

## Evaluation of a Colorimetric PCR-Based Assay To Diagnose *Plasmodium falciparum* Malaria in Travelers

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Received 23 June 1998/Returned for modification 20 August 1998/Accepted 2 November 1998

**New diagnostic tests are needed to facilitate the diagnosis of *Plasmodium falciparum* malaria in the returned traveler. We performed a blinded evaluation of a nonisotopic colorimetric PCR-based assay (Digene SHARP Signal System) and compared the results with those obtained by microscopy and nested PCR for the detection of *P. falciparum* malaria in 150 febrile travelers. By using nested PCR as the reference standard, the colorimetric assay had a sensitivity of 100% and a specificity of 95.4% for the detection of *P. falciparum*. This PCR-based nonisotopic assay is a rapid, sensitive, and specific method for the detection of falciparum malaria in returned travelers.**

The diagnosis of malaria has traditionally relied on the microscopic examination of Giemsa-stained blood smears. This process is time-consuming and labor-intensive, and accurate species identification is problematic in patients with low levels of parasitemia or those with mixed infections (1, 4, 5, 7, 11, 13). Alternative methods for the diagnosis of malaria are required. PCR-based methods have been demonstrated to be sensitive and specific for the detection of *Plasmodium falciparum* and *Plasmodium vivax* infections, with reported sensitivities approximately 10-fold greater than that of microscopy performed by experts (4–6, 11–13). Amplified products are usually detected by gel electrophoresis or by Southern or slot blotting followed by hybridization with DNA probes (1, 4–6, 9, 11–13). These approaches are often labor-intensive, require detection of radioactivity, or are otherwise poorly suited for use in general diagnostic laboratories. A non-isotopic, colorimetric PCR-based system for the detection and quantitation of PCR products in a microtiter format has been commercially developed (Digene SHARP Signal System). This method involves hybridization of a single-stranded RNA (ssRNA) probe to denatured biotinylated PCR products and capture of the RNA-DNA hybrids on a streptavidin-coated microtiter plate, followed by colorimetric detection with an enzyme-labeled antibody and a chromogenic substrate. We have previously shown this to be a sensitive and specific method for the diagnosis of *P. vivax* malaria (3).

In the present study, we undertook an evaluation of a colorimetric PCR-based assay for the diagnosis of *P. falciparum* malaria in febrile travelers. The results of this assay were compared with those of microscopic detection of *P. falciparum* and a nested PCR method for the diagnosis of falciparum malaria.

### MATERIALS AND METHODS

Patients presenting to the Tropical Disease Unit of the Toronto Hospital from June 1995 to July 1997 with a history of fever and travel to a area of endemicity for malaria were eligible for inclusion in this prospective study. All patients with positive malaria smears during the study period were enrolled. All patients with negative smears during the first 2 months of the study were enrolled to provide

a comparable control group. The prevalence of malaria during the study period was 31%. Whole-blood samples (pretreatment) were collected from all patients and placed into tubes with EDTA anticoagulant for thick and thin malaria smears, PCR, and complete blood count. Blood smears were interpreted by an expert microscopist who was unaware of the PCR results. Blood films were called negative if no parasites were seen in 500 oil-immersion fields (magnification,  $\times 1,000$ ) on a thick blood film. The parasite concentration was calculated by determining the number of parasites per 200 to 500 leukocytes in a thick blood film. The patients' baseline leukocyte counts were used to calculate the level of parasitemia (numbers of parasites per microliter). Demographic data were collected by patient interview and medical chart review. All specimens were coded, aliquoted, and frozen at  $-70^{\circ}\text{C}$  for further testing by PCR. The colorimetric and nested PCR assays were run, and the results were interpreted independently by an investigator who was blinded to the results of microscopy. This study was approved by the Ethical Review Committee of the Toronto Hospital.

The QIAamp blood kit (Qiagen, Chatsworth, Calif.) was used to extract genomic DNA from 200- $\mu\text{l}$  blood samples. For the Digene SHARP Signal System colorimetric PCR-based assay, a 206-bp segment specific for *P. falciparum* was amplified with 5  $\mu\text{l}$  of extracted genomic DNA and the K1-14 primer set, which included a biotinylated sense primer, as described previously (6, 11). A 5- $\mu\text{l}$  aliquot of the PCR mixture containing the 5' biotinylated product was hybridized with a K1-14 ssRNA probe. The resultant RNA-DNA hybrids were captured through biotin on the surfaces of streptavidin-coated microwells. Immobilized hybrids were then reacted with an antihybrid antibody conjugated to alkaline phosphatase and were detected with a colorimetric substrate. The absorbance at 405 nm was read after 2 and 20 h of substrate incubation. An absorbance reading of 0.1 was used as the cutoff value, as recommended by the manufacturer. As an independent confirmation of the species identification, a nested PCR method for the amplification of a fragment of the plasmoidal small-subunit RNA gene was performed as described previously (12). The sensitivity and specificity of the Digene kit for the diagnosis of *P. falciparum* infection were calculated by using nested PCR-based species identification as the reference standard. We used PCR as the reference standard on the basis of its reported superior performance characteristics over that of microscopy (1, 3–7, 9–13). Sensitivity was calculated as numbers of true positives/(numbers of true positives + numbers of false negatives), and specificity was calculated as numbers of true negatives/(numbers of true negatives + numbers of false positives). Steps for the prevention cross-contamination were taken as described previously (8).

### RESULTS

During the study period, 150 patients presenting with fever and travel to a area of endemicity for malaria were enrolled. The ratio of male patients to female patients was 1.6:1, and the mean age was 38 years (age range, 20 months to 80 years). Travel destinations included Africa (64.4%), the Indian subcontinent (16.1%), Latin America (14%), Oceania (3.4%), and Southeast Asia (2.3%). Patients infected with *P. falciparum* did not differ significantly from other patients with respect to age, sex, or duration of illness. Only 5% of travelers who acquired

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TABLE 1. Results of microscopy and nested PCR for diagnosis *P. falciparum* malaria in 150 febrile travelers

Malaria species <sup>a</sup>	No. of samples in which organisms were detected by the following method:	
	Microscopy <sup>b</sup>	Nested PCR
<i>P. falciparum</i>	50 Pf 1 Pf-Po 1 Pf-Pv	49 Pf, 1 Pf-Po 1 Pf 1 Pv
Non- <i>P. falciparum</i>	47 Pv 14 Po 1 Pm 1 Pv-Pm-Po 1 Pm-Po	45 Pv, 1 Po, 1 Pf 11 Po, 2 Pf-Po, 1 Pv 1 Pf-Pm 1 Pv 1 Po
<i>Plasmodium</i> spp. <sup>c</sup>	10 Psp	8 Pf, 2 Pv
Negative	24 Neg	24 Neg
Total	150	150

<sup>a</sup> Malaria species are classified according to the diagnosis obtained from the smear result.

<sup>b</sup> Pf, *P. falciparum*; Pm, *P. malariae*; Po, *P. ovale*; Pv, *P. vivax*; Psp, *Plasmodium* species; Neg, negative.

<sup>c</sup> Parasites too sparse on smears for accurate species identification.

malaria were compliant with appropriate chemoprophylaxis regimens (7).

The results of microscopic and nested PCR diagnosis of these infections are presented in Table 1. On the basis of repeatedly negative malaria smears and a negative PCR result, 24 travelers did not have malaria. Of the individuals with malaria, 39.7% (50 of 126) had *P. vivax* infection, 50% (63 of 126) had *P. falciparum* infection including 4 with mixed falciparum infections, and 10.3% (13 of 126) had *Plasmodium ovale* infection as determined by nested PCR. Complete or partial concordance between the results of microscopy and those of nested PCR was very good (98%). However, of the 52 patients with *P. falciparum* and mixed infections diagnosed by microscopy, nested PCR could not confirm one case as falciparum malaria. Of 64 samples in which non-falciparum malaria was diagnosed by microscopy, nested PCR identified an additional 4 *P. falciparum*-positive samples, with 3 of the 4 having mixed infections. Of 10 samples in which the level of parasitemia was too low for accurate species identification by microscopy, 8 were positive for *P. falciparum* by PCR. The results of the colorimetric assay in comparison with those of nested PCR are presented in Table 2. By using the nested PCR result as the reference standard, the colorimetric assay (with a 20-h substrate incubation) detected *P. falciparum* in all 63 *P. falciparum*-positive samples, giving a sensitivity of 100%. For 87 patients either without malaria or with malaria caused by a species other than *P. falciparum*, the colorimetric assay was negative for 83 of the patients (specificity, 95.4%). With a 2-h substrate incubation, the sensitivity was 88% and the specificity was 98%. The level of parasitemia in patients with *P. falciparum* infections ranged from 20 to 125,300 parasites/ $\mu$ l (mean, 23,860 parasites/ $\mu$ l). The correlation between the absorbance reading and the level of parasitemia, as determined by microscopy, was poor ( $r = 0.3$ ; data not shown).

## DISCUSSION

Microscopy is the traditional method for the diagnosis of malaria caused by *P. falciparum*. However, accurate species

determination may be difficult to achieve in all patients. In this study, as in others (1, 3–7, 10–13), diagnosis by microscopy is problematic in patients with mixed infections and for those with low levels of parasitemia. New molecular diagnostic tools are needed for *P. falciparum*. In the present study, we evaluated a new colorimetric PCR-based assay for the diagnosis of *P. falciparum* malaria in nonimmune travelers. On the basis of its previously demonstrated performance characteristics, we chose a nested PCR-based method as the reference standard for evaluation of this method. Compared to the reference method, the colorimetric assay had a sensitivity of 100% and a specificity of 95.4% for the detection of *P. falciparum* infection. All four false-positive samples were identified by nested PCR and microscopy to be infected with *P. vivax*. It is possible that these patients may have had mixed infections with *P. falciparum* at a level below that detectable by the nested PCR method. However, this seems less likely since these individuals did not receive therapy considered effective against *P. falciparum* but did not subsequently develop falciparum malaria as determined at a follow-up examination.

The colorimetric assay makes use of ssRNA probes which offer several advantages: RNA-DNA hybrids have greater thermal stability than DNA-DNA hybrids, allowing the use of higher temperatures with increased stringency (2); sensitivity is increased since the increased size of the ssRNA probes over the size of the oligonucleotide probes results in stronger and more specific hybridization and permits the incorporation of more label (14); RNA probes do not suffer from competing hybridization events as is the case with double-stranded DNA probes and therefore have increased rates of hybridization with the target; detection and quantitation of a variety of targets are possible; and the immunoassay-like system uses commercial reagents, a microtiter format, and an enzyme-linked immunosorbent assay reader, making it suitable for automation and for use in a routine diagnostic laboratory.

In conclusion, the colorimetric microtiter assay described here is a simple and accurate method for the detection of *P. falciparum*. It may be particularly useful for the identification of *P. falciparum* in patients with mixed infections or in patients with low circulating levels of parasitemia, for whom reliable species determination by microscopy is not always possible. A limitation of this test is that only falciparum malaria is detected; however, it may be combined with a similar assay for the diagnosis of *P. vivax* malaria (3).

TABLE 2. Results of colorimetric assay versus nested PCR for the diagnosis of *P. falciparum* malaria in 150 febrile travelers

Malaria species <sup>a</sup>	No. of samples with the indicated results by the following assay:	
	Nested PCR <sup>b</sup>	Digene assay <sup>c</sup>
<i>P. falciparum</i>	59 Pf 3 Pf-Po 1 Pf-Pm	59+ 3+ 1+
Non- <i>P. falciparum</i>	50 Pv 13 Po	46–, 4+ 13–
Negative	24 Neg	24–
Total	150	150

<sup>a</sup> Malaria species are classified according to the diagnosis obtained from the nested PCR result.

<sup>b</sup> Pf, *P. falciparum*; Pm, *P. malariae*; Po, *P. ovale*; Pv, *P. vivax*; Neg, negative.

<sup>c</sup> +, positive; –, negative.

## ACKNOWLEDGMENTS

This study was supported in part by the physicians of Ontario, Canada, by a grant from The Physicians Services Incorporated. K.C.K. was supported by a Career Scientist Award from the Ontario Ministry of Health.

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