Pathogen Genotyping in Polyclonal Infections: Application of a Fluorogenic Polymerase–Chain-Reaction Assay in Malaria

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Pathogen genotyping of polyclonal infections is limited by 2 major drawbacks: (1) how to establish whether multiple mutations detected in 1 gene belong to the same clone and (2) how to evaluate the proportion of different genotypes in a given sample. For drug-resistance genotyping in *Plasmodium falciparum* malaria, we address these problems by using a fluorogenic assay that combines fluorescence-resonance energy transfer, between fluorophores present on a probe and a polymerase–chain-reaction primer, and a melt-curve analysis. We demonstrate that this tool allows a more accurate insight into the *P. falciparum* populations present in complex biological samples.

Pathogen genotyping has become essential for the monitoring and control of infectious diseases. Molecular methods such as direct sequencing, allele-specific (AS) polymerase chain reaction (PCR) (AS-PCR), or restriction fragment-length polymorphism (RFLP) analysis are currently used. However, both the performance of these methods and their practical applications may diminish significantly when infections are polyclonal, because it becomes difficult both (1) to establish whether multiple mutations detected in 1 gene are located in the same clone and (2) to evaluate the proportions of the different genotypes within a given sample. This problem can be illustrated by the genotyping of point mutations linked to drug resistance in *Plasmodium falciparum* malaria. In many countries, resistance to the antifolate sulfadoxine-pyrimethamine (SP) is rapidly spreading, and several AS-PCR assays to detect the different mutations present in the *P. falciparum* dihydrofolate reductase–thymidylate synthase (DHFR-TS) gene and in the dihydropteroate synthase (DHPS) gene are used [1]. Field studies aiming to establish a relationship between discrete polymorphism in the candidate genes and parasite resistance to SP in vitro and in vivo have reported contrasting results, particularly in areas of intense transmission (mainly in sub-Saharan Africa), where polyclonal infections are extremely common [2]. This is probably attributable to the reliability of the methods used to detect mutations.

The use of fluorogenic hybridization probes in a real-time PCR assay offers an attractive alternative for the genotyping of polyclonal infections. The basis of this assay is fluorescence-resonance energy transfer (FRET), which requires 2 fluorophores, referred to as "donor" and "acceptor." At hybridization of the fluorogenic probes on the amplicon, these fluorophores are brought into close proximity. The fluorescence emitted from the donor is then absorbed by the acceptor, resulting in fluorescence emission by the acceptor, which can be detected. Progressive increase of the temperature during melt-curve analysis (MCA) leads, at specific temperatures, to the dissociation of the fluorogenic probes from the target, resulting in the loss of the fluorescent signal visualized on a melt curve. The specific temperature at which the probe melts depends on the thermodynamic stability and allows discrimination of perfectly complementary and mismatched probe-target duplexes [3, 4]. We applied this principle to genotype-critical mutations linked to antifolate drug resistance in *P. falciparum* malaria. We present here an innovative FRET/MCA assay performed on the iCycler (Bio-Rad) and using a fluorogenic primer/probe design. Our assay allows both (1) identification of the genotype at multiple codons simultaneously in a mutation–hot-spot region (codons 50–60) of the *P. falciparum* DHFR gene and (2) quantification of different genotypes present in a polyclonal malaria infection. The significant advantages demonstrated by our method could be useful in the study of other infectious diseases.

MATERIALS AND METHODS

Informed consent was obtained from either the patients or their parents or guardians. Human-experimentation guidelines of Prince Leopold Institute of Tropical Medicine, Antwerp (IT-

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Table 1. Detection of genotype of dihydrofolate reductase, by sequencing and fluorescence-resonance energy transfer/melt-curve analysis.

	Sequence, codons 49–60[a]												Melting temperature, °C	
Sample	49	50	51	52	53	54	55	56	57	58	59	60	Observed	Predicted
FR-3D7[b]	5′-A	TGT	AAT	TCC	CTA	GAT	ATG	AAA	TAT	TTT	TGT	G-3′	62.4	63.8
N15	5′ A	TGT	TAT	TCC	CTA	GAT	ATG	AAA	TAT	TTT	TGT	G 3′	59.4	61.1
N5	5′ A	TGT	ATT	TCC	CTA	GAT	ATG	AAA	TAT	TTT	TGT	G 3′	58.3	60.0
FR-50	5′ A	CGT	ATT	TCC	CTA	GAT	ATG	AAA	TAT	TTT	TGT	G 3′	57.6	56.1
FR-V1S	5′ A	TGT	ATT	TCC	CTA	GAT	ATG	AAA	TAT	TTT	CGT	G 3′	53.7	55.2
N11	5′ A	TGT	ATT	TCC	CTA	GAT	ATG	AAA	TAT	TTT	CGT	G 3′	53.7	55.2
N13	5′ A	TGT	ATT	TCC	CTA	GAT	ATG	AAA	TAT	TTT	CGT	G 3′	53.7	55.2
N7	5′ A	TGT	ATT	TCC	CTA	GAT	ATG	AAA	TAT	TTC	TGT	G 3′	52.8	55.8
N2	5′ A	TGT	ATT	TCC	CTA	GAT	GTG	AAA	TAT	TTT	CGT	G 3′	51.6	52.9
N4	5′ A	CGT	ATT	TCC	CTA	GAT	GTG	AAA	TAT	TTT	CGT	G 3′	51.3	47.4

NOTE. Six genotypes were defined on the basis of significant and reproducible (interexperiment SD, <0.4°C) differences in T_m: 62.4°C (wild type), 59.4°C (1 mutated base, at codon 51 [TAT]), 57.6°C–58.3°C (1 mutated base, at codon 51 [ATT]), 53.7°C (2 mutated bases, at codons 51 [ATT] and 59 [CGT]), 52.8°C (2 mutated bases, at codons 51 [ATT] and 58 [TTC]), and 51.3°C–51.6°C (3 mutated bases, at codons 51 [ATT], 55 [GTG], and 59 [CGT]).

[a] Base mutations (determined on the basis of sequencing results) are underlined.

[b] Wild type.

MA) were followed. Ethical clearance was obtained from the research-ethics committee at ITMA and from the Uganda National Council for Science and Technology.

Plasmids containing a DHFR insert with a known genotype were obtained from MR4/American Type Culture Collection. FR-3D7, with the genotype 50 Cys (TGT)–51 Asn (AAT)–59 Cys (TGT), was used as the wild-type control, and FR-V1S, with the genotype 50 Cys (TGT)–51 Ile (ATT)–59 Arg (CGT), was used as the mutant control. A third plasmid, FR-50, with the genotype 50 Arg (CGT)–51 Ile (ATT)–59 Cys (TGT), also was used to evaluate FRET/MCA.

Field samples were obtained in the frame of a malariometric cross-sectional survey performed in Uganda during September–December 1999. Duplicate thick and thin blood films were collected for parasitological examination. The presence of *P. falciparum* in peripheral blood was determined by microscopic examination of 200 fields, with a ×100 oil-immersion objective. For molecular analysis, a blood sample from each individual was collected onto 3M Whatman filter paper, stored at room temperature in an individual zip-lock dry polyethylene bag, and extracted as described elsewhere [5].

The primer/probe tandem used in FRET was specifically designed to achieve a melting temperature (T_m) difference between the most prevalent genotypes (51A-59T, 51T-59T, and 51T-59C), as calculated by Meltcalc (developed by E. Schütz and N. von Ashen and available at http://www.meltcalc.com/) [6]. The probe (5′-CACAAAAATATTTCATATCTAGGGAA-TTACAT-3′) complements the wild-type antisense strand of the PCR product and was labeled, with FAM, at the 3′ end. The sense primer (5′-GGAAATAAAGGAGTATTACAATGGA-AA-3′) was labeled, with ROX, at a position 6 nt from the 3′ end, so that extension of the primer during PCR would not be obstructed. A point mutation, C→A, at a position 8 nt from the 3′ end, was introduced to avoid formation of primer dimers. The reverse primer (5′-TATAAACATCTTCATCAAAATCTTC-3′) was chosen so as to have a T_m similar to that used for the forward primer and to avoid formation of primer dimers. Primers and probe were synthesized by Eurogentec.

The target strand to which the FAM-labeled probe binds was produced in excess by an asymmetric PCR. Reaction mixtures of 50 μL, containing 1 μL of primary PCR product resulting from amplification with AMP1 and AMP2 [5], 1× iQ supermix (Bio-Rad), sense primer (500 nmol/L), and antisense primer (100 nmol/L), were made. PCR conditions were as follows: initial denaturation for 5 min at 94°C, followed by 35 cycles with denaturation for 30 s at 95°C, annealing for 1 min at 52°C, extension for 1 min at 72°C, and a final extension for 8 min at 72°C. The probe was added immediately after amplification, in a final concentration of 160 nmol/L

The iCycler was used to perform the MCA, with a 490/20X FAM excitation filter and a 620/30M ROX emission filter. Because of the unusual filter combination, well factors had to be taken into account before MCA was performed. These factors are used by the system to compensate for any system or pipetting nonuniformity, in order to optimize postrun data analysis. Well factors were taken into account by means of an external 96-well plate containing 50 μL of 1× external-well solution (Bio-Rad) in each well. The MCA protocol consisted of 2 steps: (1) 1 min at 94°C and (2) 110 repeated heatings (each for 30 s), starting at 48°C and with increments of 0.3°C. Change in fluorescence

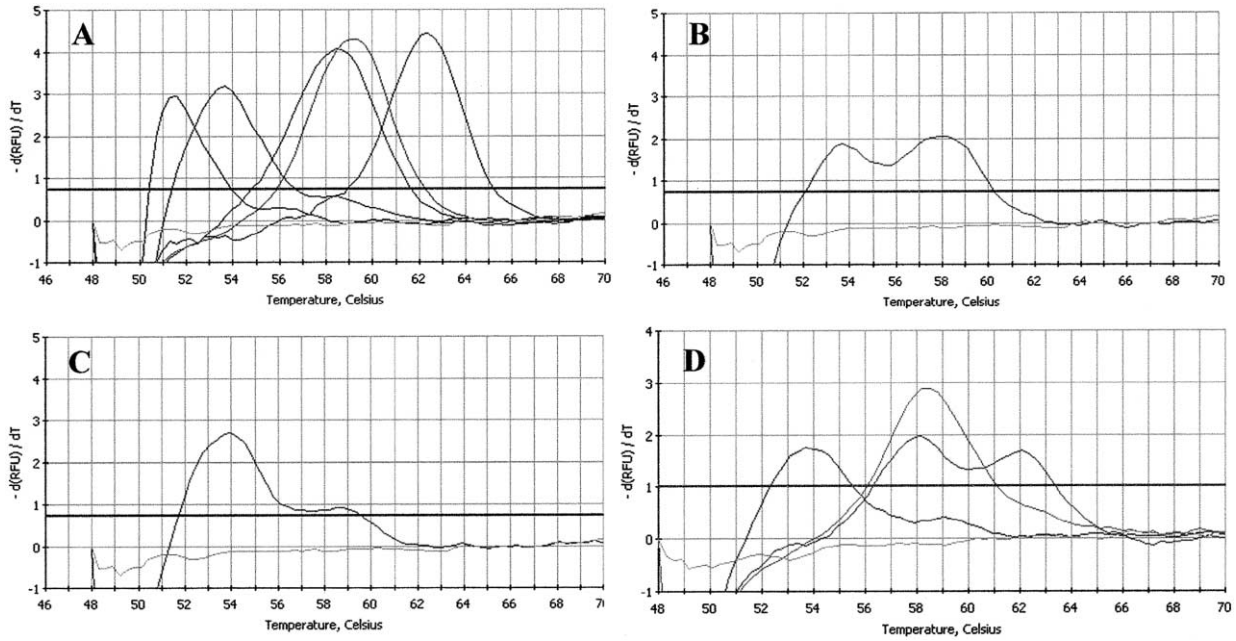

Figure 1. Fluorescence-resonance energy transfer/melt-curve analysis of the gene for *Plasmodium falciparum* dihydrofolate reductase. In all 4 panels, the thicker horizontal line denotes the threshold for background fluorescence, and the curve entirely below that thicker horizontal line denotes the results for the nontemplate control. −dF/dT, fluorescence/temperature function. *A,* Cloned sequence variants, from lower to higher melting temperature: N2, 3 mutations (at codons 51 [ATT], 55 [GTG], and 59 [CGT]); FR-V1S, 2 mutations (at codons 51 [ATT] and 59 [CGT]); N5, 1 mutation (at codon 51 [ATT]); N15, 1 mutation (at codon 51 [TAT]); and FR-3D7 (wild type). *B* and *C,* Experimental mixtures of cloned sequences: *B,* 50% FR-V1S and 50% FR-50; *C,* 90% FR-V1S and 10% FR-50. *D,* Blood samples from patients with malaria, from lower to higher melting temperature: KH36, monoclonal, melting temperature of 53.7°C for FR-V1S–like double mutant 51 (ATT) and 59 (CGT); KH100, monoclonal, melting temperature of 58.5°C for N5-like single mutant 51 (ATT); and C32 *(curve with 2 peaks),* biclonal, melting temperatures 61.8°C and 58°C, respectively, for wild type (FR-3D7 like) and single mutant 51 (ATT) (N5 like).

appears as a positive peak on a plot of the first negative derivative of the fluorescence/temperature function. All experiments using FRET/MCA were performed in triplicate, to ensure reproducibility. AS-PCR was performed as described elsewhere [5, 7].

RESULTS

We targeted, in the DFHR gene, a small region (codons 50–60) containing several mutations, with the 2 most important mutations being located in codons 51 (AAT→ATT) and 59 (TGT→CGT), which have been reported to significantly increase in vitro resistance to pyrimethamine [8]. Our innovative FRET design involves (1) a sense primer labeled, with ROX (acceptor), near the 3′ end, and (2) an adjacent probe specific for wild-type DHFR, encompassing codons 50–60 and labeled, with FAM (donor), at the 3′ end. The genotyping assay consists of 2 steps. The first step consists of a standard PCR using the ROX-labeled sense primer, which amplifies a 304-bp fragment of the *Plasmodium* DHFR gene. During this PCR, the product is labeled with ROX as the sense primer is extended. After amplification, the FAM-labeled probe hybridizes, and, as a result of FRET between the 2 fluorophores, ROX starts to emit fluorescence. The second step consists of MCA of this probe/amplicon hybrid, to differentiate, within the targeted region, the different genotypes of the PCR product. This was verified with reference plasmids and DHFR recombinant clones containing sequence variants encountered in field samples and submitted to bidirectional sequencing (table 1). The wild-type genotype showed the highest T_m; for the other genotypes, the more mutations, the lower the T_m (figure 1*A* and table 1). Mutations in the penultimate base of the targeted region led to slight differences in T_m (e.g., note both the T_m for FR50 vs. that for N5 and the T_m for N4 vs. that for N2; table 1); hence, they were not considered for further genotyping. In total, 6 different genotypes could reproducibly be resolved (table 1).

We also performed stability calculations with the spreadsheet application Meltcalc running under Microsoft Excel 2000, to predict, with an error of <2.4°C, the T_m of oligonucleotide sequences with or without base-pair mismatches (table 1) [6]. The predicted T_ms match the observed T_ms—with the exception of clone N4, which is to be expected, because the influence of terminal and penultimate mismatches cannot be properly reflected by the thermodynamic model on which Meltcalc is based [6].

To check whether the presence of different genotypes in a polyclonal infection can be detected and quantified, we tested experimental mixtures of plasmids containing either (1) 50% FR-V1S and 50% FR-50 or (2) 90% FR-V1S and 10% FR-50. The resulting melt curves clearly contain the 2 expected peaks that correspond to the 2 different genotypes, as is shown in figure 1*B* and *C;* the estimated percentage of each genotype in the mixture is represented by the area underneath the peak for that genotype.

Blood samples from 26 malarial infections were analyzed by FRET/MCA and AS-PCR, to characterize codons 51 and 59. The FRET/MCA assay resulted in clearly interpretable melt curves for all samples (see examples in figure 1*D*): 20 infections were typed as monoclonal, and 6 were typed as biclonal, with the range in the relative proportions of clones being 10/90–45/55; and comparison with the T_m of reference DNA allowed the genotypes that were present to be identified as belonging to 1 of the 6 categories defined above. Interestingly, FRET/MCA typed fewer samples as polyclonal than did AS-PCR (12 vs. 26, respectively). We cannot exclude the possibility that our fluorogenic assay can miss clones that have frequencies of <10%. However, in light of the fact that AS-PCR is based on mispriming of the 3′-end primer, it seems more likely that AS-PCR resulted in false positives because of inadequate concentrations of template [9, 10]. We verified this in the present study by using 10-fold–dilution series of the reference recombinant clone FR-V1S, which contains a mutation at codon 51. Both wild-type and mutation AS-PCR for codon 51 gave positive results at high concentrations of DNA, whereas only mutation AS-PCR should give positive results for it. This finding proves that AS-PCR can result in false positives and that its reliability is indeed dependent on template concentration. FRET/MCA, on the other hand, identified codon 51 as containing a mutation (the expected genotype) in the entire 10-fold–dilution series, proving that the reliability of this assay is independent of template concentration (data not shown). Altogether, our results suggest that FRET/MCA is an adequate genotyping tool in monoclonal and polyclonal infections.

DISCUSSION

A carefully scrutinized design of probes/primers is an absolute requirement for the successful development of a FRET/MCA assay. Fortunately, supporting software, such as Meltcalc, is now available, which allows the prediction of the outcome of a FRET/MCA assay quite accurately before fluorogenic probes are purchased. Using this software, we developed a FRET/MCA assay that is innovative in several aspects. First, we demonstrated that a FRET/MCA assay can be performed successfully by use of a standard PCR cycler equipped with an optical module (such as Bio-Rad's iCycler). Thus far, most other FRET/MCA assays reported have used the LightCycler (Roche), which offers the advantage of rapid-cycle PCR but works with expensive capillaries and has low capacity [11]. Second, our assay did not use 2 adjacent fluorogenic probes, as do most reported FRET/MCA assays, but a fluorogenic primer/probe pair. This type of design is particularly interesting for difficult genomes, such as the AT-rich *Plasmodium* genome, where it is not always possible to choose 2 adjacent probes with the 5°C–10°C T_m difference that is required for a successful FRET/MCA [12]. Third, and most important, we have proven that FRET/MCA can verify whether point mutations in different codons of a gene are present within the same parasite clone: 6 different genotypes could be resolved. Furthermore, our assay permits the quantification of the different genotypes that may be present, without the drawbacks (e.g., dependence on template concentration) that are associated with techniques, such as AS-PCR, that are based on mispriming. In conclusion, FRET/MCA should open new doors in the research field of malaria and other infectious diseases, especially when polyclonal infections are present. The next challenge will be to expand this genotyping concept to the analysis of different gene loci.

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References

1. Plowe CV, Djimde A, Wellems TE, Diop S, Kouriba B, Doumbo OK. Community pyrimethamine-sulfadoxine use and prevalence of resistant *Plasmodium falciparum* genotypes in Mali: a model for deterring resistance. Am J Trop Med Hyg **1996**; 55:467–71.
2. Arnot D. Unstable malaria in Sudan: the influence of the dry season: clone multiplicity of *Plasmodium falciparum* infections in individuals exposed to variable levels of disease transmission. Trans R Soc Trop Med Hyg **1998**; 92:580–5.
3. Wittwer CT, Hermann MG, Moss AA, Rasmussen RP. Continuous fluorescence monitoring of rapid cycle DNA amplification. Biotechniques **1997**; 22:130–1, 134–8.
4. Cardullo RA, Agrawal S, Flores C, Wamecnik PC, Wolf DE. Detection of nucleic acid hybridization by nonradiative fluorescence resonance energy transfer. Proc Natl Acad Sci USA **1988**; 85:8790–4.
5. Plowe CV, Djimde A, Bouare M, Doumbo O, Wellems TE. Pyrimethamine and proguanil resistance–conferring mutations in *Plasmodium falciparum* dihydrofolate reductase: polymerase chain reaction methods for surveillance in Africa. Am J Trop Med Hyg **1995**; 52:565–8.
6. Schütz E, von Ashen N. Spreadsheet software for thermodynamic melting point prediction of oligonucleotide hybridization with and without mismatches. Biotechniques **1999**; 27:1218–24.
7. Plowe CV, Cortese JF, Djimde A, et al. Mutations in *Plasmodium falciparum* dihydrofolate reductase and dihydropteroate synthase and epidemiologic patterns of pyrimethamine-sulfadoxine use and resistance. J Infect Dis **1997**; 176:1590–6.
8. Peterson DS, Milhous WK, Wellems TE. Molecular basis of differential

resistance to cycloguanil and pyrimethamine in *Plasmodium falciparum* malaria. Proc Natl Acad Sci USA **1990**; 87:3018–22.
9. Chaparro J, Rojas MO, Wasserman M. *Plasmodium falciparum:* underestimation of dihydrofolate reductase and dihydropteroate synthase polymorphism in field samples: a technical shortcoming of nested PCR assays with mutation-specific primers. Exp Parasitol **2001**; 99:115–22.
10. Bottema CD, Sarkar G, Cassady JD, Ii S, Dutton CM, Sommer SS. Polymerase chain reaction amplification of specific alleles: a general method of detection mutation, polymorphisms, and haplotypes. Methods Enzymol **1993**; 218:388–402.
11. Bell AS, Ranford-Cartwright, LC. Real-time quantitative PCR in parasitology. Trends Parasitol **2002**; 18:337–42.
12. Lyon E. Mutation detection using fluorescent hybridization probes and melting curve analysis. Expert Rev Mol Diagn **2001**; 1:92–101.