APPLICATION OF REAL-TIME POLYMERASE CHAIN REACTION (PCR) ANALYSIS FOR DETECTION AND DISCRIMINATION OF MALARIA PARASITE SPECIES IN THAI PATIENTS

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Abstract. A TaqMan real-time PCR system was used to detect and discriminate the 4 species of human malaria parasites in clinical blood samples. A 150-base pair (bp) region of the small subunit ribosomal RNA (SSU rRNA) gene of each malaria parasite, including species-specific sequences to be detected by TaqMan probe, was used as a target for PCR analysis. The PCR method used universal primers and species-specific TaqMan probes for *Plasmodium falciparum, P. vivax, P. ovale*, and *P. malariae*. The detection threshold for the method, as determined with serial dilution of cultured *P. falciparum*-infected erythrocytes, was 5 parasite-infected erythrocytes per reaction. Fifty blood samples of falciparum malaria and a second set of 50 samples of vivax malaria, diagnosed by microscopic examination at the Hospital for Tropical Diseases, Mahidol University, Thailand, were analyzed by real-time PCR. In the 50 samples of microscopically-diagnosed falciparum malaria, 40 were regarded as *P. falciparum* single infection, 7 were *P. falciparum* and *P. vivax* mixed infections, and 3 were *P. vivax* single infection by real-time PCR. In the second set of 50 samples of microscopically diagnosed vivax malaria, all were considered *P. vivax* single infection by PCR. Neither *P. ovale* nor *P. malariae* infection was identified in the 100 blood samples. Real-time PCR analysis was shown to be more sensitive and accurate than routine diagnostic methods. Application and extension of the PCR method reported here will provide a powerful tool for further studies of malaria.

INTRODUCTION

Accurate diagnosis of malaria and identification of the *Plasmodium* species responsible are essential for optimal patient management and for understanding the epidemiology of malaria. Microscopic examination of blood smears is the standard method for diagnosis of malaria. Although this method is sensitive and specific, it is subjective and time-consuming, particularly for detecting a small number of parasites. In addition, it is often very difficult to identify mixed infections (Snounou *et al*, 1993a; Rubio *et al*, 2002).

Various alternative diagnostic methods have been developed. These include fluorescence microscopy (Kawamoto and Billingsley, 1992), concentration techniques, such as the quantitative buffy coat method (Petersen and Marbiah, 1994), and immunological antigen capture assays such as malaria rapid diagnostic tests (Tham *et al*, 1999; Wongsrichanalai *et al*, 2003). However, persistence of the antigens in the blood-streams of patients might result in false positive results in an antigen capture assay. Thus, use of more than one test is recommended to confirm diagnosis.

In this study, we applied real-time PCR with TaqMan technology (Bell and Ranford-Cartwright, 2002) to analyze malaria parasite infections in clinical blood samples.

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MATERIALS AND METHODS

Parasite culture

The FCR-3 strain of *P. falciparum* was cultured by the method of Trager and Jensen (1976) followed by synchronization with sorbitol treatment (Lambros and Vanderberg, 1979). When the parasites reached the ring stage, the culture was diluted serially, and blood samples containing 5×10^5 to 5×10^1 parasite-infected erythrocytes were prepared. Genomic DNA was extracted from the samples, and of the amount, 1/10 was used as a template for PCR. The minimum number of parasite-infected erythrocytes per reaction was 5.

Preparation of plasmid DNA

Approximately 150-bp of the small subunit ribosomal RNA (SSU rRNA) gene sequence was amplified from the parasite DNA with the universal

primers shown in Table 1. Genomic DNA served as the PCR template and was isolated from the clinical blood samples that contained *P. vivax*, *P. ovale*, or *P. malariae*, or from cultured blood for *P. falciparum*. The DNA fragment was cloned into the plasmid vector pCR® 2.1-TOPO® (Invitrogen Corp, Carlsbad, CA, USA), and the sequence was confirmed by cycle sequencing (Applied Biosystems, Foster City, CA, USA).

TaqMan PCR

The universal primers and TaqMan probes used are shown in Table 1. The TaqMan probes were labeled with a reporter dye, 5-carboxyfluorescein (FAM), and conjugated with minor groove binder (MGB) for increased sensitivity and decreased background fluorescence (Kutyavin *et al*, 2000). The probes were designed with Primer Express software (Applied Biosystems) based on the SSU rRNA gene sequence (Genbank accession numbers of the 4 parasite species are shown in Table 1). The primers and probes were synthesized and HPLC purified by Applied Biosystems Japan (Tokyo, Japan).

Real-time PCR was performed according to the manufacturer's instructions (Applied Biosystems). Briefly, 5 µl template DNA, 300 nM forward primer, 300 nM reverse primer, 100 nM TaqMan MGB probe and 1x Universal Master Mix in a total reaction volume of 25 µl were amplified on ABI PRISM 7700 or 7900 Sequence Detection Systems (SDS) (Applied Biosystems). The following PCR conditions were used: optimization of AmpErase uracil-N-glycosylase (Applied Biosystems) activity at 50°C for 2 minutes, initial hot start at 95°C for 10 minutes and 50 cycles of 95°C for 15 seconds and 60°C for 1 minute. The fluorescence signal was detected and analyzed with ABI PRISM SDS software (Applied Biosystems). Plasmid DNA in the range of 0.001fg -10pg was used as a quantification standard for the SDS calibration curve.

Clinical blood samples

One hundred blood samples were obtained from patients at the Hospital for Tropical Diseases, Mahidol University, Thailand, in 1997. The patients provided written consent, and this study was approved by the Ethics Committee of Mahidol University. The blood was collected with EDTA, and thin blood smears were prepared. The blood smears were examined microscopically by the laboratory staff of the hospital. *P. falciparum* was confirmed in 50 samples, and *P. vivax* was confirmed in a second set of 50 samples. The blood samples were stored at -20°C until DNA extraction.

DNA extraction from blood samples

The frozen blood samples were thawed on ice, and 250 µl were taken for DNA extraction. Five hundred µl DNAzol® BD reagent (Invitrogen) were mixed in, 200 µl isopropanol were added, and the reaction was incubated for 5 minutes at room temperature. The samples were centrifuged at 6,000*g* for 6 minutes to sediment the precipitated DNA. The DNA pellets were washed with DNAzol® BD reagent and then washed with 75% ethanol. After removing the ethanol, the DNA pellets were dissolved in 50 µl 8 mM NaOH and frozen at -20°C until use.

Table 1
Sequence of primers and oligonucleotide probes used for the detection of SSU rRNA genes of the malaria parasites.

Primers/probes	Sequence (5'→3')	Position[a]	Origin	
Primer				
Forward	ACGATCAGATACCGTCGTAATCTT	1062-1085	M19172,	Kimura *et al*, 1997
Reverse	GAACCCAAAGACTTTGATTTCTCAT	1180-1204	M19172,	Kimura *et al*, 1997
Probe[b]				
Pf-probe	CATCTTTCGAGGTGACTT	1138-1155	M19172	
Pv-probe	TCTCTTCGGAGTTTAT	1471-1486	X13926	
Po-probe	TTTCCCCGAAAGGA	1152-1165	L48987	
Pm-probe	AGAGACATTCTTATATATGAGTG	1173-1195	M54897	

[a]Positions correspond to the 18S rRNA sequence.
[b]Pf = *P. falciparum;* Pv = *P. vivax;* Po = *P. ovale;* Pm = *P. malariae*

Statistical analysis

Nonparametric analysis was performed with the Mann-Whitney *U* test. p-values < 0.05 were considered to be statistically significant.

RESULTS

Specificity of PCR method

To test the specificity of the probes for the SSU rRNA genes of the 4 species of *Plasmodium*, PCR was performed with a species-specific gene fragment in the plasmid as a template. The amplification plot with the *P. falciparum*-specific probe is shown in Fig 1. Only signal from the *P. falciparum* plasmid DNA was detected, whereas signals from other species were not detected. The probe detected *P. falciparum* plasmid DNA in a concentration-dependent manner. The specificity of the probe was confirmed with the use of cultured parasite DNA as a template. Equivalent specificity of the probes for the other 3 parasite species was obtained with the use of species-specific genes in the plasmid as the template DNA (data not shown).

Sensitivity of PCR method

The sensitivity of the method was determined with a 10-fold dilution of cultured *P. falciparum*-infected erythrocytes. *P. falciparum* DNA could be detected in a reaction containing as few as 5 parasite-infected erythrocytes.

Application of PCR method to clinical blood samples

DNA extracted from patient blood was analyzed by real-time PCR (Table 2). Parasite DNA was detected in 100 blood samples. In 1 group of 50 samples, which were diagnosed as *P. falciparum* infection by microscopic examination, *P. falciparum* DNA alone was detected in 40 samples, and *P. vivax* DNA alone was detected in 3 samples. Both *P. falciparum* DNA and *P. vivax* DNA were found in 7 samples. In the second group of 50 samples, which were diagnosed as *P. vivax* infection by microscopic examination, *P. vivax* DNA alone was detected in all 50 samples. DNA of other parasite species was not detected in these samples.

DISCUSSION

Several techniques for the detection of malaria parasite DNA and RNA have been developed. These techniques, including standard PCR (Snounou *et al*, 1993a), nested PCR (Snounou *et al*, 1993b; Kimura *et al*, 1997; Rubio *et al*, 2002), and DNA probe assay (McLaughlin *et al*, 1993), are objective and have advantages in specificity and sensitivity. In the present study, we applied real-time PCR with TaqMan technology for the detection and discrimination of human malaria parasite species. An approximate 150-bp region of the SSU rRNA gene of *Plasmodium* (Waters and MacCutchan, 1989) was amplified with interspecies conserved universal primers and was detected with species-specific TaqMan MGB probes. In experiments with the plasmid-cloned SSU rRNA gene fragment as a template, probes specific for each parasite species only amplified DNA corresponding to each species, with no cross-reactivity with that of other species. In experiments with DNA from cultured *P. falciparum*-infected erythrocytes as the template, the detection and discrimination threshold for the PCR method was 5 parasite-infected erythrocytes per reaction. Equivalent sensitivity was determined for the 3 other parasite species. It has been reported that microscopy can routinely detect as few as 10-100 malaria parasites per µl of blood (Rubio *et al*, 2002).

Table 2
Comparison of real-time PCR and microscopic examination for diagnosis of malaria in blood samples from Thai patients.

Microscopy		Real-time PCR[b]				
		Pf	Pv	Pf + Pv	Po	Pm
Pf	50	40	3[a]	7	0	0
Pv	50	0	50	0	0	0
Total	100	40	53	7	0	0

[a] Numbers underlined indicate discrepant results between the 2 methods.
[b] Pf: *P. falciparum*; Pv: *P. vivax*; Po: *P. ovale*; Pm: *P. malariae*

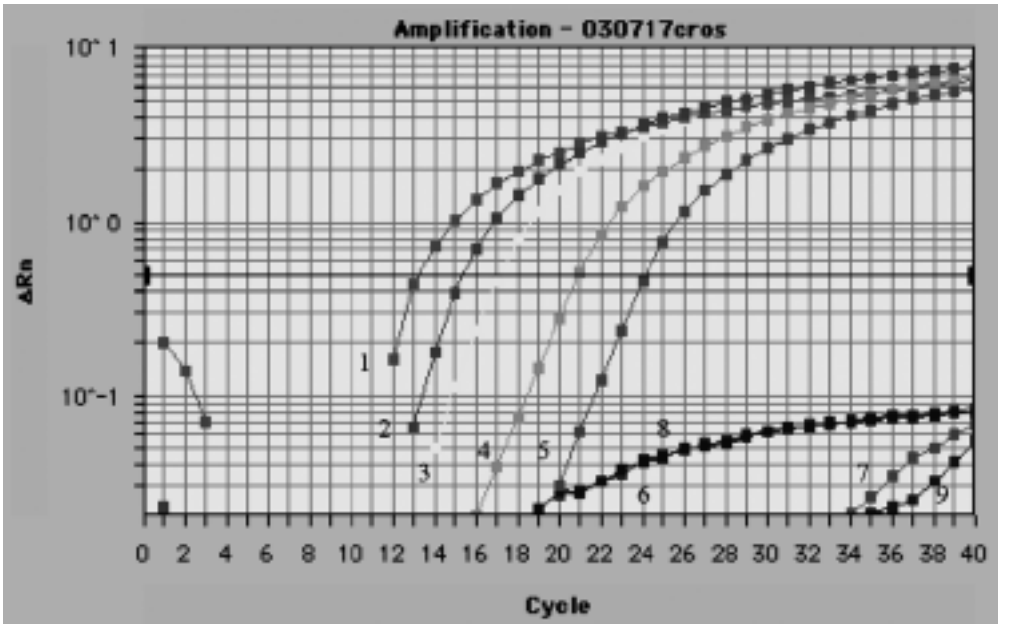

Fig 1- Amplification plot of fluorescence against cycle number for 4 SSU rRNA genes of human malaria parasites, *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*. ΔRn is relative fluorescence units. One hundred nM TaqMan MGB probes specific for *P. falciparum* and 300 nM universal primers for *Plasmodium* SSU rRNA gene were used. The templates were composed of plasmid DNA with a species-specific SSU rRNA gene. Each curve indicates the template used:

1-5 = plasmid DNA with the *P. falciparum* gene, 10 ng, 1ng, 100 pg, 10 pg, and 1 pg, respectively; 6 = plasmid DNA with the *P. vivax* gene, 10 pg; 7 = plasmid DNA with the *P. ovale* gene, 10 pg; 8 = plasmid DNA with the *P. malariae* gene, 10 pg; 9 = No template control.

Thus, the sensitivity of the real-time PCR method reported here is apparently higher than that of microscopic examination.

To evaluate the real-time PCR method, blood samples from Thai patients who were diagnosed by microscopy were utilized. Interestingly, comparison of methods for the 100 blood samples showed that the PCR method could detect mixed infection in 7% of the samples, whereas microscopic observation detected only a single infection for the same samples (Table 2). All samples diagnosed as mixed infection by PCR showed relatively low parasite counts by microscopy. Mean parasitemia in the group with *P. falciparum* single infection was 0.82%, whereas that in the group with mixed infection was 0.17% ($p<0.05$). It is known that *P. vivax* and *P. falciparum* in the ring/early trophozoite stage are difficult to distinguish, particularly when the level of parasitemia is low (Snounou *et al*, 1993b), and microscopic diagnosis may only be successfully performed when the late asexual stages of *P. vivax* are present in the sample (Rubio *et al*, 2002).

Recently, the real-time PCR method, with other systems, such as SYBR® Green I have been used in malaria research (Polanco *et al*, 2002; Cheesman *et al*, 2003; de Monbrison *et al*, 2003). However, nonspecific binding of SYBR® Green I to primer-dimers and spurious amplicons must be minimized by careful optimization of the reaction conditions (Bell and Ranford-Cartwright, 2002). In the present study, we found that real-time PCR with target-specific TaqMan MGB probes allowed for high-performance detection and discrimination of parasite DNA without complicated optimization procedures. Because this PCR method uses standard cycling conditions and reagent concentrations, it permits simultaneous assay of multiple targets. This method may be useful in monitoring the effectiveness of malaria chemotherapy in situations where drug-resistant strains are prevalent. Drug-resistant genes of the parasite, such as *pfcrt* and *pfmdr-1*, would be used as targets. Further application of the real-time PCR method with allele-specific probes for other genetic markers of malaria parasites, or for human genetic markers associated with susceptibility, may increase the efficacy of treatment as well as the prevention of this most globally prominent infectious disease.

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