EVALUATION OF A REAL-TIME POLYMERASE CHAIN REACTION ASSAY FOR THE DIAGNOSIS OF MALARIA IN PATIENTS FROM THAILAND

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Abstract. We compared the diagnosis of malaria in 297 patients from Thailand by a real-time polymerase chain reaction (PCR) assay using the LightCycler with conventional microscopy using Giemsa-stained thick and thin blood films. The PCR assay can be completed in one hour and has the potential to detect and identify four species of Plasmodium in a single reaction by use of melting temperature curve analysis (however, we did not detect Plasmodium ovale in this study). Blood was collected, stored, and transported on IsoCode STIX, which provide a stable matrix for the archiving and rapid simple extraction of DNA. A genus-specific primer set corresponding to the 18S ribosomal RNA was used to amplify the target sequence. Fluorescence resonance energy technology hybridization probes were designed for P. falciparum over a region containing basepair mismatches, which allowed differentiation of the other Plasmodium species. The PCR results correlated with the microscopic results in 282 (95%) of 297 patient specimens. Most of these were single-species infections caused by P. vivax (150) and P. falciparum (120), along with 5 P. malariae, 2 mixed infections (P. falciparum and P. vivax), and 5 negative specimens. No negative microscopy specimens were positive by PCR (100% specificity for detection of any Plasmodium). The 15 discrepant results could not be resolved, but given the subjective nature of microscopy and the analytical objectivity of the PCR, the PCR results may be correct. The ability of the PCR method to detect mixed infections or to detect P. ovale could not be determined in this study. Within the limitations of initial equipment costs, this real-time PCR assay is a rapid, accurate, and efficient method for the specific diagnosis of malaria. It may have application in clinical laboratories, as well as in epidemiologic studies and antimalarial efficacy trials.

INTRODUCTION

Malaria is a major tropical disease infecting approximately 500 million people and causing 1.5–2.7 million deaths annually. Ninety percent of the deaths occur in sub-Saharan Africa and most of these occur in children less than five years old; it is the leading cause of mortality in this age group. This disease is also widespread in Central and South America, Hispaniola, the Indian subcontinent, the Middle East, Oceania, and Southeast Asia. Microscopy of Giemsa-stained thick and thin blood films is the standard laboratory method for the diagnosis of malaria. However, in resource-poor areas, microscopic diagnosis has been shown to be insensitive and nonspecific, especially when parasitemias are low or mixed infections are present. Under optimal conditions, the sensitivity of thick film microscopy is 10–30 parasites per microliter of blood. In field conditions, sensitivities and specificities as low as 71–72% have been reported. A variety of polymerase chain reaction (PCR) methods have been developed and shown to increase the sensitivity and specificity of detection, as well as the identification of mixed infections. In particular, use of a PCR was shown to increase the detection of placental malaria from 42% to 97% and asymptomatic parasitemia from 17% to 47%. However, utility of PCR is limited by the complex methodologies and need for specially trained technologists. The introduction of automated real-time PCR that is rapid and does not require multiple complex procedures or skilled technologists has raised the possibility that these methods may have wider application in the sensitive and specific diagnosis of malaria. We have developed a real-time PCR assay using fluorescence resonance energy technology (FRET) and the LightCycler (Roche Applied Science, Indianapolis, IN) instrument. This method has the advantage of allowing amplification and detection of four species of Plasmodium using one set of primers and probes for the 18S ribosomal RNA (rRNA) gene in a single run of the assay, which takes less than one hour to complete. Identification of the species present is accomplished by melt curve analysis of the amplified product. This assay was evaluated using blood from 297 patients from Thailand who were suspected of having malaria. The blood was collected, stored, and transported on IsoCode STIX (Schleicher & Schuell, Keene, NH), which provide a stable matrix for the archiving and rapid, simple extraction of DNA. The STIX have been shown to be comparable to the QIAmp Mini Kit (Qiagen, Valencia, CA) in the extraction of DNA from Bacillus anthracis and had a sensitivity ≥ 96% for the detection of single-species Plasmodium infection by PCR. Real-time PCR results were compared with those of conventional microscopy performed on site in Thailand.

MATERIALS AND METHODS

Blood samples for PCR. This study was reviewed and approved by the Institutional Review Board of the Mayo Foundation and by the Ethics Committee, Faculty of Tropical Medicine, Mahidol University. Blood samples were collected from 297 patients with clinical symptoms of malaria at an outpatient clinic in Bangkok, Thailand. Blood from a finger stick on each of the patients was applied to each of four triangles (10–12 μL per triangle) of the IsoCode STIX (Schleicher & Schuell) for preservation and transport to the Mayo Clinic clinical microbiology laboratory in Rochester, Minnesota. The IsoCode STIX was folded into a tent-like formation and allowed to air-dry at least three hours. After complete preservation and transport to the Mayo Clinic clinical microbiology laboratory in Rochester, Minnesota. The IsoCode STIX was folded into a tent-like formation and allowed to air-dry at least three hours.
Thick and thin blood smears were prepared from finger sticks at the same time that the IsoCode STIX samples were obtained. The slides were stained with Giemsa and examined microscopically by experienced technologists in the Hospital for Tropical Diseases, Faculty of Tropical Medicine, Mahidol University in Bangkok. The parasitemia was recorded as number of Plasmodium counted per 200 white blood cells present in the thick smear.

Positive control plasmid for PCR. A patient sample shown to be positive by microscopy and a second PCR assay were used to generate a plasmid containing a portion of the Plasmodium 18S rRNA gene for use as a positive control. A plasmid containing the PCR product was constructed using the PCR 2.1-TOPO Vector five minute PCR cloning kit (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions. Plasmid purification was performed with the High Pure Plasmid Isolation Kit (Roche Applied Science). Dilutions of the positive control (1–100 copies/μL) were prepared using PCR-grade water and stored at 4°C. These dilutions were spiked into normal blood samples for determining analytical sensitivity.

Extraction method. Two triangles of the IsoCode STIX from each patient specimen were placed in a 2.0-ml screw-capped tube containing 500 μL of sterile water. The tubes were vortexed at full speed three times for five seconds. After a brief centrifugation, the supernatant was removed and discarded. Sterile water (100 μL) was added to each tube and placed in a 95°C dry heat block for 30 minutes. The tubes were briefly centrifuged and 100 μL of S.T.A.R. buffer (Roche Applied Science) was added. The supernatant (approximately 200 μL) was transferred to a MagNa Pure sample cartridge for automated DNA extraction on the MagNa Pure LC System (Roche Applied Science) using the Total Nucleic Acid Isolation Kit (Roche Applied Science) with a final elution volume of 100 μL. Positive plasmid controls (Plasmodium falciparum and P. vivax) at a concentration of 100 targets/μL in S.T.A.R. buffer and a blank buffer control were included with each extraction run. Although simple extraction from the IsoCode STIX has been found to be adequate for PCR amplification, the additional processing with the MagNa Pure was performed because this has been found to confer greater stability on extracts that are stored for long periods of time (Muyombwe A, Rosenblatt J, unpublished data).

Amplification and detection. A genus-specific primer set corresponding to the 18S rRNA was used to amplify the target sequence. FRET hybridization probes were designed for Plasmodium species targeting Plasmodium falciparum over a region containing base pair mismatches allowing for differentiation of the other Plasmodium species by the use of melt curve analysis. The primer and hybridization probes (TIB Molbio, Berlin, Germany) and melting temperatures are listed in Table 1. All PCRs were performed using the LightCycler-FastStart DNA Master Hybridization Probes kit (Roche Applied Science). Each reaction had a final concentration of 4 mM MgCl2, 0.5 μM primer PF1, 1.0 μM primer PF2, 0.2 μM probe PF3, and 0.4 μM probe PF4. The final volume of each LightCycler reaction was 20 μL (15 μL of PCR master mixture and 5 μL of sample). The amplification and detection was carried out using the LightCycler System with the following thermal profile: denaturation at 95°C for 10 minutes, amplification for 45 cycles at 95°C for 10 seconds, 55°C for 15 seconds (single acquisition), and 72°C for 15 seconds, melting at 95°C for 0 seconds, 59°C for 20 seconds, 40°C for 20 seconds at 0.2°C/second, 85°C for 0 seconds at 0.2°C/second (continuous acquisition), and cool down at 40°C for 30 seconds.

Amplification of the different Plasmodium species yield products with different melting temperature curves. A melting temperature of 60 ± 2.0°C indicates P. falciparum, a melting temperature of 57 ± 1.0°C indicates P. malariae, a melting temperature of 51.8–55.5°C indicates P. ovale, and a melting temperature of 49.5 ± 1.0°C indicates P. vivax. Figure 1 shows melting temperature analyses of positive control plasmids of

![Fluorescence vs Temperature](image)

**Figure 1.** Melting temperature curves of positive control plasmids of the four species of Plasmodium following amplification by the LightCycler polymerase chain reaction assay. Fluorescence peaks occurring at different melt temperatures permit differentiation of the amplicons of each species.

<table>
<thead>
<tr>
<th>Primer-probe</th>
<th>Nucleotide sequence (5’ → 3’)</th>
<th>Parasite targeted</th>
<th>Melting temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PF1</td>
<td>CATTYGATTACAGATGTC</td>
<td>Plasmodium species</td>
<td></td>
</tr>
<tr>
<td>PF2</td>
<td>TCTTTTTAACTTTCCTGC</td>
<td>Plasmodium species</td>
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</tr>
<tr>
<td>PF3</td>
<td>GATACCGGTGTAATCTTAAACCTAAGC TAT-FL</td>
<td>P. falciparum</td>
<td></td>
</tr>
<tr>
<td>PF4</td>
<td>LC RED640 GACTAGGTGGTTGATGAAAGTG-PO</td>
<td>P. malariae</td>
<td>60.0 ± 2.0</td>
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<tr>
<td></td>
<td>GACTAGGTGGTTGATGAAAGTG</td>
<td>P. falciparum</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GACTGGHITGGATGAAAGTG</td>
<td>P. vivax</td>
<td>51.8–55.5</td>
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<tr>
<td></td>
<td>GACTGGHITGGATGAAAGTG</td>
<td>P. ovale</td>
<td>49.5 ± 1.0</td>
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</tbody>
</table>

* Lower case letters indicate base pair mismatches in the LC RED640 hybridization probe that result in the appropriate temperature shift.

**Table 1.** Characteristics of Plasmodium primer and hybridization probe sets

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the four species of \textit{Plasmodium}, following amplification by the LightCycler PCR assay. Mixed infections present as a double curve with appropriate species temperatures. Figure 2 shows melting temperature analysis from one of the patients with a mixed infection of \textit{P. vivax} and \textit{P. falciparum}. Two separate temperature peaks are present that correspond to the peaks for the plasmid controls for each organism.

**RESULTS**

**Analytical sensitivity and specificity of the PCR assay.** Based on studies with the plasmid control, the minimum detectable level of DNA was 10 targets/μL of blood. Although the assay was not developed to be quantitative, it routinely was positive with clinical specimens whose parasitemia was reported as low as a single ring form per 200 white blood cells. Microorganisms other than \textit{Plasmodium} were tested with this assay to determine specificity. These included \textit{Anaplasma phagocytophilum}, Babesia microti, Bartonella henselae, \textit{B. quintana}, Borrelia burgdorferi, \textit{B. afzelii}, \textit{B. garinii}, \textit{B. andersonii}, \textit{B. japonica}, \textit{Ehrlichia risticii}, \textit{E. canis}, \textit{Escherichia coli}, Epstein-Barr virus, herpes simplex virus, \textit{Staphylococcus aureus}, \textit{S. epidermidis}, \textit{Streptococcus pyogenes}, \textit{Toxoplasma gondii}, and \textit{Tropheryma whippelii}. None were positive. Twenty-eight blood specimens from healthy persons were also tested; none were positive These results indicated an analytical specificity of 100%.

**Clinical specimens.** The microscopic results from the laboratory in Thailand were blinded from the technologist running the LightCycler PCR assay. Two hundred ninety-two blood samples were positive for the presence of any \textit{Plasmodium} species by light microscopy and five were negative. Overall, the LightCycler PCR results correlated with microscopic results in 282 (95.9%) of 292 patient specimens (Table 2). Most were single species infections caused by \textit{P. vivax} (150) and \textit{P. falciparum} (120). Results of both microscopy and PCR correlated in 270 specimens, along with 5 \textit{P. malariae}, 2 of the 4 mixed infections (\textit{P. falciparum} and \textit{P. vivax}) that were diagnosed by microscopy, and 5 negative specimens. No specimens negative by microscopy were positive by PCR (specificity = 100% for detection of any \textit{Plasmodium}).

Fifteen specimens were discrepant when we compared microscopy with PCR. All of these specimens were restested in the PCR assay using both the original DNA extract and a fresh DNA extract with the two remaining triangles from the IsoCode STIX kit. Eight of the 15 specimens were PCR negative and microscopy positive for one of the \textit{Plasmodium} species (284 of 292; sensitivity = 97% for detection of any \textit{Plasmodium} compared with microscopy). These included 3 \textit{P. falciparum}, 3 \textit{P. vivax}, and 2 \textit{P. ovale} that were reported by microscopy but had negative PCR results. Of five specimens that were identified as \textit{P. falciparum} by melting temperature in the LightCycler PCR assay, three were identified as \textit{P. vivax} by microscopy and two were identified as 4 mixed infections with \textit{P. falciparum} and \textit{P. vivax} microscopically. Another discordant specimen was identified as \textit{P. vivax} by PCR melting temperature and as \textit{P. falciparum} by microscopy. The final discrepant specimen had double melting temperature curves, which indicated a mixed infection with \textit{P. falciparum} and \textit{P. vivax} and was identified as \textit{P. vivax} by microscopy. Thus, the sensitivity of the PCR compared with microscopy was 97% (124 of 128) for detection of \textit{P. falciparum} and 95% (153 of 161) for detection of \textit{P. vivax}.

**DISCUSSION**

We have demonstrated that compared with conventional microscopy, the real-time LightCycler PCR assay is a sensitive and specific method for the detection of \textit{Plasmodium} in

<table>
<thead>
<tr>
<th>Microscopy</th>
<th>\textit{P. falciparum}</th>
<th>\textit{P. malariae}</th>
<th>\textit{P. ovale}</th>
<th>\textit{P. vivax}</th>
<th>Mixed infections</th>
<th>Negative</th>
<th>Total</th>
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<tr>
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<td></td>
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<tr>
<td>\textit{P. falciparum}</td>
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<td></td>
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<td></td>
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<tr>
<td>\textit{P. malariae}</td>
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<td>5</td>
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<td></td>
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<td></td>
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<td>\textit{P. ovale}</td>
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<tr>
<td>\textit{P. vivax}</td>
<td>1</td>
<td></td>
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<td></td>
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<tr>
<td>Mixed infections</td>
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<td></td>
<td>151</td>
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<td>124</td>
<td>5</td>
<td>2</td>
<td>157</td>
<td>4</td>
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blood specimens that have been stored and transported and whose DNA has been extracted from IsoCode STIX. We have obtained similar results using fresh whole blood and blood in EDTA (Sloan L, Rosenblatt J, unpublished data). Moreover, the PCR assay is rapid (turnaround time of one hour), simple to perform, and does not require technologists skilled in malaria diagnosis. It also relies on standardized objective criteria for determining results rather than the considerable subjectivity inherent in microscopy. In this study, PCR results were compared with those of conventional microscopy to determine a sensitivity and specificity of 97% and 100% for detection of any Plasmodium species. Because microscopy cannot be considered a true gold standard, it is difficult to resolve the discrepancies between the two methods that occurred. The eight microscopy-positive PCR-negative results suggest a lack of sensitivity for the PCR at lower levels of parasitemia, but our own experience (Stauffer W, Sloan L, Rosenblatt J, unpublished data) and that of others suggests that PCR is more sensitive than microscopy in detecting low levels of parasitemia in asymptomatic patients from malaria-endemic areas. These may have been false-positive results by microscopy. In addition, other factors, such as inadequate sample or deterioration in storage and transport, may have affected PCR sensitivity. It was not possible to determine if each sample contained the same volume of blood and if each was handled the same way throughout the entire process. The remaining seven discrepancies represented different species identifications by the two methods, resulting in sensitivity calculations of 97% and 95% for detection of P. falciparum and P. vivax, respectively, by PCR. These included three mixed infections detected by one method but not the other (two by microscopy and one by PCR). It is difficult to resolve these discrepancies, but the objective characteristics of distinct species sites by melting curve analysis compared with the subjectivity of microscopy suggests that the PCR results were correct. Although both methods identified P. malariae in five specimens, this is insufficient to ensure the accuracy of the PCR with this species. Two P. ovale microscopy-positive specimens were negative by PCR. However, using plasmid controls and other sets of specimens, we have successfully detected and identified this species (Sloan L, Rosenblatt J, unpublished data). Therefore, the assay has potential usefulness for species identification.

We have discussed the possible reasons for the lack of sensitivity of the PCR. The question of detection of mixed infections by this assay remains unresolved. Greater experience with such specimens is needed. We have demonstrated the ability to detect multiple melting curve peaks in a single specimen. However, since the FRET probes were designed for P. falciparum (although over a target sequence region that would detect all four species), detection of P. falciparum might overshadow that of another species present in a mixed infection. Another question that was not resolved in this study is the performance of PCR when only gametocytes are present. Although we were not able to assess this question, we presume that the assay results would be positive in such cases and one must be cognizant of this possibility.

The added capability of species identification by melting curve analysis is a significant advantage of our method compared with similar assays. This ability to provide accurate species identification in the same sample used for detection of the parasite is the single greatest attribute of this method. Because prognosis and treatment are different, it is extremely important to rapidly and correctly identify the infecting species. It is especially important to be sure that P. falciparum is present, although this identification may be difficult for unskilled microscopists. In addition, the assay could be potentially designed to quantitate the level of parasitemia in patient samples. This can be done by correlating crossing points (PCR cycle number at which an amplicon is first detected) with parasitemia determined by microscopy of patient specimens or blood seeded with cultured Plasmodium. Although we did not perform this in our study, others have made initial attempts to quantitate parasitemia using real-time PCR.

The exact role of this LightCycler PCR assay in the clinical diagnosis of malaria has not yet been determined. It is unlikely to replace microscopy in most laboratories, particularly those whose resources do not justify widespread application of PCR for clinical diagnosis. Because the LightCycler is a closed system with each individual assay contained in its own sealed microtube, the risk of contamination with foreign nucleic acid is low and the procedure may be carried out on the open bench in the general laboratory area (although reagent preparation should be conducted in separate clean rooms). Availability of reagent-grade water and a reliable source of electricity are the only absolute requirements. Although the initial investment in LightCycler hardware is substantial, the equipment can be used for multiple different molecular assays and the labor and reagent costs are estimated to be similar to those for performing conventional microscopy. Using the simple extraction method described in this study, we determined that the cost of reagents for this PCR assay ($4–5 per test) is 3–4 times greater than that for stained smears. Conversely, the substantial costs of technologists’ time for the staining and microscopic examination of smears is one-third greater than for PCR. These do not include the costs of obtaining and transporting specimens and would be greater for PCR if a commercial (manual or automated) method of nucleic acid extraction was used. The turnaround times for staining and PCR are similar (the analytical time for performing individual tests is approximately one hour). The actual turnaround times will vary depending upon the need to batch specimens and fit testing into the laboratory work flow. Practically speaking, results of both methods should be available within several hours of arrival of the specimen in the laboratory. Beyond individual patient diagnosis, the assay could also play an important role in large-scale epidemiologic studies and antimalarial clinical trials, although the maximum of 32 samples per run would be a limiting factor in some situations. This assay would convey advantages of accuracy and efficiency in any diagnostic setting where resources were available to establish and maintain the methodology.

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