

Quantitative real-time PCR with *TaqMan* and SYBR green for estimation of *Pfmdr1* copy number and application for monitoring of antimalarial drug resistance

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Abstract

Amplification of *Plasmodium falciparum* multi-drug resistant gene 1 (*Pfmdr-1*) has been linked with resistance of antimalarial drugs, particularly mefloquine and artemisinin derivatives. This could be used as a molecular marker for monitoring and surveillance of antimalarial drug resistance. The objective of the study was to compare the correlation between copy number of *Pfmdr-1* gene analyzed by two different techniques, *i.e.*, real-time polymerase chain reaction (PCR) with *Taqman* and real-time PCR with SYBR green assay. Gene copy number was determined in of a total of 18 dried blood spot samples collected onto filter paper from patients infected with *P.falciparum* malaria. DNA extraction Kit was used to extract genomic *P.falciparum* DNA. The results showed good correlation of the copy number analyzed by the two methods (Spearman correlation test, $r = 0.852$). Further study should be performed with more number of *P.falciparum* isolates, together with clinical (*in vivo*) response, to confirm the association between *Pfmdr-1* amplification and resistance to mefloquine-artemisinin combination.

Keywords: *Pfmdr-1* copy number, SYBR green1, *Taqman*, drug resistance

Introduction

Multidrug resistance *Plasmodium falciparum* is a serious problem throughout the world particularly in Southeast Asia where strains are mostly resistant to chloroquine, antifolates, quinine, and mefloquine. Surveillance for drug-resistant malaria is based on *in vivo* criteria for treatment failure, measurement of the activities of antimalarial drugs against cultured parasites *in vitro* and more effectively by using molecular markers (1). Recently, *Pfmdr-1* (*P. falciparum* multidrug resistance gene-1) copy number, has proved as useful molecular marker in assessing resistance to mefloquine and artemisinin derivatives (2). The *Pfmdr-1*, a member of the ATP-binding cassette(ABC) superfamily, has been proposed to function as drug transporter, using the energy from ATP hydrolysis to catalyze drug efflux from a cell or cellular compartment containing the relevant drug target, thereby promoting drug resistance (3). Two techniques are being used for DNA quantification, real-time PCR has been developed using either *TaqMan* probe with reporter and quencher dye, or SYBR Green dye were used for sources of the fluorescence (4). The first method has been more widely used as a standard method due to its high sensitivity. However, the technique is relatively expensive and requires PCR machine with filter with specific wavelength to detect fluorescence signal. In addition, it also requires specific probe in PCR reaction. The objective of this study was to preliminarily investigate the correlation between *Pfmdr-1* gene copy number analyzed by these two methods in a total of 18 *P.falciparum* isolates.

Methods

DNA extraction from dried blood spot on filter paper

A total of 18 of dried blood spot samples collected from patients infected with *P. falciparum* from different malaria endemic areas of Thailand along the Thai-Mynamar border (14 from Kanchanaburi, 2 from Mae Hongson and 2 from Ranong) during 2009, were included in the study. Parasite genomic DNA was extracted from all samples by QIAmp[®] DNA extraction kit (Qiagen, Crawley, UK).

Quantification of *Pfmdr-1* by real-time PCR

The amplification of *Pfmdr-1* was determined by Taqman and SYBR green real-time PCR as follows:

Taqman: Amplification reactions were done in 25 μ L, containing *TaqMan* buffer (8% glycerol, 0.625 U DNA polymerase, 5.5 mM MgCl₂, 300 μ M dNTP, 600 nM passive reference dye ROX(5-carboxy-X-rhodamine), pH 8.3), 300 nM of each forward and reverse primer, 100 nM of each probe and 5 μ L of template DNA (5).

SYBR green: Individual PCR reaction was prepared in a total volume of 25 μ L containing 0.5x of Platinum[®]SYBR[®] Green qPCR Supermix-UDG (Invitrogen[®]), 10 μ M of each forward and reverse primer, and 2 μ L of DNA template.

For each experiment, *Taqman* and SYBR green real-time PCR, *Pfmdr-1* copy numbers of DNA of the *P. falciparum* clone Dd2 and 3D7 were determined in parallel as control strains. Cycle threshold (Ct) and melting curve were generated at the end of each reaction for further data analysis. The results were analyzed by a comparative C_t method (5), based on the tested assumption that the target (*pfmdr1*) and reference gene were amplified with the same efficiency within an appropriate range of DNA concentrations.

Statistical analysis

Correlation of *Pfmdr-1* copy number analyzed by the two methods were determined using Spearman Correlation Test at a statistical significance level of $p = 0.05$.

Results

Total of 18 DNA samples of *P. falciparum* isolates were successfully analyzed for *Pfmdr-1* copy number by Taqman and SYBR green assay and results are shown in Table 1.

Table 1 Analysis of *Pfmdr-1* copy number by Taqman and SYBR green assay. Data are presented as number of *P.falciparum* isolates.

<i>Pfmdr-1</i> copy number		Taqman			Total
		1.00	2.00	3.00	
SYBR green	1.00	14	1	0	15
	2.00	0	2	1	3
Total		14	3	1	18

Statistical analysis of *Pfmdr-1* copy number by Spearman Correlation Test showed good correlation with $r = 0.852$.

Discussion and Conclusion

The preliminary results from this study, based a limited number of *P.falciparum* isolates included, showed a good and significant correlation between the two techniques currently being used for estimation of *Pfmdr-1* copy number (quantitative real-time PCR with *Taqman* and SYBR green). Inconsistent results were found only in 2 samples where higher number of gene copy number was obtained with the *Taqman* method (3 vs 2 and 2 vs 1 copies for *Taqman* vs SYBR green method). It was noted however that the sampling procedure (4) including sample collection and DNA concentration in each reaction (6) could greatly affect to the results. In one report, *Taqman* assay was found to be more specific than SYBR green in general since it needs hybridization with fluorescence probe (4). Further study should be performed with larger number of *P.falciparum* isolates to confirm this finding. Real-time PCR with SYBR green would thus be used to replace *Taqman* to investigate the association between *Pfmdr-1* amplification and resistance to mefloquine-artemisinin combination. In this preliminary study, two isolates collected from Kanchanaburi and Ranong Provinces were observed to carry *Pfmdr-1* copy number greater than 1 (2 and 3 copies). It is interesting to investigate the relationship between clinical response and amplification of this gene in large number of patients throughout the endemic areas of Thailand where mefloquine –artesunate combination therapy has been used as first-line treatment.

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