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Multiplex PCR–RFLP methods for *pfcrt*, *pfmdr1* and *pfdhfr* mutations in *Plasmodium falciparum*

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Abstract

Plasmodium falciparum drug resistance is a major factor for the death toll of malaria. Resistance has been associated with specific single nucleotide polymorphisms (SNPs) in the parasite genes *pfmdr1* (N86Y) and *pfcrt* (K76T) associated with quionoline antimalarial resistance, and *pfdhfr* (N51I, C59R, S108N) correlated with resistance of the antifolate combination sulfadoxine–pyrimethamine. These SNPs constitute the basis for the surveillance of drug resistance through high sensitive molecular methods in malaria endemic countries. In this work, we developed a multiplex PCR–RFLP protocols for the diagnosis of these molecular markers, leading to significant decreases in reagent costs, time, number of manipulations and hence human resources.

Keywords: Falciparum; pfmdr1; pfcrt; pfdhfr; Multiplex PCR; Malaria

1. Introduction

Malaria kills one child in Africa approximately every 30 s [1]. This burden is largely due to the capacity of the lethal malaria agent *Plasmodium falciparum* to develop resistance against most antimalarial drugs currently used, including the old mainstay drugs chloroquine (CQ) and sulfadoxine–pyrimethamine (SP).

Antimalarial drug resistance has been associated with the presence of particular gene single nucleotide polymorphisms (SNPs) of *P. falciparum*. These include: (a) the *pfcrt* (*P. falciparum* CQ resistance transporter) K76T a.a. change, significantly correlated to CQ and possibly amodiaquine (AQ) resistance [2–4]; (b) *pfmdr1* (*P. falciparum* multidrug resistance 1) N86Y alteration associated to the response of the parasite to aminoalcool quinolines as mefloquine (MQ) [5] and lumefantrine (LUM) [6]; and (c) *pfdhfr* (*P. falciparum* dihydrofolate reductase) N51I, C59R, S108N/T SNPs. constitute the main variants in this key enzyme member of folic acid pathway associated to resistance to SP [7].

These SNPs are believed in the near future to represent molecular epidemiology surveillance tools of antimalarial resistance, which may replace the more conventional and logistically complex in vitro or in vivo phenotyping approaches [8,9].

PCR–RFLP protocols constitute the most common methodological approach for the analysis of these SNPs [9]. Technologies based on quantitative PCR (Q-PCR) or DNA microarray formats are emerging but these are presently too costly for malaria control centers in the endemic countries, particularly in terms of equipment and logistics. This leaves PCR–RFLP still as the method of choice.

In the context of the expected future establishment of drug resistance sentinel networks in malaria settings [10–12], we propose two multiplex PCR–RFLP methodologies targeted for the aforementioned key gene SNPs. These involve the simultaneous nest PCR amplifications of *pfmdr1* N86Y and *pfcrt* K76T SNP and the amplification of DNA fragments comprising the three main resistance associated SNPs of *pfdhfr* (N51I, C59R, S108N/T). These are further associated to double digestion conditions allowing the simultaneous scoring of two SNPs per reaction tube. The proposed protocols lead to a significant decrease of reagent costs, time and number of manipulations.

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2. Materials and methods

2.1. Biological material

DNA was obtained from blood samples preserved in filter papers (Whatman, Brentford, UK) from *P. falciparum* carriers originated in Zanzibar and Iran, as well as a set of 10 samples of Kenya with a known range of parasitaemia. All samples were part of molecular epidemiological studies, all approved by the Ethics Committee of the Karolinska Institute and the responsible local authorities.

2.2. DNA extraction methods

Genomic DNA was extracted with two different methods: QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer recommendations, and through a rapid TE buffer-based protocol [13].

2.3. Primer design

The primers design was based on published *pfmdr1*, *pfcrt* and *pfdhfr* sequences of *P. falciparum* (GenBank accession numbers X56851, NC_004328, M22159, respectively) aided by the Primer Express software (Applied Biosystems, Fresno, CA, USA). Primers synthesis was effectuated by Thermo Electron Corporation (Ulm, Germany).

A set of first and internal (nest) primers were designed for the amplification of DNA fragments containing *pfmdr1* N86Y to work with the *pfcrt* K76T PCR–RFLP method described by Djimde and collaborators (2001), as it is the most widely applied protocol for the analysis of this SNP.

For the *pfdhfr* SNPs analysis, a 1st amplification was designed harboring the SNPs present on position 51, 59 and 108. The nest primers were design to amplify two DNA fragments, one containing the S108N/T SNP and another harboring the C59R and N51I SNPs. For N51I and C59R SNPs detection, trough restriction enzymes discrimination, mismatches were introduced to create DNA recognition sites. Amplicons primer sequences and lengths are listed in Table 1.

2.4. PCR reaction protocols

All PCR reactions were targeted for a final volume of 25 μL. Reagents concentration for *pfcrt* K76T/*pfmdr1* N86Y multiplex was 0.4 mM dNTPs, 2.5 mM $MgCl_2$, 1.4 U of *Taq* DNA polymerase (unless stated otherwise, *Taq* polymerase, $MgCl_2$ and dNTPs were from Promega (Madison, WI, USA)), 0.8 μM of M1 primers, 1.2 μM of C1 primers and 3 μL DNA template. The nest PCR was performed over 2 μL of 1st amplification product with similar reaction components, excepted for the primers M2 and C2 (0.6 μM of primer M2 and 1.2 μM of primer C2).

Table 1
Sequences of the primer and PCR conditions used for the detection of the *pfcrt* K76T, *pfmdr1* N86S, and *pfdhfr* N51I, C59R, S108N/T SNPs

Primer		Sequence (5′–3′)[a]	Size (bp)	PCR
1st amplification				
Pfmdr1	M1Fw	AAGAGGTTGAAAAAGAGTTGAAC	447	94 °C, 3′ followed by 45 cycles (94 °C, 30″; 56 °C, 30″; 60 °C, 30″); 60 °C, 3′
	M1Rev	CCGTTAATAATAAATACACGCAG		
Pfcrt	C1Fw	ATTTCGTACCAATTCCTGAACT	538	
	C1Rev	CGGATGTTACAAAACTATAGT-TACC		
Nest				
Pfmdr1	M2Fw	AGAGTACCGCTGAATTATTTAG	418	94 °C, 3′ followed by 40 cycles (94 °C, 30″; 47 °C, 30″; 68 °C, 1′); 64 °C, 3′
	M2Rev	CCTGAACTCACTTGTTCTAAAT		
Pfcrt	C2Fw	TGTGCTCATGTCTTTAAACTT	145	
	C2Rev	CAAAACTATAGTTACCAATTTTG		
1st amplification				
Pfdhfr	D1Fw	ATGATGGAACAAGTCTGCGAC	515	94 °C, 2′ followed by 10 cycles (92 °C, 30″; 54 °C, 30″; 68 °C, 45″), followed by 15 cycles (92 °C, 30″; 52 °C, 30″; 68 °C, 45″), followed by 10 cycles (92 °C, 30″; 51 °C, 30″; 68 °C, 45″), followed by 10 cycles (92 °C, 30″; 50 °C, 30″; 68 °C, 1′)
	D1Rev	CTTGATAAACGGAACCTCC		
Nest				
pfdhfr 108	D108Fw	CAAAGAAACTGTGGATAATG-TAAATGATATGC	262	94 °C, 1′ followed by 10 cycles (94 °C, 20″; 61 °C, 30″; 72 °C, 10″), followed by 10 cycles (94 °C, 20″; 63 °C, 30″; 72 °C, 30″), followed by 10 cycles (94 °C, 20″; 61 °C, 30″; 72 °C, 30″), followed by 15 cycles (94 °C, 20″; 58 °C, 30″; 72 °C,30″)
	D108Rev	AACAACGGAACCTCCTATAA-TAAAACATT		
Pfdhfr 51/59	D51/59Fw	CTAGGAAATAAAGGAGTATTAC-CATGGAAATGgA	94	
	D51/59Rev	CATATTTTGATTCATTCACA-TATGTTGTAACTGCtC		

[a] Mismatches that were engineered into the primers are indicated in lowercase.