

Use of Molecular Beacon Probes for Real-Time PCR Detection of *Plasmodium falciparum* and Other *Plasmodium* Species in Peripheral Blood Specimens

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We describe the development and evaluation of a novel pair of real-time, fluorescence-based PCR assays using molecular beacon probes for rapid, sensitive, and specific detection and quantification of *Plasmodium falciparum* and other *Plasmodium* species organisms.

Diagnosis of human malaria has traditionally rested on microscopic examination of stained peripheral blood films. While relatively inexpensive, this “gold standard” method is labor-intensive and time-consuming, requires an experienced microscopist, and may be insufficiently sensitive in detecting low-level parasitemia. Molecular diagnosis of malaria has recently attracted attention as a possible alternative to microscopy. Conventional PCR assays, while demonstrating increased sensitivity and specificity, remain labor-intensive, slow, and prone to potential amplicon contamination problems. The recent emergence of real-time PCR technology addresses these concerns by allowing for rapid, simultaneous amplification, detection, and quantification of target DNA through the use of specific fluorophore-labeled probes or nonspecific DNA-binding dyes. We describe a novel pair of molecular beacon probe-based real-time PCR assays for the sensitive and specific detection and quantification of human malaria parasites, with simultaneous differentiation of *Plasmodium falciparum* from other members of the genus.

Assay development and evaluation were performed at Calgary Laboratory Services, an integrated laboratory serving an urban population of 1.25 million people. Peripheral blood specimens for malaria diagnosis are routinely processed and analyzed according to CLSI standards (1, 15). Positive samples are further retained at -80°C for conventional species-specific PCR confirmation by a reference laboratory (Toronto General Hospital, Toronto, Canada). In the present study, 28 frozen nonduplicate microscopy-positive peripheral blood specimens and 50 randomly selected microscopy-negative specimens collected over a 25-month period from May 2001 to June 2003 were tested in a blind fashion. Template DNA was purified using QIAamp DNA blood mini kits (QIAGEN Inc., Alameda, CA). Two sets of oligonucleotide primers were designed to specifically amplify, respectively, a 120-bp region of the cytochrome *c* oxidase subunit 1 (*cox1*) mitochondrial gene unique to *P. falciparum* (forward primer, 5'-TTACATCAGGAATGTATTGC-3', and reverse primer, 5'-ATATTGGATCTCCTG

CAAAT-3') and a 105-bp conserved region of the 18S rRNA gene found in all *Plasmodium* species (forward primer, 5'-TAGTGTGTATCAATCGAGTT-3', and reverse primer, 5'-CTCTCCGGAATCGAACT-3'). Molecular beacon probes (5'-FAM-CTGGCCCTGGAGGAGTATTAATGTTATTATCGGCCAG-BHQ1-3' and 5'-FAM-CTCGCGCTTTTGATGTTAGGGTATTGGGCGAG-BHQ1-3') (where FAM is 6-carboxy-fluorescein, BHQ1 is black hole quencher-1, and underlined bases form the molecular beacon probe stem) were designed to hybridize to the aforementioned *cox1* and 18S rRNA amplicons, respectively. Real-time PCR was performed on the Stratagene Mx4000 system (Stratagene Inc., La Jolla, CA). Singleplex reactions were performed in 50- μl final volumes based on a previously described protocol (5), except for concentrations of MgCl_2 and the beacon probe of 6 mM and 0.4 μM , respectively. Thermocycling parameters were as previously described (5), except for an annealing temperature of 50°C and no final extension stage. Fluorescence data were measured during the annealing stage of amplification. The presence of a specific amplicon was established when the fluorescence intensity of the probe exceeded background fluorescence as defined by no-template controls included in each run. Diagnosis of non-*P. falciparum* malaria was based on a positive *Plasmodium* genus-specific PCR test and negative *P. falciparum* PCR test. Analytical sensitivity was determined by testing serial 10-fold dilutions of sequential peripheral blood specimens from patients with acute malaria and known parasitemia.

A total of 78 specimens were tested by real-time PCR, of which 11 were positive for *P. falciparum* and 16 for non-*P. falciparum* spp. There were no patients with mixed infections as determined by microscopy or conventional PCR. All 50 microscopy-negative specimens were negative by both real-time and conventional PCR. Complete concordance was noted apart from two discrepancies. One patient was presumed to have *P. falciparum* malaria on the basis of microscopy but had negative conventional and real-time PCR results; after reexamination of the blood films, no malarial parasites were observed. Additionally, one patient presumed to have *P. ovale* malaria on the basis of microscopy was found to be positive for *P. falciparum* by conventional and real-time PCR (Table 1). There was no significant amplification of no-template controls. Figure 1 shows representative fluorescence-versus-cycle curves for the 18S rRNA gene PCR assay. The sensitivity limit of our

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TABLE 1. Comparison of microscopy, conventional PCR, and real-time PCR for malaria diagnosis

Result	No. of patients with infection detected by indicated method		
	Microscopy	Conventional PCR	Real-time PCR
<i>P. falciparum</i> infection	11 ^a	11 ^b	11 ^b
<i>P. vivax</i> infection	14	14	16 ^c
<i>P. ovale</i> infection	3 ^b	2	
<i>P. malariae</i> infection	0	0	
Negative for malaria	50	51 ^a	51 ^a
Total	78	78	78

^a One patient presumed to have a *P. falciparum* infection by microscopy proved to be negative by conventional and real-time PCR assays.

^b One patient presumed to have a *P. ovale* infection by microscopy was determined to be infected with *P. falciparum* by conventional and real-time PCR assays.

^c The real-time PCR determined only the presence of *Plasmodium* species other than *P. falciparum* in 16 patients. The assay did not specifically determine *P. vivax* infection as opposed to *P. ovale* or *P. malariae* infection.

P. falciparum-specific real-time PCR assay was determined to be 0.004 parasite/ μ l blood, while that for the genus-specific assay was at least 0.16 parasite/ μ l, depending on the species tested.

Plasmodium falciparum and *P. vivax* account for the majority of malaria cases worldwide (19). *Plasmodium falciparum* typically causes the most severe disease and is usually rapidly fatal if untreated, while *P. vivax* and *P. ovale* produce dormant liver stages that may result in relapse of infection months to years later. Hence, it is essential to rapidly diagnose human malaria and differentiate diseases caused by *P. falciparum* from other *Plasmodium* species. The limit of detection of standard microscopy is estimated to be five parasites/ μ l blood, while traditional

and seminested-PCR assays have detection limits of ≤ 1 parasite/ μ l (8, 13, 14). Notwithstanding the robustness of conventional PCR methods, these assays have several limitations and are only semiquantitative in nature. The recent emergence of real-time PCR technology has overcome these pitfalls, while allowing for continuous monitoring of the PCR. Our experience demonstrates the power, speed, and ease of real-time PCR for malaria diagnosis. With a total assay time of 2 h, our assays are sufficiently robust for routine clinical use. To date, no other molecular beacon probe-based real-time PCR assays for malaria diagnosis have been reported prior to ours, although other investigators have described their experience with TaqMan probes (9, 10, 17, 18) and SYBR green dye (4, 6, 12). The *Plasmodium* 18S (small-subunit) rRNA gene has served as the prototypic target for genus-based malaria PCR assays (2–4, 6, 7, 9–12, 14, 16, 18), although the *cox1* gene has not previously been used for *P. falciparum* malaria identification. The main limitations of our method are the need for two separate PCRs, the lack of an internal control, and no specimens with *P. malariae*. While combining these two assays would enhance the attractiveness of our approach, multiplexing the *cox1* and 18S rRNA assays results in lower analytic sensitivity, especially if fluorophores other than FAM are used (unpublished observations). While recommended to ascertain adequate sample preparation and PCR amplification, we found that inclusion of an internal control (beta-globin gene) resulted in greatly decreased assay sensitivity (data not shown). While not intended to completely replace conventional microscopy, our assays would find the greatest utility in helping to rule out the presence of *P. falciparum* in patients with low-level parasitemia, as well as confirming the diagnosis of malaria in clinically compatible cases with negative microscopy results.

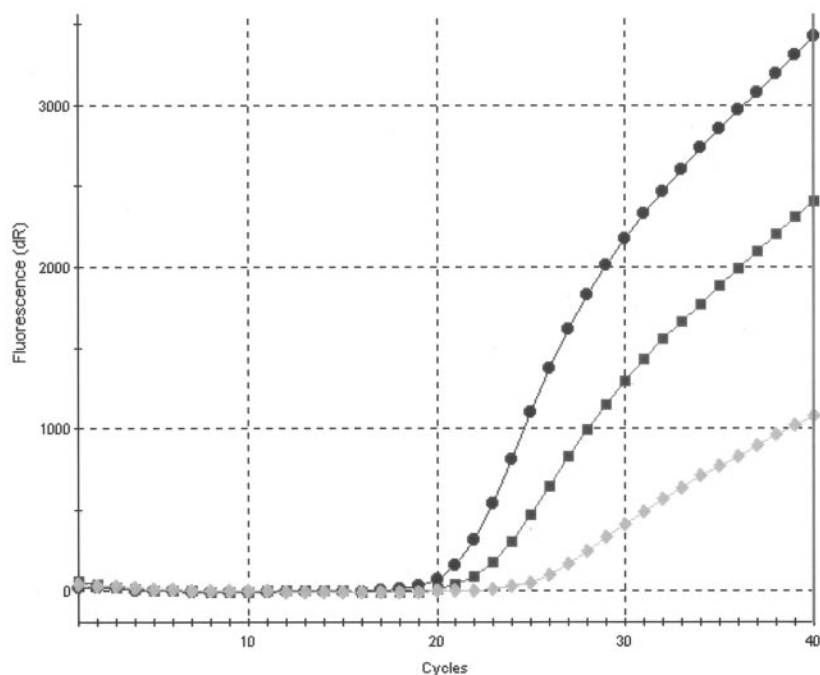


FIG. 1. Representative singleplex 18S rRNA gene (*Plasmodium* genus-specific) PCR fluorescence-versus-cycle curves for three clinical samples with various parasitemias as determined by microscopy.

While mixed infections cannot be diagnosed with our set of assays, this is of lesser immediate clinical importance, especially if *P. falciparum* can be ruled out, although this may be remedied by future enhancements to real-time PCR methodologies and to our existing assay.

Based on our observations (data not shown), probe fluorescence appears to correlate well with parasitemia, suggesting that real-time PCR may become the new "gold standard" for monitoring therapeutic responses to antimalarial agents. Despite the very high sensitivity of real-time PCR, repeat testing is indicated, based on clinical suspicion, if initial results are negative. At this time, however, the costs associated with routine implementation of molecular diagnostics for malaria diagnosis would be too prohibitive for countries where malaria is endemic.

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