr1* at codon 86 using PCR-RFLP method among the migrant population and also in native Qatar population visiting malaria endemic region to stall the potential spread of *P. falciparum* in the state of Qatar.

In the present study, a total of 448 patients with malaria parasite were positive (*P. falciparum* (26%), *P. vivax* (71%) or a mixed *P. falciparum* and *P. vivax* (3%). Majority of patients infected with *P. falciparum* (71%, *n* = 92) were from Africa followed by Indian Sub-continent (20.3%, *n* = 24) and GCC (1.7%, *n* = 2). However, majority of *P. vivax* malaria (83.3%, *n* = 265) samples

Table 2. The distribution of *Pfmdr1*, *Pfcr* genotype frequencies in different nationalities in Qatar.

Nationality (<i>n</i>)	<i>pfmdr1</i>			<i>Pfcr</i>		
	Wild <i>n</i> (%)	Mutant <i>n</i> (%)	Mixed <i>n</i> (%)	Wild <i>n</i> (%)	Mutant <i>n</i> (%)	Mixed <i>n</i> (%)
East Africa (71)	44 (62)	18 (25.3)	9 (12.7)	17 (23.9)	45 (63.4)	9 (12.7)
Indian subcontinent (24)	21 (87.5)	3 (12.5)	0	5 (20.8)	16 (66.6)	3 (12.5)
West and Central Africa (21)	18 (85.7)	2 (9.5)	1 (4.8)	5 (23.8)	12 (57.1)	4 (19)
GCC (2)	2 (100)	0	0	0	2 (100)	0
Total (118)	85 (72)	23 (19.5)	10 (8.5)	27 (22.9)	75 (63.6)	16 (13.5)

Table 3. Relationship between mean parasite density and wild/mutant/mixed alleles of *Pfcr* and *Pfmdr1* genes.

Genes	Genotypes	Total no. patients	Parasitemia median (95% CI)*	Mann Whitney Test (<i>Z</i>)	<i>p</i> value
<i>Pfcr</i>	Wild	27	2.35 (0.83–4.3)	–	–
	Mutant	75	0.65 (0.45–1.0)	2.760	0.006
	Mixed	16	0.80 (0.40–2.3)	1.569	0.117
<i>Pfmdr1</i>	Wild	85	0.80 (0.63–1.43)	–	–
	Mutant	23	0.64 (0.28–1.13)	1.285	0.199
	Mixed	10	1.35 (0.28–6.32)	0.547	0.585

*Median and its 95% CI are based on 1000 bootstrap samples.

were from patient's origin from the Indian subcontinent. Although India have only 33% *P. vivax* cases but majority of imported cases were coming from the area where *P. vivax* is dominant. In addition, the rest were belonging to Africa (16.7%, $n = 53$). Overall, the ratio of *P. vivax* and *P. falciparum* malaria cases were 73:27, which is in agreement and reflects the precise situation of *P. vivax/P. falciparum* proportion in their origin countries [1].

Resistance to CQ has been associated with N86Y mutation of the *Pfmdr1* gene and mutations exert an added effect on the CQ response in the presence of the K76T mutation in the *Pfcr*t gene [15]. In this study, we report a high level of frequency of drug resistance molecular markers for CQ *Pfcr*t T76 (77.1%) and a low level of *Pfmdr1* Y86 (28%). This finding is in agreement with the other studies carried out in the malaria endemic countries, confirming that *Pfcr*t is a useful marker for the detection and surveillance of chloroquine resistance in *P. falciparum* [15].

Although the CQ is already discontinued worldwide for the treatment of *P. falciparum*, hence, this finding is most likely explained by the fixation of *Pfcr*t T76 in the *P. falciparum* population with moderate prevalence of the *Pfmdr1* Y86 mutation. It has been also suggested that *Pfmdr1* mutations may also play a role in modulating the level of resistance to not only CQ but also other antimalarial drugs [16]. In addition, Kublin et al. [17] also suggested that withdraw of CQ can lead to decreased the prevalence of the *Pfmdr1* 86Y mutation.

The *Pfcr*t T76 along with *Pfmdr1* Y86 mutations are very useful molecular markers of CQ resistance in areas where resistance rates are low to mild [6,18]. In the present study, prevalence of *Pfcr*t K76T mutation from different endemic country was 77.1% showing a widespread distribution of mutant allele as the sample used in this study were from several countries. Although the numbers of samples studied were relatively small, our results showed high to moderate level of *Pfcr*t and *Pfmdr1* mutations respectively, which is consistent with previous reports from Ethiopia [19,20]. In addition, Kebede et al. [20], correlated the mutant genotypes with the low CQ efficacy in Ethiopia, however, this was not further evaluated because of the limitations of the present study. Several studies from India and Pakistan have demonstrated the high level of mutant *Pfcr*t allele and complete saturation of *Pfcr*t K76T indicates that CQ resistant *P. falciparum* is almost fixed particularly in the Indian subcontinent patients [21,22]. Although, in the Indian subcontinent, CQ is recommended only for *P. vivax* infection but *P. falciparum* infections are also often exposed to this treatment as well. Self-medication with counterfeit medicines could be a leading cause for the maintaining this high prevalence of T76 mutant parasites as a result of continued use of CQ [23].

Overall, 23% samples showed the wild type genotype for *Pfcr*t genes and this prevalence was almost

equal from different continent [Indian (21%) and Africa (24%). Although, mutant genotype in the present study was high however, when it was compared to the studies carried out during year 2000, the mutant genotype was 100% [24]. Similar reductions in the prevalence of the *Pfcr*t 76T genotype have also been reported from African countries [25,26]. Furthermore, unlike the mutation at 76T in *Pfcr*t a low level of *Pfmdr1* mutant genotype and the rational of this observed research, particularly in the African countries may be due to withdraw of drug pressure.

Patients infected with *P. falciparum* parasites carrying drug-resistant mutations sometimes overcome infection after treatment [27]. The ability to recover has been associated with host age [28,29] and transmission intensity [30,31], reflecting an effect of acquired host immunity. In addition, age and parasite density have also shown association with mutant *Pfcr*t T76 and *Pfmdr1* Y86 alleles [30,32–34]. However, the present study found no significant association between the *Pfcr*t and *Pfmdr1* mutation alleles with age and parasitaemia, our findings are consistent with previous reports from Sudan and Gabon [35,36].

In conclusion, molecular surveillance continues to be a valuable tool for monitoring the emergence and spread of drug resistant parasite worldwide and also on imported malaria cases in non-endemic countries. However, the utility of these markers in correlating with *in vivo* antimalarial resistance varies on the basis of many factors including local malaria transmission and immunity. The present study shows the high prevalence of resistance pattern of CQ and to a lesser extent of *Pfmdr1* gene among imported *P. falciparum* malaria, which might be helpful for enrichment of molecular surveillance of antimalarial resistance.

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Disclosure statement

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References

- [1] World Malaria Report. World Health Organization. Geneva; 2016 Available from: <http://www.who.int/malaria/publications/world-malaria-report-2016/report/en/>
- [2] WHO; 2004. Available from: <http://www.who.int/csr/resources/publications/drugresist/malaria.pdf>
- [3] Shah NK, Dhillon GP, Dash AP, et al. Antimalarial drug resistance of *Plasmodium falciparum* in India: changes over time and space. *Lancet Infect Dis.* 2011;11:57–64.
- [4] Verlinden BK, Louw A, Birkholtz LM. Resisting resistance: is there a solution for malaria? *Expert Opin Drug Discov.* 2016;11:395–406.
- [5] Dorsey G, Vlahos J, Kanya MR, et al. Prevention of increasing rates of treatment failure by combining sulfadoxine-pyrimethamine with artesunate or amodiaquine for the sequential treatment of malaria. *J Infect Dis.* 2003;188:1231–1238.
- [6] Djimdé A, Doumbo OK, Cortese JF, et al. A molecular marker for chloroquine-resistant *falciparum* malaria. *N Engl J Med.* 2001;344:257–263.
- [7] Beljaev AE. The malaria situation in the WHO Eastern Mediterranean Region. *Med Parazitol (Mosk).* 2000;2:12–15.
- [8] Al-Kuwari MG. Epidemiology of imported malaria in Qatar. *J Travel Med.* 2009;16:119–122.
- [9] Mikhail MW, Al-Bursheed KM, Abd El-Halim AS, et al. Studies on mosquito borne diseases in Egypt and Qatar. *J Egypt Soc Parasitol.* 2009;39:745–756.
- [10] Khan FY, Elshafie SS, Almaslamani M, et al. Epidemiology of bacteraemia in Hamad general hospital, Qatar: A one year hospital-based study. *Travel Med Infect Dis.* 2010;8:377–387.
- [11] World Malaria Report. World Health Organization. Geneva; 2014 Available from: <http://www.emro.who.int/annual-report/2014/table-2-parasitologically-confirmed-cases-in-countries-with-no-or-sporadic-transmission-and-countries-with-low-malaria-endemicity.html>
- [12] Tatem AJ, Jia P, Ordanovich D, et al. The geography of imported malaria to non-endemic countries: a meta-analysis of nationally reported statistics. *Lancet Infect Dis.* 2017;17:98–107.
- [13] Singh GB, Bobogare A, Cox-Singh J, et al. A genus- and species-specific nested polymerase chain reaction malaria detection assay for epidemiologic studies. *Am J Trop Med Hyg.* 1999;60:687–692.
- [14] Duraisingh MT, Von Seidlein LV, Jepson A, et al. Linkage disequilibrium between two chromosomally distinct loci associated with increased resistance to chloroquine in *Plasmodium falciparum*. *Parasitology.* 2000;121:1–7.
- [15] Jelinek AT, Aida SAO, Peyerl-Hoffmann TG, et al. Diagnostic value of molecular markers in chloroquine-resistant *falciparum* malaria in Southern Mauritania. *Am J Trop Med Hyg.* 2002;67:449–453.
- [16] Duraisingh MT, Cowman AF. Contribution of the *pfmdr1* gene to antimalarial drug-resistance. *Acta Trop.* 2005;94:181–190.
- [17] Kublin JG, Cortese JF, Njunju EM, et al. Re-emergence of chloroquine-sensitive *Plasmodium falciparum* malaria after cessation of chloroquine use in Malawi. *J Infect Dis.* 2003;187:1870–1875.
- [18] Mockenhaupt FP, Ehrhardt S, Eggelte TA, et al. Chloroquine-treatment failure in northern Ghana: roles of *pfprt* T76 and *pfmdr1* Y86. *Ann Trop Med Parasitol.* 2005;99:723–732.
- [19] Mula P, Fernández-Martínez A, de Lucio A, et al. Detection of high levels of mutations involved in anti-malarial drug resistance in *Plasmodium falciparum* and *Plasmodium vivax* at a rural hospital in southern Ethiopia. *Malar J.* 2011;10:214.
- [20] Kebede F, Taffa N, Tedla T. An *in vivo* study of *falciparum* malaria sensitivity to chloroquine in unstable malaria endemic areas of central Ethiopia. *Ethiop Med J.* 1999;37:97–109.
- [21] Chatterjee M, Ganguly S, Saha P, et al. Polymorphisms in *Pfprt* and *Pfmdr-1* genes after five years withdrawal of chloroquine for the treatment of *Plasmodium falciparum* malaria in West Bengal, India. *Infect Genet Evol.* 2016;44:281–285.
- [22] Khattak AA, Venkatesan M, Jacob CG, et al. A comprehensive survey of polymorphisms conferring anti-malarial resistance in *Plasmodium falciparum* across Pakistan. *Malar J.* 2013;12:300.
- [23] Frosch AE, Venkatesan M, Laufer MK. Patterns of chloroquine use and resistance in sub-Saharan Africa: a systematic review of household survey and molecular data. *Malar J.* 2011;10:116.
- [24] Frosch AE, Laufer MK, Mathanga DP, et al. Return of widespread chloroquine-sensitive *plasmodium falciparum* to Malawi. *J Infect Dis.* 2014;1(210):1110–1114.
- [25] Mwai L, Ochong E, Abdirahman A, et al. Chloroquine resistance before and after its withdrawal in Kenya. *Malar J.* 2009;8:106.
- [26] Ndiaye M, Faye B, Tine R, et al. Assessment of the molecular marker of *Plasmodium falciparum* chloroquine resistance (*Pfprt*) in Senegal after several years of chloroquine withdrawal. *Am J Trop Med Hyg.* 2012;87:640–645.
- [27] Dorsey G, Kanya MR, Singh A, et al. Polymorphisms in the *Plasmodium falciparum* *pfprt* and *pfmdr-1* Genes and Clinical Response to Chloroquine in Kampala, Uganda. *J Infect Dis.* 2001;183:1417–1420.
- [28] Omar SA, Adagu IS, Gump DW, et al. *Plasmodium falciparum* in Kenya: high prevalence of drug-resistance-associated polymorphisms in hospital admissions with severe malaria in an epidemic area. *Ann Trop Med Parasitol.* 2001;95:661–669.
- [29] Djimde AA, Doumbo OK, Traore O, et al. Clearance of drug-resistant parasites as a model for protective immunity in *Plasmodium falciparum* malaria. *Am J Trop Med Hyg.* 2003;69:558–563.
- [30] Khalil IF, Alifrangis M, Tarimo DS, et al. The roles of the *pfprt* 76T and *pfmdr1* 86Y mutations, immunity and the initial level of parasitaemia, in predicting the outcome of chloroquine treatment in two areas with different transmission intensities. *Ann Trop Med Parasitol.* 2005;99:441–448.
- [31] Francis D, Nsoyba SL, Talisuna A, et al. Geographic differences in antimalarial drug efficacy in Uganda are explained by differences in endemicity and not by known molecular markers of drug resistance. *J Infect Dis.* 2006;193:978–986.
- [32] Al-Mekhlafi AM, Mahdy MA, Al-Mekhlafi HM, et al. High frequency of *Plasmodium falciparum* chloroquine resistance marker (*pfprt* T76 mutation) in Yemen: An urgent need to re-examine malaria drug policy. *Parasit Vectors.* 2011;4:94.
- [33] May J, Meyer CG. Association of *Plasmodium falciparum* chloroquine resistance transporter variant t76 with age-related plasma chloroquine levels. *Am J Trop Med Hyg.* 2003;68:143–146.

- [34] Happi CT, Gbotosho GO, Folarin OA, et al. Association between mutations in *Plasmodium falciparum* chloroquine resistance transporter and *Plasmodium falciparum* multidrug resistance 1 genes and *in vivo* amodiaquine resistance in *Plasmodium falciparum* malaria-infected children in Nigeria. *Am J Trop Med Hyg.* [2006](#);75:155–161.
- [35] Giha HA, Elbashir MI, A-Elbasit IE, et al. Drug resistance-virulence relationship in *Plasmodium falciparum* causing severe malaria in an area of seasonal and unstable transmission. *Acta Trop.* [2006](#);97:181–187.
- [36] Mayengue P, Kalmbach Y, Issifou S, et al. No variation in the prevalence of point mutations in the *pfprt* and *pfmdr1* genes in isolates from Gabonese patients with uncomplicated or severe *Plasmodium falciparum* malaria. *Parasitol Res.* [2007](#);100:487–493.