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Real-Time Fluorescence-Based PCR for Detection of Malaria Parasites

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A fluorescence-based real-time 5' nuclease PCR capable of detecting all four human malaria parasites was developed to screen large numbers of samples during an outbreak to prevent further transmission of malaria. The effectiveness of antimalarial therapy for malaria patients can be monitored by determining the reduction of parasitemia by this method.

Malaria is a major tropical infection causing up to 500 million clinical cases and 2.7 million deaths per year worldwide. For proper treatment of malaria patients, accurate and rapid diagnosis of malaria is essential. The microscopic examination of a blood smear is the "gold standard" for malaria diagnosis. The method is sensitive and specific but laborious and time-consuming (6). Alternative methods, such as PCR and rapid antigen capture assays (ParaSight F test, OptiMAL rapid malaria test, and ICT Malaria P.f/P.v), have been developed. A PCR to detect *Plasmodium falciparum* and *P. vivax* was previously developed and evaluated (1, 7, 8, 9). We have developed a method for malaria diagnosis that can be adapted for high-throughput rapid screening of hundreds of samples with a sensitivity and specificity comparable to those of the microscopic method. The method is a fluorescence-based real-time 5' nuclease PCR based on the TaqMan technology (Roche Molecular Diagnostics Systems) (3, 4), with primers and a probe from the small-subunit (SSU) rRNA gene.

Malaria samples. Clinical blood samples were obtained from the Malaria Reference Center in the Department of Microbiology, National University of Singapore. Clinical samples of patients undergoing treatment were obtained from the National University Hospital, Singapore. The Gombak A strain of *P. falciparum* (5) was cultured by the method of Trager and Jensen (10). Two *P. ovale* (Po1 and Po2) and three *P. malariae* (Pm1, Pm2, and Pm3) samples were obtained.

DNA extraction. DNA was extracted from 200 μ l of EDTA-treated blood samples with a QIAamp DNA blood minikit (Qiagen GmbH, Hilden, Germany) and stored at 4°C.

Sequences of primers and probes. Primers (Mach 60 [5'-ACATGGCTATGACGGGTAACG-3'] and Mach 61 [5'-TGCCTTCCTTAGATGTGGTAGCTA-3']) and a probe (Mach 62 [5'-TCAGGCTCCCTCTCCGGAATCGA-3']) were designed to detect the SSU rRNA genes of all four human malarial

species (accession numbers M19172 for *P. falciparum*, X13926 for *P. vivax*, M54897 for *P. malariae*, and L48987 for *P. ovale*). The probe was labeled with a reporter dye, FAM (5-carboxy-fluorescein), and a quencher dye, TAMRA (*N,N,N,N*-tetramethyl-6-carboxyrhodamine) (Applied Biosystems, Foster City, Calif.).

5' nuclease PCR. Five microliters of DNA, 900 nM Mach 60, 300 nM Mach 61, 200 nM Mach 62, and 1 $\times$ TaqMan universal PCR master mix (Applied Biosystems) in a total reaction volume of 25 μ l were amplified on an iCycler (Bio-Rad Laboratories, Hercules, Calif.). The following conditions were used for PCR: an initial denaturation at 50°C for 2 min, 95°C for 10 min, and 45 cycles of 95°C for 15 s and 60°C for 1 min. The cycle threshold value (C_T) indicates the cycle where the fluorescence detected from the amplification of the quantity of the target gene exceeded the preset threshold.

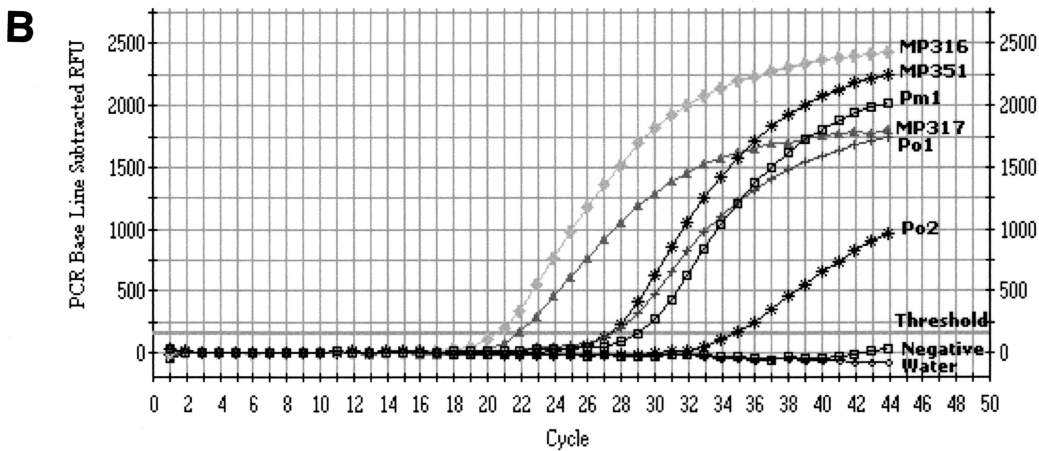
Specificity. The primers amplified an 84-bp fragment from DNA prepared from *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale* samples (Fig. 1A). The probe, designed specifically to detect the amplified PCR fragment, gave an increased fluorescent signal as the PCR progressed (Fig. 1B). The specificity of the method was evaluated with 153 blood samples, and the method was compared with blood film microscopic examination (Table 1). One hundred twenty-five samples were positive by both blood film examination and the fluorescence-based PCR method. Two other samples were positive by PCR but negative by blood film examination. These two samples were from patients who were undergoing antimalarial therapy, and the blood film method failed to detect the small amount of parasite DNA present on the last day of treatment; the PCR method, being more sensitive, could detect the parasites. All 26 samples that were negative by blood film were also negative by PCR. The positive samples had C_T values of 16 to 37, while the negative samples had C_T values greater than 38.

Sensitivity. The sensitivity of the method was determined by fivefold dilution of genomic DNA isolated from the cultured Gombak A strain of *P. falciparum*. Fluorescence can be detected from 90 ng (C_T , 16) to 0.002 pg (C_T , 35) of *P. falciparum*

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FIG. 1. Specificity of the PCR-based method for detecting four species of human malaria parasites. (A) Agarose gel electrophoresis of PCR-amplified products with SSU rRNA primers. Lanes: MP 317 and MP316, *P. falciparum* samples; MP 351, *P. vivax* sample; Po1 and Po2, *P. ovale* samples; Pm3, *P. malariae* sample; NC, noninfected sample (negative control); H₂O, no-template control; M, molecular size markers. (B) Amplification plot for the samples listed in the legend for panel A, with the threshold being set at 150 relative fluorescence units (RFU).



DNA. The amount of 0.002 pg of *P. falciparum* DNA is equivalent to 0.1 parasite, based on one genome equivalent of the parasite being 0.02 pg (2). The assays were repeated seven times and found to be reproducible, with the C_T value varying from 34 to 36 for 0.002 pg of DNA.

Monitoring antimalarial treatment. The PCR method was used to monitor the progress of the antimalarial treatment of three patients and compared with the microscopic examination of blood film. The numbers of parasites in the patients undergoing treatment were determined daily with fluorescence-based PCR and blood film methods. The parasitemia determined from the blood film for one patient fell from 0.45 to 0% from days 1 to 5 of treatment. The fluorescence PCR method indicated that the number of parasites detected fell from 2×10^4 parasites to 0.17 parasite per 5 μ l of blood from days 1 to

5, with the serially diluted cultured Gombak A strain being used as the standard. Both methods could monitor the reduction of parasitemia during the course of treatment, though the fluorescence-based PCR method was more sensitive and could detect very small numbers of parasites from the samples on the day before the patients were discharged. Similar results were obtained for two other patients undergoing antimalarial therapy (data not shown).

In summary, a real-time fluorescence PCR method with specific primers and a labeled probe of the SSU rRNA gene was developed for the detection of malaria parasites. This method has a sensitivity of 0.002 pg of *P. falciparum* DNA, which is equivalent to 0.1 parasite. The method is also specific for all *P. falciparum*, *P. malariae*, *P. ovale*, and *P. vivax* parasites, since all the samples that tested positive by blood film were also positive by real-time PCR.

The method would be useful in monitoring the effectiveness of antimalarial therapy, especially in situations where drug-resistant strains of the parasites are prevalent. Determining parasitemia by microscopy could be subjective, depending on the technician counting the numbers of parasites and red blood cells, and could vary from person to person and from day to day. The real-time PCR will provide an objective method for determining the parasitemia in the samples.

The method developed in this study will be useful in countries where malaria is not endemic and there is a lack of skilled technicians. A large number of negative samples may need to be screened for malaria as a measure to prevent further transmission of malaria. This was demonstrated in a recent malaria outbreak in Singapore, where 242 samples from asymptomatic

TABLE 1. Comparison of fluorescence-based PCR assays with microscopic blood film examination for detection of malaria parasites in blood samples

Species	No. of samples detected by:	
	Blood film	Fluorescence-based PCR
<i>P. falciparum</i>	42	42
<i>P. vivax</i>	74	76
<i>P. ovale</i>	2	2
<i>P. malariae</i>	3	3
Mixed species	4	4
None (negative)	28	26
Total	153	153

individuals suspected of harboring the malaria parasites were screened. One positive malaria case was detected by PCR but not from blood film examination (data not shown).

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