Detection and Species Determination of Malaria Parasites by PCR: Comparison with Microscopy and with ParaSight-F and ICT Malaria Pf Tests in a Clinical Environment

JILL M. THAM,1,2* SZU HEE LEE,3 THERESA M. C. TAN,1† ROBERT C. Y. TING,1 AND URSULA A. K. KARA2

Institute of Molecular and Cell Biology, Singapore 117609,1 Division of Haematology, National University Hospital, Singapore 119074,2 and Molecular Parasitology Laboratory, Department of Biological Sciences, National University of Singapore, Singapore 1192602

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A rapid procedure for the diagnosis of malaria infections directly from dried blood spots by PCR amplification was evaluated with samples from 52 patients. Plasmodium infections were identified with a genus-specific primer set, and species differentiation between Plasmodium falciparum and Plasmodium vivax was analyzed by multiplex PCR. The PCR test with any of the three primer sets was able to detect as few as four parasites per microliter by gel electrophoresis or by nonisotopic paper hybridization chromatography. The diagnoses obtained by PCR correlated closely with those obtained by Giemsa staining except for two samples observed to have mixed P. falciparum-P. vivax infections. These were initially missed by microscopic analysis. In comparison with antigen-capture assays for P. falciparum, the PCR assays were able to detect three infections that were missed by the ParaSight-F test. The PCR test was negative for nine ParaSight-F-positive samples and one ICT Malaria Pf-positive sample, and these were confirmed to be false-positive results. The PCR thus gave no false-negative or false-positive results. Patients undergoing antimalarial therapy were also monitored by the PCR assay. Four of seven patients who were PCR positive for P. vivax at the time of discharge were later readmitted to the hospital with a recurrence of P. vivax infection. We would like to propose that PCR is a sensitive and easy method that can serve as a useful addition to microscopy for the diagnosis and the clinical monitoring of treatment of malaria.

Microscopy has historically been the mainstay of the diagnosis of malaria. A clinical diagnosis of malaria currently depends on the visualization of parasites by light microscopy of Giemsa-stained thick and thin blood smears. This procedure is cheap and simple, but it is labor intensive and requires personnel who are well trained in the morphological differentiation of the Plasmodium species (11) for successful diagnosis, which leads to proper treatment.

In recent years, alternative methods for the identification of malaria infections have been developed, and these have had various specificities and sensitivities. Several malaria diagnostic kits based on antigen detection of Plasmodium falciparum have been developed, such as ParaSight-F (Becton Dickinson, Cockeysville, Md.) and ICT Malaria Pf (ICT Diagnostics, Sydney, Australia). At the same time, several PCR assays have been developed for the diagnosis of malaria. The 18S rRNA gene has been used as a DNA target for the differentiation of plasmodial species by nested PCR (14, 15) and reverse transcription-PCR (1). Other DNA targets such as the circumsporozoite protein gene (5, 13, 15) have also been investigated for species-specific regions. Tan et al. (16) demonstrated that the large-subunit rRNA gene is extensively conserved within Plasmodium species and is suitable as a genus-specific DNA target region. In this paper, we describe a sensitive and reliable two-step PCR-based amplification assay for the diagnosis of malaria. Plasmodium infections were diagnosed by use of a genus-specific primer set. In addition, two distinct primer sets were designed to specifically detect either P. falciparum or Plasmodium vivax, the two major Plasmodium species which infect and cause malaria in humans. We also present the results of a clinical study of this PCR-based assay in which it was compared with the ParaSight-F and ICT Malaria Pf diagnostic kits performed with samples from patients in Singapore. One advantage of studying malaria in Singapore is the virtual absence of local transmission of malaria, thereby excluding the compounding factor of reinfection as a source of relapse.

MATERIALS AND METHODS

Microscopic diagnosis and sample collection. All patients who had a history of fever and persistent headaches and who had recently traveled outside of Singapore were examined for malaria at the National University Hospital of Singapore. Thick and thin blood films were prepared from venous blood collected in tubes containing EDTA. The use of heparin was avoided because of its known high-level inhibitory effect on Taq polymerase (5). The slides were stained with Giemsa and analyzed for the presence of parasites and parasite species. Quantitative Buffy Coat (QBC; Becton Dickinson) analysis for malaria, which is a fluorescent microscopic examination of capillary-centrifuged blood, was performed in tandem with the thick-film Giemsa stain analysis. Parasites were quantified by counting the number of parasites per 200 leukocytes (3, 7). The parasite density (number of parasites per microliter) was then calculated from the automated leukocyte count obtained with a Bayer Technicon H*A automated cell counter (Bayer Tarrytown, N.Y.). Five microliters of blood from each patient was spotted onto grade 1 Whatman filter paper (Whatman International Ltd., Maidstone, United Kingdom) and air dried at room temperature for PCR analysis. Blood was obtained daily from the patients for monitoring the progress of the antimalarial therapy. Four methods were used to examine these samples: mi-
TABLE 1. Comparison of PCR assays with Giemsa staining and ParaSight-F and ICT Malaria Pf (P. falciparum antigen-capture) tests for detection of Plasmodium infection in patients

<table>
<thead>
<tr>
<th>Cause of infection</th>
<th>Microscopy</th>
<th>PCR detection</th>
<th>P. falciparum antigen-capture assays</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Genus specific</td>
<td>Pf specific</td>
</tr>
<tr>
<td>P. falciparum</td>
<td>16</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>P. vivax</td>
<td>34</td>
<td>34</td>
<td>0</td>
</tr>
<tr>
<td>Mixed (P. falciparum and P. vivax)</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Total Plasmodium spp. detected</td>
<td>52</td>
<td>52</td>
<td>18</td>
</tr>
</tbody>
</table>

* Pf, P. falciparum; Pv, P. vivax.

Results and three samples had false-negative results. By the ParaSight-F assay, 24 of 52 samples were observed to be P. falciparum positive. When the results were compared with those of film diagnosis, nine samples had false-positive results and three samples had false-negative results. By the ICT Malaria Pf assay 19 samples were P. falciparum positive, and only 1 of these was observed to have a false-positive result, while the results for the remaining 18 positives samples correlated with those of blood film diagnosis.

PCR analysis for detection of the Plasmodium genus and species determination were run as independent assays. With the species-specific primers L1 and L2, a 100% detection rate for the presence of Plasmodium infection was achieved. With the L1 and L2 primers, a 595-bp PCR product was observed. Amplification with the species-specific primers gave rise in 16 samples to a 422-bp PCR product which correlates to the P. falciparum primer set Pf1-Pf2. In the other 34 samples, a 332-bp PCR product was observed and this product correlates to the P. vivax primer set Pv1-Pv2. In the course of these diagnostic tests, two samples were observed to have both the 422-bp and the 332-bp fragments. These two blood samples were from the two patients with mixed infections, which were initially misdiagnosed as only P. falciparum infections by light microscopy. Paper chromatography hybridization detection of the multiplex PCR products obtained with the species-specific primers was performed with probes designed to detect the two plasmodial species fixed on nitrocellulose strips (Fig. 1). Thus, colorimetric detection was able to differentiate the two species in the same PCR.

When analyzing the sensitivity of the PCR assay with serially diluted infected blood, it was found that the PCR assay could detect as few as three to four parasites per microliter of blood with either the genus- or the species-specific primers (Fig. 2). This corresponds to parasitemias of 0.0005 to 0.0015% obtained with fresh infected venous blood diluted with uninfected human blood. This level of sensitivity was observed both by aga-
rose gel electrophoresis and by the paper chromatography hybridization method. PCR amplification with the three primer sets was also performed with 115 random hospital blood samples that were not infected with malaria parasites. No amplicons were observed. Human β-actin primers were used as a positive control as described previously (16).

Blood samples from 21 patients receiving treatment were examined by PCR with the genus- and the species-specific primers. The patients were monitored for an average of 4 days following initial presentation. Response to treatment could readily be monitored by agarose gel electrophoresis and ethidium bromide staining (Fig. 3). No PCR amplification products were observed from the blood of 14 patients upon discharge from the hospital. However, for the remaining 7 patients, the PCR product obtained with the L1-L2 primer set was still detectable when they were discharged from the hospital. For all of these patients, the corresponding Giemsa-stained films and QBC test were negative at the time of discharge. Four of these patients were later readmitted to the hospital with a recurrence of \textit{P. vivax} infection. One of these patients had initially been diagnosed with a \textit{P. falciparum} infection by thick and thin blood film analysis. However, the PCR assay originally detected a mixed \textit{P. falciparum} and \textit{P. vivax} infection. The patient was initially treated only for \textit{P. falciparum} infection and was then discharged from the hospital. When the patient was readmitted 3 weeks later, \textit{P. vivax} infection with a parasite load of 0.1% was diagnosed. Microscopy detected a predominance of gametocytes. Analysis by the ParaSight-F, ICT Malaria Pf, and PCR assays showed no \textit{P. falciparum} infection.

**DISCUSSION**

Here we have reported on the development and application of a PCR-based test for the diagnosis of malaria and the differentiation between \textit{P. falciparum} and \textit{P. vivax} infections in a clinical environment. This method permits the detection of four parasites per microliter, which is equivalent to a 0.0015% parasitemia. One of the major advantages of the technique is the minimal need for sample preparation. Infected blood directly spotted on filter paper was used immediately for PCR amplification. Within our assay system, no inhibition of the PCR by any of the blood components was observed. It was possible to amplify old blood spot samples that had been stored at room temperature up to 3 years by using the L1

**FIG. 1.** Detection of multiplex PCR products with species-specific primers. Random samples of patients’ blood were analyzed. Lanes 1 and 6, blood infected with \textit{P. vivax} (parasitemias, 1.3 and 0.3%, respectively); lanes 2, 4, 5, and 7, blood infected with \textit{P. falciparum} (parasitemias, 1.1, 0.8, 0.075, and 0.15%, respectively); lanes 3 and 8, blood from non-malaria-infected patients; lane M, 100-bp marker (Promega). (A) PCR-amplified products were run on a 1% agarose gel in 1× TAE electrophoresis buffer. (B) Paper chromatography hybridization. Oligonucleotide probes specific for \textit{P. falciparum} (Pf) and \textit{P. vivax} (Pv) were spotted onto 5-mm-wide nitrocellulose strips. The \textit{P. falciparum}-specific probe was spotted on the top right corner. The \textit{P. falciparum}-specific probe was spotted on the bottom left corner.

**FIG. 2.** Assay of sensitivities of genus- and species-specific primer sets for the detection of malaria by PCR. Infected blood was serially diluted with noninfected blood. (A) Genus-specific L1 and L2 primers (initial parasitemia, 0.016%). (B) \textit{P. vivax}-specific Pv1 and Pv2 primers (initial parasitemia, 0.05%). (C) \textit{P. falciparum}-specific Pf1 and Pf2 primers (initial parasitemia, 0.075%). The numbers above each lane indicate the number of parasites present per microliter of blood; lane M, 100-bp ladder marker (Promega).

**FIG. 3.** PCR monitoring of blood samples from patients undergoing treatment for malaria by using the genus-specific primers L1 and L2. Five microliters of blood spotted on filter paper was assayed in each reaction. Blood samples were obtained daily. For patient A (A; initial parasitemia, 0.35%) and patient B (B; initial parasitemia, 0.5%), PCR products could still be detected after day 5. No PCR product was observed after 4 days for patient C (C; initial parasitemia, 0.3%). Lane M, 100-bp ladder (Promega) used as a molecular size marker.
and L2 primers as well as the multiplex Pf1-Pf2 and Pv1-Pv2 primers (17).

Our strategy for the PCR amplification was to use two regions from the extrachromosomal DNA of Plasmodium. Detection of a malarial infection was done with genus-specific primers made from the conserved large-subunit rRNA gene, and detection of the two main human Plasmodium species, P. falciparum and P. vivax, was done with primers made from the cod1 gene. These primers were then used in a multiplex PCR system. The ability to perform multiplex PCR to differentiate the species decreases the number of PCR assays required to be performed with each blood sample. Most nonisotopic PCR methods require liquid hybridization and capture on microtiter plates for detection (7, 10). The microtiter plate system has limited application in that each well is assigned microtiter plates for detection (7, 10). The microtiter plate PCR methods require liquid hybridization and capture on PCR system. The ability to perform multiplex PCR to differentiate species and detect mixed infections. The production cost of our PCR assay for the diagnosis of malaria is comparable to that of the commercially available antigen-capture assays. Further development and evaluation of the dipstick detection system are under way. This PCR diagnostic assay can easily be developed for mass screenings through automation and could thus be an effective diagnostic tool that is sensitive, specific, and less labor intensive than currently used methods. We would like to present this system as a simple and reliable test for the diagnosis of malaria.

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ERRATA

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JILL M. THAM, SZU HEE LEE, THERESA M. C. TAN,
ROBERT C. Y. TING, AND URSULA A. K. KARA
Institute of Molecular and Cell Biology, Singapore 117609, Division of Haematology,
National University Hospital, Singapore 119074, and Molecular Parasitology
Laboratory, Department of Biological Sciences, National
University of Singapore, Singapore 119260

Volume 37, no. 5, p. 1269–1273, 1999. Page 1270, column 1, line 30 from bottom: “(5’-TGT TAA TAC AHC TCC AAT-3’)” should read “(5’-CTG GTA ATA TTA AAA TAT AC-3’).”

Nucleic Acid Sequence-Based Amplification, a New Method for
Analysis of Spliced and Unspliced Epstein-Barr Virus
Latent Transcripts, and Its Comparison
with Reverse Transcriptase PCR

ANTOINETTE A. T. P. BRINK, MARCEL B. H. J. VERVOORT, JAAP M. MIDDELDORP,
CHRIS J. L. M. MEIJER, AND ADRIAAN J. C. VAN DEN BRULE
Section Molecular Pathology, Department of Pathology, University Hospital Vrije Universiteit,
1007 MB Amsterdam, and Bioresearch Unit, Organon Teknika,
5281 RM Boxtel, The Netherlands

Volume 36, no. 11, p. 3164–3169, 1998. Page 3166, Table 2, column 2, line 5: “1.1-2.2” should read “1.2-2.1.”
Page 3167, Fig. 3d: “252 bp” should read “203 bp.”
Page 3168, Fig. 4b: “252 bp” should read “203 bp.”

Typing of Clinical Herpes Simplex Virus Type 1 and Type 2
Isolates with Monoclonal Antibodies

JAN-ÅKE LILJEQVIST, BO SVENNERHOLM, AND TOMAS BERGSTROM
Department of Virology, University of Gotenburg, S-413 46 Gotenburg, Sweden

Volume 37, no. 8, p. 2717–2718, 1999. Page 2718, column 1, lines 4 and 5: “gG-2” should read “gC-2.”