Available online at www.sciencedirect.com
SCIENCE DIRECT®
Acta Tropica 93 (2005) 97–106
ACTA TROPICA
www.elsevier.com/locate/actatropica

Prevalence of pfcrt mutations in Congolese and Malawian *Plasmodium falciparum* isolates as determined by a new Taqman assay

Paul E. Wilson[a], Walter Kazadi[b], Deborah Demster Kamwendo[c], Victor Mwapasa[d], Anne Purfield[a], Steven R. Meshnick[a,e,*]

[a] *Department of Microbiology and Immunology, University of North Carolina School of Medicine, CB 7290, Chapel Hill, NC 27599, USA*
[b] *Programme National de Lutte contre le Paludisme (PNLP), Kinshasa, Democratic Republic of the Congo*
[c] *Department of Epidemiology, University of Michigan, Ann Arbor, MI, USA*
[d] *Department of Community Health, College of Medicine, Blantyre, Malawi*
[e] *Department of Epidemiology, University of North Carolina School of Public Health, Chapel Hill, NC, USA*

Received 1 July 2004; received in revised form 16 September 2004; accepted 23 September 2004

Abstract

A real-time PCR assay was developed to detect the K76T point mutation in the *Plasmodium falciparum* putative chloroquine resistance transporter gene. The assay was used with malaria positive clinical isolates from Rutshuru in the eastern part of the Democratic Republic of the Congo (DRC) and from Malawi. The K76T mutation was found in 52/56 (93%) clinical isolates from the DRC, where chloroquine resistance is high, but in none of the 12 isolates tested from Malawi where chloroquine is now rarely used. Sixteen percent of specimens from the DRC had detectable levels of both wild-type and mutant alleles. The real-time PCR results were compared to results from a nested allele-specific PCR assay and from direct DNA sequencing. Using allele-specific PCR as the reference method, the new assay is 100% sensitive and specific towards the mutant allele. In addition to its low per-test cost, the new assay is fast, easily automated, sensitive and well-suited to large-scale epidemiological studies.

Keywords: *Plasmodium falciparum*; Single nucleotide polymorphism; Membrane proteins/genetics; Genotype; Chloroquine; Chloroquine resistance transporter; Polymerase chain reaction; Drug resistance; Democratic Republic of the Congo; Malawi

Abbreviations: FAM, 6-carboxy-fluorescein; PCR, Polymerase chain reaction; MGB, minor groove binding non-fluorescent quencher; pfcrt, *Plasmodium falciparum* chloroquine resistance transporter; ASPCR, allele-specific PCR; DHPS, dihydropteroate synthase; DHFR, dihydrofolate reductase; Pfmdr1, *Plasmodium falciparum* multidrug resistance gene 1; TE, Tris-EDTA buffer; rTime, real-time PCR; EDTA, Ethylenediaminetetraacetic acid; DBS, dried blood spots; SNP, single-nucleotide polymorphism; VIC, Applied Biosystems VIC™ fluorescence probe; WBC, white blood cell

* Corresponding author. Tel.: +1 919 966 7414; fax: +1 919 962 8103.
E-mail address: meshnick@email.unc.edu (S.R. Meshnick).

0001-706X/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.actatropica.2004.09.010

1. Introduction

Malaria kills over 1 million people annually (Suh et al., 2004). Most of the deaths are in *Plasmodium falciparum*-infected African children. Chloroquine has served as an effective anti-malarial, but the emergence of chloroquine-resistant parasites has diminished its utility (Wellems and Plowe, 2001; Wongsrichanalai et al., 2002). The *Plasmodium falciparum* putative chloroquine resistance transporter (pfcrt) is a 424 amino-acid transmembrane protein encoded by the pfcrt gene (accession no. AF495378.1) and is thought to participate in the malaria parasite's digestive processes (Carlton et al., 2001). Research suggests that a single-nucleotide polymorphism (SNP) at codon 76, resulting in replacement of threonine for lysine in the gene product, helps confer chloroquine resistance on the parasite (Fidock et al., 2000).

While some studies exploit the K76T SNP as a direct marker for chloroquine resistance (Djimde et al., 2001a,b; Jelinek et al., 2002; Maguire et al., 2001), others only concede that the mutation is a necessary, but not sufficient condition for resistance (Mayor et al., 2001; Pillai et al., 2001). At least one study considers the marker of limited use (Vinayak et al., 2003). Despite the controversy, several methods have been developed to detect the K76T SNP. Such methods include direct sequencing (Hunt et al., 2004; Pickard et al., 2003), restriction-fragment length polymorphism analysis (Syafruddin et al., 2003), allele-specific PCR (Contreras et al., 2002; Kyosiimire-Lugemwa et al., 2002; Vinayak et al., 2003), PCR followed by fluorescence detection with allele-specific molecular beacons (Durand et al., 2002), and melting analysis with sensor probes (de Monbrison et al., 2003). Herein, we report a 5′-nuclease real-time PCR (TaqMan®) assay to detect the K76T pfcrt mutation. The assay was performed on *P. falciparum* clinical isolates from both the Democratic Republic of the Congo where chloroquine was until recently used as a first-line anti-malarial drug and where chloroquine resistance is high and from Malawi where chloroquine is now rarely used (Kublin et al., 2003; Mita et al., 2003). The isolates were also tested with an allele-specific nested PCR method and selected samples were directly sequenced.

2. Materials and methods

2.1. Ethical approvals

Ethical approval was obtained from Institutional Review Boards at the University of North Carolina, the Programme National de Lutte contre le Paludisme (PNLP) in Kinshasa, Democratic Republic of Congo, and The University of Malawi College of Medicine.

2.2. Specimen collection

Eight *P. falciparum* DNA reference samples (Table 1) were provided by MR4 (ATCC, Manassas Virginia). Strains HB3 and W2 were used for assay optimization and mixture analysis. Reference DNA stocks were provided at a 3 ng/μl concentration and were diluted 100-fold in TE buffer prior to use.

Dried-blood spots (DBS) were prepared from the blood of *P. falciparum* infected study participants living in Rutshuru in the Democratic Republic of the Congo between June and September 2002. Study participants had an average age of 28 months in a range of 6–58 months. The average asexual parasitemia was 1600 parasites per 200 WBCs in a range of 36–7200. Blood was applied to IsoCode® Stix (Schleicher & Schuell, 10495015) and dried prior to desiccated storage.

Table 1
Real-time PCR genotype of MR4 DNA reference standards. The wild-type codon 76 is lysine

MR4#	Strain	Codon 76 identity (rTime-PCR)	Chloroquine phenotype	Phenotype reference	Accession no.
388	Dd2	Thr	Resistant	MR4	AF030694.2
390	W2	Thr	Resistant	MR4	
391	FCR3	Thr	Resistant	(Geary et al., 1990)	
392	K1	Thr	Resistant	(Stocks et al., 2002)	AF495378.1
395	V1/S	Thr	Resistant	(Muregi et al., 2004)	
386	3D7	Lys	Sensitive	(Gilberger et al., 2000)	AL844506
389	HB3	Lys	Sensitive	MR4	AF233068.1
396	D10	Lys	Sensitive	(Saliba et al., 1998)	