Drug Resistance Genotyping in Malaria Using FRET/Melt-Curve Analysis

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Introduction

Sulfadoxine-pyrimethamine (SP) is an antifolate drug for the treatment of malaria. Pyrimethamine binds and inhibits *Plasmodium falciparum* dihydrofolate reductase (DHFR), a key enzyme for DNA synthesis. However, the widespread use of SP has led to a rapid emergence of SP-resistant parasites. Mutation of the DHFR gene at codon 108 (A→T) causes resistance to pyrimethamine. Additional point mutations at codons 51 (A→T) and 59 (T→C) increase resistance to the drug (Peterson et al. 1988). Molecular tools for genotyping such mutations could be used for the surveillance of SP resistance in countries where malaria is endemic.

In this report, we present a new assay based on fluorescence resonance energy transfer (FRET) during melt-curve analysis that is able to determine the genotype of parasites at codons 51 and 59 of the DHFR gene. The FRET assay design involves (1) a probe specific for the DHFR gene that encompasses codons 51 and 59 labeled with FAM at the 3' end, and (2) a primer for amplification of the DHFR gene placed adjacent to the probe and labeled with ROX near its 3' end. Consequently, upon hybridization of the probe to the PCR product and excitation, FRET can occur from FAM to ROX (Figure 1).

The assay basically consists of two steps (Figure 1). The first step is a standard PCR using the ROX-labeled forward primer and a reverse primer that amplifies a fragment of the *Plasmodium* DHFR gene. During this PCR, the product is labeled with ROX as the forward primer is extended. Following amplification, the FAM-labeled probe is added to the obtained PCR products. The resulting mixture is denatured and then cooled to 10°C below the melting temperature (T_m) of the probe, allowing the probe to anneal adjacent to the ROX fluorophore of the PCR product. The temperature is subsequently slowly increased, while ROX fluorescence resulting from FRET is continually monitored. When the T_m of the probe/PCR product hybrid is reached, ROX fluorescence will decrease as FRET can no longer occur between the FAM label of the probe and the ROX label of the PCR product.

The change in ROX fluorescence appears as a positive peak on a plot of the –dF/dT (first negative derivative of the fluorescence with respect to temperature function) vs. temperature. However, the melting temperature of the probe/PCR product hybrid will depend on the nucleotide sequence of codons 51 and 59. The probe was designed as an exact complement of the 51A 59T (wild-type) sequence. If a mutation is present at codon 51, 59, or both in the PCR product, the mismatches make the hybrids weaker, resulting in a lower melting temperature compared to the hybrid with no mismatches. Thus, a 51A 59T PCR product will have a much higher T_m than a 51T 59C PCR product, while a 51T 59T PCR product will have an intermediate T_m. Analysis of the melt curve allows determination of the DHFR genotype of codons 51 and 59 of the parasite population present in the sample.

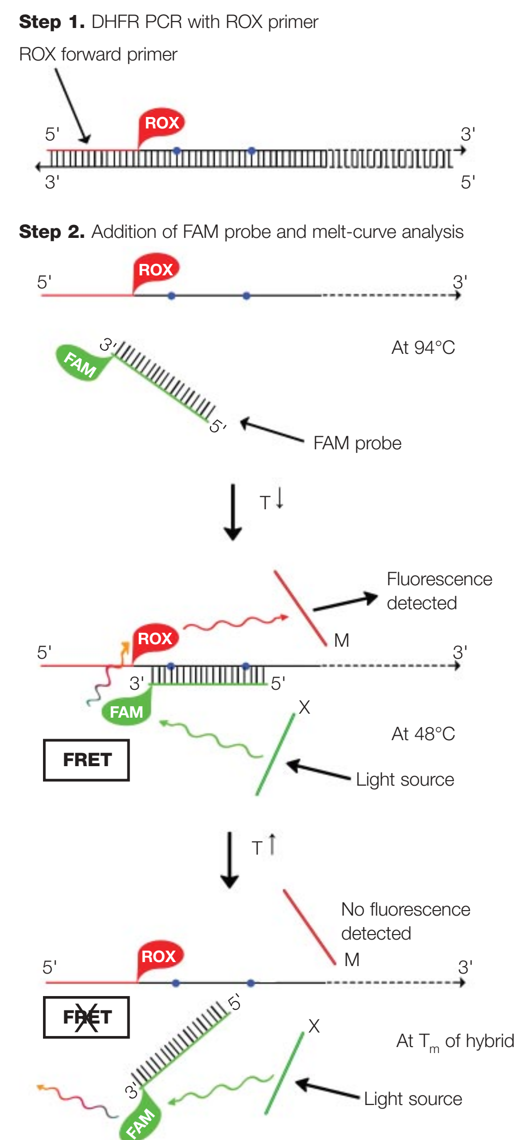

Fig. 1. Schematic representation of the FRET/melt-curve assay. The assay was designed to genotype codons 51 and 59 of *Plasmodium falciparum* DHFR.

• = codons 51 and 59 of *Plasmodium falciparum* DHFR; X = 490/20X FAM excitation filter; M = 620/30M ROX emission filter.

Methods

Design of FRET Primer and Hybridization Probe

The forward primer was labeled 6 nucleotides from the 3' end with ROX, in order not to obstruct extension of the primer during PCR.

The probe was specifically designed to achieve a maximum difference in T_m between the different genotypes as calculated with Meltcalc software (developed by E Schütz and N von Ahsen; downloadable at http://meltcalc.com) (Schütz and von Ahsen 1999). The probe complements the wild-type antisense strand of the PCR product and was labeled at the 3' end with FAM. Primers and probe were synthesized by Eurogentec.

PCR and Melt-Curve Analysis Conditions

The target strand to which the FAM-labeled probe binds was produced in excess using an asymmetric PCR. Amplification reactions (50 µl) contained 1x iQ™ supermix (Bio-Rad), 500 nM forward primer, and 100 nM reverse primer. The probe was added immediately after amplification at a final concentration of 160 nM.

The DNA template used in these experiments consisted of DHFR clones with a *Plasmodium* DHFR insert with known genotype at codons 51 and 59.

Bio-Rad's iCycler iQ® system was used to perform the PCR and melt-curve analysis. PCR conditions were: initial denaturation at 94°C for 5 min, followed by 35 cycles at 95°C for 30 sec, 52°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 8 min. The melt-curve protocol that followed addition of the probe consisted of two steps: (1) 1 min at 94°C, (2) 110 repeats of heating for 30 sec, starting at 48°C with 0.3°C increments.

All experiments were carried out in triplicate to verify reproducibility.

Results

The plots of –dF/dT of the melt curves for three different clones containing respectively 51A 59T DHFR (plasmid FR-3D7), 51T 59T DHFR (clone N5), and 51T 59C DHFR (plasmid FR-V1S) are shown in Figure 2. The three different genotypes had distinct melting peaks, with 51A 59T at 62.4°C, 51T 59T at 58.3°C, and 51T 59C at 53.7°C.

Since a *Plasmodium* population in a sample might be polyclonal (that is, some parasites have 51T 59T and some have 51T 59C), this assay should also be capable of identifying and quantitating multiple DHFR genotypes in one sample. Therefore, experimental mixtures containing different plasmids were also assayed. The following mixtures were tested: (1) 50% FR-V1S/50% FR-50 and (2) 90% FR-V1S/10% FR-50. Plasmid FR-V1S contains a DHFR gene with a point mutation in codons 51 and 59. Plasmid FR-50 has a DHFR insert that also contains the mutated codon 51, and a point mutation in codon 50 at the penultimate position of the 3' end of the probe, which has only a minor influence on the T_m of the probe. The melt curves of the mixtures (Figure 3) contained the two expected peaks that correspond to the two different genotypes. The percentage of each genotype in the mixtures roughly concurred with the area underneath the peak that represents that genotype, as expected.

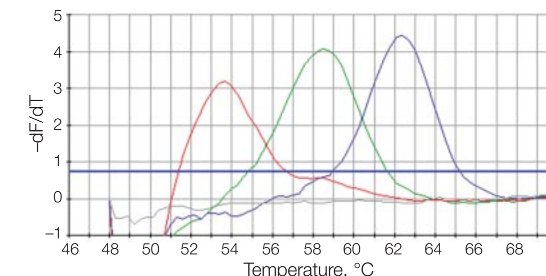

Fig. 2. First negative derivative of melt curves for DHFR clones. Plasmids analyzed were 51T 59C DHFR (FR-V1S, red), 51T 59T DHFR (N5, green), and 51A 59T DHFR (FR-3D7, blue); also shown is no-template control (gray). Melting peaks were identified at 53.7°C, 58.3°C, and 62.4°C, respectively. Horizontal blue line indicates threshold for background fluorescence.

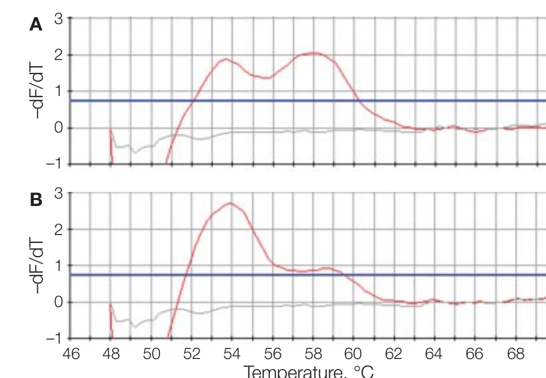

Fig. 3. First negative derivative of melt curves of experimental mixtures. Red curves: A, 50% FR-V1S and 50% FR-50; B, 90% FR-V1S and 10% FR-50. Gray curve represents no-template control; horizontal blue line indicates threshold for background fluorescence.

Discussion

Melt-curve analysis using FRET technology is rapidly emerging as an efficient genotyping method. However, most FRET genotyping assays can only detect one point mutation. The new DHFR genotyping assay presented here clearly demonstrates that with a simple FRET primer/probe design it is feasible to detect mutations at two (or more) nucleotides simultaneously. Furthermore, we show that it is possible to quantitate the different genotypes present in a polyclonal parasite population, which is not possible with any other molecular tool. Consequently, FRET/melt-curve analysis promises to be a very useful genotyping tool for all infectious diseases.

References

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Schütz E and von Ahsen N, Spreadsheet software for thermodynamic melting point prediction of oligonucleotide hybridization with and without mismatches, Biotechniques 27, 1218–1224 (1999)

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