DEVELOPMENT AND OPTIMIZATION OF POLYMERASE CHAIN REACTION–BASED MALARIA DIAGNOSTIC METHODS AND THEIR COMPARISON WITH QUANTITATIVE BUFFY COAT ASSAY

HAIANA CHARIFKER SCHINDLER, LILIAN MONTENEGRO, ROSANA MONTENEGRO, ALEXANDRE B. CARVALHO, FREDERICO G. C. ABATH, AND GINETTE JAUREGUIBERRY

Departamento de Imunologia, Centro de Pesquisas Aggeu Magalhães, Fundação Oswaldo Cruz, Recife, Pernambuco, Brazil; Laboratoire de Biologie Parasitaire et Chimiotherapie, EP 1790 CNRS, Museum National d’Histoire Naturelle, Paris, France

Abstract. Polymerase chain reaction (PCR)–based assays targeting the small-subunit rRNA were developed and evaluated, allowing for the simultaneous diagnosis of *Plasmodium falciparum* and *Plasmodium vivax* DNA in human blood samples. The PCR methods and quantitative buffy coat (QBC) were compared in 402 patients. The heminested PCR method showed a sensitivity of 97.4%, which was superior to the sensitivity of the QBC method (91.7%, $P < 0.05$), to simple PCR (84.6%, $P < 0.001$), and to PCR with digoxigenin labeling (PCR-DIG) (88.5%, $P < 0.001$). The PCR-DIG and QBC analyses were more sensitive than simple PCR ($P < 0.003$ and $P < 0.05$, respectively). There was no significant difference between the sensitivities of the QBC assay and the PCR-DIG assay. The specificity for the 3 PCR-based methods was 100%, superior to the specificity calculated for the QBC assay (88.95%, $P < 0.009$). The frequency of a positive result in groups from endemic areas but without detectable parasitemia increased, in order, from simple PCR, QBC test, PCR-DIG, to heminested PCR. An association between a positive PCR result and a history of malaria was also found. Taken together, these data suggest that this technology could be further developed to screen people with oligoparasitemia and to monitor malaria treatment.

INTRODUCTION

Malaria is a major public health problems in Brazil, with >2 million cases diagnosed 1992–1997. Most of these cases are due to either *P. vivax* or *P. falciparum*. Transmission of malaria by blood transfusion is a significant issue in regions of the world where this disease is endemic. Currently, the conventional method of diagnosis of acute malaria is by microscopic examination of Giemsa-stained thick and thin blood films. Although the method is cheap, convenient, and relatively accurate, it requires highly skilled technicians. Although this is ideal as a rapid and inexpensive diagnostic tool, it is labor intensive and not sensitive enough to detect low parasitemia in asymptomatic donors. Thus, it is not suitable for screening a large number of donor blood samples or for large-scale epidemiology studies. The sensitivity limit of the thick blood film examination is estimated at ~5 parasites/μL and requires counting 100 microscope fields.

Immunologic tests have been proposed as an alternative to microscopic diagnosis of malaria. However, these are not sensitive enough, and they cannot discriminate between past and present infection. Concentration of malaria parasite–infected red blood cells by centrifugation coupled with acridine orange staining and fluorescence microscopy (quantitative buffy coat [QBC] System, Becton Dickinson) is easier to use and more sensitive, although this method is not generally accepted. DNA hybridization methods show a modest improvement in sensitivity compared with blood films, and these hybridization methods are suitable for screening many samples. In fact, a number of candidate DNA probes was found to be less useful than first thought. To minimize the risk of malaria transmission when large volumes of blood are delivered to a transfusion recipient, an extremely sensitive test is required. Sensitive alternative methods to detect low parasitemia in donor blood is therefore required to support and to replace microscopy because blood transfusion is an important component of transmission of the disease.

In this report, we describe a highly sensitive detection procedure, one based on heminested PCR, that does not require complex blood sample processing and is able to detect both *P. falciparum* and *P. vivax* DNA. The results of this test were compared with microscopic examination of blood, QBC analysis, and other PCR–based methods performed with samples from patients who were from malaria-endemic areas of Brazil.

MATERIAL AND METHODS

Patients and sample collection. All patients with malaria were examined in one of the following institutions: Hemo-centro de Manaus, Amazonas; Hemo-centro de Porto Velho, Rondonia; Instituto de Medicina Tropical de Manaus, Manaus; and Fundação Nacional de Saúde, Manaus. The patients were grouped as parasitemic or not on the basis of conventional blood smear microscopy. The project was approved by the ethical committee of Centro de Pesquisas Ag-geu Magalhães-FIOCRUZ, and all enrolled patients provided written consent. All the above areas are endemic for malaria and are located in northern Brazil. The 402 patients selected for the study were placed in one of the following groups: 1) 156 symptomatic and parasitemic patients living in endemic areas (59% boys and men and 41% girls and women, aged 3–72 years, average 30 years); 2) 39 symptomatic patients living in endemic areas without detectable parasitemia (56% boys and men and 44% women and girls, aged 16–63 years, average 32 years); 3) 144 asymptomatic patients living in endemic areas (79% boys and men and 21% girls and women, aged 3–67 years, average 28 years), including 70 blood donors, who were selected on the basis of the current Bra-zilian regulations for blood donation in endemic areas; 4) 33 healthy people living in Recife, Brazil, an area not endemic for malaria (48% men and 52% women, aged 18–78 years, average 40 years); and 5) 30 patients from regions in which malaria transmission is absent but that have other parasitic diseases, including Chagas disease ($n = 10$), visceral leish-maniasis ($n = 10$), cutaneous leishmaniasis ($n = 10$) (60%...
boys and men and 40% girls and women, aged 3–62 years, average 27 years). Typical malarial symptoms include periodic attacks of chills, fever, and sweating; headache; myalgia; splenomegaly; and anemia. The parasitemia levels in Group 1 range from 200 parasites/µL to 500,000 parasites/µL.

**Processing of samples.** Blood samples (5 mL) were collected by venipuncture by means of a Vacutainer containing ethylene diamine tetra-acetic acid (Becton Dickinson, Inc., Franklin Lakes, NJ). For PCR, the samples were centrifuged at 1,000 × g for 10 min. Plasma was discarded, and the pellet was lysed with 5 mL 0.4% saponin and centrifuged immediately at 10,000 × g for 15 min. The supernatant was discarded, and the pellet was washed twice with PBS pH 7.2. The pellet was suspended in 50 µL of sterile Milli-Q water and stored at −20°C until use. In preliminary studies, the pellet harvested after saponin lysis was further purified by means of the classical phenol-chloroform extraction with no significant advantages over the simplified saponin method described above (data not shown).

**Microscopy.** Thick and thin blood smears were stained by Giemsa and examined independently by 2 different expert microscopists; 100 microscope fields were examined under oil immersion optics for 10 min before concluding a negative result. The number of infected erythrocytes per 200 white blood cells was determined, and parasitemia was expressed as the number of parasites per microliter. Slides with discrepancies were reexamined.

**QBC analysis.** Blood (55–65 µL) was collected into commercially supplied malaria detection tubes (Becton Dickinson, Inc., Franklin Lakes, NJ) following the manufacturer’s instructions; the tubes were centrifuged at 10,000 × g for 5 min and examined with a Leitz HM Lux 3 microscope fitted with Paralens (Becton Dickinson) UV Microscope Adaptor (10× wide-field eyepieces and a 60× oil-immersion lens).

**Oligonucleotides.** The oligonucleotides GJ1 (5′-GGCTT-AAGTTACGATTAAATAG-3′), GJ2 (5′-ACACTTTCATCC-AACACCTA-3′) and HR842 (5′-CCAATCCCTTGTGTTTAG-3′) were based on the primary sequence of the 18S RNA gene of *P. falciparum*, targeting variable regions that differentiate between human and *Plasmodium* ribosomal RNAs. HR842 is highly conserved in the genus *Plasmodium*, whereas the primers GJ1 and GJ2 amplify *P. falciparum* and *P. vivax* DNA.

**PCR amplification and analysis of PCR products.** Purified *P. falciparum* DNA for positive controls was prepared from parasites (Brazilian strain PFB) cultured in vitro by standard methods. The purified DNA was serially diluted to 10−6, 10−7, 10−8, and 10−9 mg/mL and examined with a Leitz HM Lux 3 microscope fitted with Paralens (Becton Dickinson) UV Microscope Adaptor (10× wide-field eyepieces and a 60× oil-immersion lens). The number of infected erythrocytes per 200 white blood cells was determined, and parasitemia was expressed as the number of parasites per microliter. Slides with discrepancies were reexamined.

**RESULTS**

The DNA detection limit of the PCR approaches. The detection limits of the simple PCR, PCR-DIG, and heminested PCR were 100 pg, 10 pg, and 0.01 pg, respectively, of purified *P. falciparum* DNA (Figure 1). These results indicate that the heminested PCR is more sensitive than the other PCR-based methods evaluated. Thus, we decided to study the performance of these tests in human populations in field conditions.

**Sensitivities of the diagnostic methods evaluated.** The sensitivities were calculated by taking into account the symptomatic patients with parasitemia whose malaria had been confirmed by conventional microscopy (Group 1; Figure 2). The heminested PCR showed a sensitivity of 97.4%, which is greater than QBC (91.7%, P < 0.05), simple PCR (84.6%, P < 0.001), and PCR-DIG (88.5%, P < 0.001). The PCR-DIG and QBC assays were more sensitive than the simple PCR (P < 0.003 and P < 0.05, respectively). There was no significant difference between the QBC assay and the PCR-DIG.

To determine whether the diagnostic tests were preferentially detecting a particular species of *Plasmodium*, patients with confirmed parasitemia were subgrouped as infected with *P. falciparum* or *P. vivax* on the basis of conventional
microscopical examination of blood smears. Neither mixed infections nor infection with other species of Plasmodium could be detected. The results shown in Figure 2 indicate that both the QBC assay and the PCR-based methods can detect P. falciparum and P. vivax in a nonpreferential pattern.

Specificities of the diagnostic methods evaluated. The specificity of the assays was determined by examining healthy people and patients with other parasitic diseases (Groups 4 and 5) who live in malaria-free areas (Figure 3). The specificity for the 3 PCR-based methods was 100%, superior to the specificity for the QBC assay (88.95%, \( P < 0.009 \)).

Concordance of the tests. The concordance of the PCR methods and the QBC assay with the gold standard was analyzed on the 402 selected people (Groups 1–4). The results concerning the positive concordance, negative concordance, general concordance, and kappa index are shown in the Table 1. The highest kappa index was found for simple PCR and PCR-DIG; heminested PCR presented the lowest value. However, these results should be interpreted carefully because the low sensitivity of conventional microscopy renders it an imperfect gold standard. We observed that 3.2, 6.0, 4.4, and 1.0% of all people we studied were positive by microscopic examination but negative by QBC, simple PCR, PCR-DIG, and heminested PCR assays, respectively. On the other hand, 10.0, 3.5, 5.6, and 15.4% were negative by micro-
scopical examination but positive by QBC, simple PCR, PCR-DIG, and heminested PCR assays, respectively.

With particular reference to the groups without detectable parasitemia who lived in endemic areas—Group 2 (symptomatic patients) and Group 3 (asymptomatic people)—it is noteworthy that in the symptomatic group, the heminested PCR, PCR-DIG, simple PCR, and QBC tests had positivity frequencies of 53.8, 13.5, 2.6, and 10.3%, respectively, whereas in the asymptomatic group, the positivity frequencies were 28.5, 12.3, 9.2, and 20.1%, respectively, for these tests. The group of blood donors (part of Group 3) deserves special attention. In this group, heminested PCR was positive in 17 (24%) of 70, PCR-DIG in 12 (17%) of 70, and simple PCR in 10 (14.3%) of 70 blood donors. The QBC method was negative for all blood donors.

Positivity of heminested PCR correlates with the level of parasitemia and previous history of malaria. As noted in the previous section, 1% (4 samples) of the people studied were negative by heminested PCR but positive by microscopic examination. Analysis of the parasitemia distribution showed that most of these samples were from people harboring < 1000 parasites/μL (data not shown). On the other hand, analysis of the malaria history of people without detectable parasitemia (Groups 2–5) but with positive heminested PCR showed that there is a dependency between positivity by heminested PCR and malaria antecedents ($\chi^2 = 42.06, P < 0.001$). All patients who tested positive lived in malaria-endemic areas.

**DISCUSSION**

In malaria-endemic areas in Brazil, the predominant *Plasmodium* species are *P. falciparum* and *P. vivax*. Although *P. falciparum* is the most virulent species, and therefore the most important cause of severe disease, a method allowing detection of both species is preferable, considering the morbidly also associated with *P. vivax* infection. Thus, we developed and evaluated PCR-based assays (simple PCR, PCR-DIG, and heminested PCR) targeting appropriate regions of the SSU rRNA, allowing for the simultaneous diagnosis of both species in human blood samples. In this report, we compared the PCR methods and QBC in 402 people living either in endemic or nonendemic areas and compared sensitivity, specificity, and simplicity. The aim was to evaluate the possibility of transferring the technology to blood banks in endemic areas.

Microscopy has historically been the mainstay of malaria diagnosis and continues to be the gold standard. However, disadvantages include the low sensitivity, subjectivity, and inadequacy for large-scale epidemiology studies. Thus, parasite densities of 4–40 parasites/μL blood (1/200 to 1/2,000 parasites per leukocytes) are rarely detected, and in busy, routine examination, the sensitivity is 10-fold lower. In addition, the number of false-negative findings in oligoparasiticemic patients and false-positive findings due to artifacts is not negligible. As a result of these limitations, alternative techniques for the diagnosis of malaria have been developed. The QBC method is more sensitive, rapid, and practical than thick blood film for the diagnosis of malaria. The main concerns of this test are cost and the need for special equipment (centrifuge, fluorescence microscope, and capillary tubes).

These features are clearly undesirable for fieldwork conditions. In our hands, the QBC assay had a sensitivity of 91.7% and a specificity of 88.9%, indicating the possibility of false positivity in higher proportion than the PCR-based methods, a finding that is in agreement with those of previous studies. Moreover, the discrimination of *Plasmodium* species by the QBC assay posed some difficulties because the discrimination of *P. vivax* from *P. falciparum* was impossible in 14.2% of the patients with parasites (data not shown). In general, these results are corroborated by previous studies.

The most promising antigen detection methods for malaria diagnosis are the immunochromatographic dipstick tests. These tests are based on the immunologic detection of 2 parasite antigens: the histidine-rich protein 2 (HRP-2) of *P. falciparum* (Parasight-F (Becton Dickinson, Cockeysville, MD) and ICT Malaria Pf (ICT Diagnostics, Sydney, Australia), and the lactate dehydrogenase enzyme, pLDH, produced by all 4 *Plasmodium* species infecting humans (OptiMAL). These tests are simple to use, easy to interpret, and produce results in < 15 min. The major limiting feature is the inability to detect low parasitemia density. Studies that used microscopy and PCR as standards showed that sensitivity and specificity of these tests are comparable to those of microscopy at a parasitemia > 100 parasites/μL, declining considerably for samples with lower grade parasitemia. Although HRP-2–based serologic tests permit rapid diagnosis of falciparum malaria, they are of limited clinical usefulness because HRP-2 is only present in *P. falciparum* and may persist in the blood long after the parasites have been cleared from the host.

In this context, OptiMAL presents some advantages as it differentiates falciparum from nonfalciparum malaria. However, disadvantages include the low sensitivity, subjectivity, and inadequacy for large-scale epidemiology studies. Thus, parasite densities of 4–40 parasites/μL blood (1/200 to 1/2,000 parasites per leukocytes) are rarely detected, and in busy, routine examination, the sensitivity is 10-fold lower. In addition, the number of false-negative findings in oligoparasiticemic patients and false-positive findings due to artifacts is not negligible. As a result of these limitations, alternative techniques for the diagnosis of malaria have been developed. The QBC method is more sensitive, rapid, and practical than thick blood film for the diagnosis of malaria. The main concerns of this test are cost and the need for special equipment (centrifuge, fluorescence microscope, and capillary tubes). These features are clearly undesirable for fieldwork conditions. In our hands, the QBC assay had a sensitivity of 91.7% and a specificity of 88.9%, indicating the possibility of false positivity in higher proportion than the PCR-based methods, a finding that is in agreement with those of previous studies. Moreover, the discrimination of *Plasmodium* species by the QBC assay posed some difficulties because the discrimination of *P. vivax* from *P. falciparum* was impossible in 14.2% of the patients with parasites (data not shown). In general, these results are corroborated by previous studies.

Table 1

<table>
<thead>
<tr>
<th>Diagnostic test</th>
<th>Microscopic examination of blood smears</th>
<th>Heminested PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pos. concordance</td>
<td>Neg. concordance</td>
</tr>
<tr>
<td>QBC</td>
<td>35.6%</td>
<td>51.2%</td>
</tr>
<tr>
<td>Simple PCR</td>
<td>33.0%</td>
<td>57.5%</td>
</tr>
<tr>
<td>PR-DIG</td>
<td>33.8%</td>
<td>56.2%</td>
</tr>
<tr>
<td>Heminested PCR</td>
<td>37.8%</td>
<td>45.8%</td>
</tr>
</tbody>
</table>

402, 400, 340, and 402 individuals were simultaneously tested by microscopic examination and QBC analysis, simple PCR, PCR-digoxigenin labeling (PCR-DIG), and heminested PCR, respectively.

Table 2

<table>
<thead>
<tr>
<th>Malaria history</th>
<th>Negative</th>
<th>Positive</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N (%)</td>
<td>N (%)</td>
<td>N (%)</td>
</tr>
<tr>
<td>No</td>
<td>160 (84.7)</td>
<td>29 (15.3)</td>
<td>189 (100.0)</td>
</tr>
<tr>
<td>Yes</td>
<td>24 (42.1)</td>
<td>33 (57.9)</td>
<td>57 (100.0)</td>
</tr>
</tbody>
</table>

N = number of individuals, $\chi^2 = 42.061, P < 0.001$.
ciparum malaria, and it is able to monitor the results of patient antimalarial treatment. The relatively high cost of immunochemical tests mandates careful consideration of the role of such tools in malaria control programs. These tests cost approximately US$1.20 in developing countries.

Recently, DNA detection techniques based on amplification by PCR followed by hybridization with *P. falciparum* DNA-specific probes have detected as little as 0.01 pg of specific DNA (a *P. falciparum* merozoite contains ≈ 0.02 pg of DNA). However, the hybridization step after PCR is time-consuming and unsuitable for large numbers of samples. Methods that use laborious procedures to detect DNA, such as blot hybridization or radioisotope handling, are also not suitable for epidemiological purposes. On the other hand, a number of highly sensitive PCR-based tests have been reported. The major advantage of a PCR-based technique is the ability to detect infection in patients with low parasitemia. However, in general, these methods require either time-consuming processing of blood samples or DNA purification, including washing of blood, proteinase K digestion, and DNA extraction to remove inhibitors present in blood components, which make these methods impractical for field use.

Very recently, 2 simplified PCR-based methods were reported that target the 18S SSU rRNA gene. This gene is well characterized and allows the choice of conserved and hypervariable regions to be amplified. One method aims at discriminating species by use of heminested PCR, and the other uses either genus- or species-specific primers for the nest 2 amplification. However, the latter method requires 4 independent reactions for each of the human *Plasmodium* species.

In the present study, a simple saponin-based method was used for PCR sample processing. The heminested PCR was designed to detect both *P. falciparum* and *P. vivax* by means of a simple sample processing. The DNA quantity detected by the heminested PCR (0.01 pg) is equivalent to 50% of a simple sample processing. Thus, although the primers used target conserved regions for *P. falciparum* and *P. vivax*, we investigated the possibility of preferential species amplification by analyzing the specific sensitivities for both species; we did not find any significant difference. The results indicate that both the QBC assay and the PCR-based methods detect *P. falciparum* and *P. vivax* in a nonpreferential pattern. No mixed infections were detected microscopically in our study, although the possibility of misdiagnosis exists because of the tendency for one species to be dominant over another species. Primers can be designed for preferential detection of a single species. In this context, Roy and others and Barker, who performed hybridization with oligoprobes, found that the assay was 10-fold more sensitive for *P. falciparum* than for *P. vivax*, depending on the probe.

Although very sensitive, our heminested PCR method was unable to detect 4 of 156 patients with confirmed parasitemia (all of them displaying < 1,000 parasites/μL). Barker and others extensively studied why microscopic examination and PCR diagnosis do not always agree. They reported several possible reasons to explain the situation: samples containing parasites may fail to amplify during PCR; the target sequence may be absent, deleted, or mutated; DNA may degrade during sample preparation and storage; incorrect microscopic identification may occur; and the target may be inaccessible because of the presence of PCR inhibitors or inadequate cellular lysis during sample processing. Although none of the above-mentioned hypotheses can be discarded, it is of note that the blood smear of the samples with diagnostic disagreements were reexamined to confirm the microscopic result.

The frequency of a positive result in groups from endemic areas but without detectable parasitemia increased, in order, from simple PCR, QBC test, PCR-DIG, to heminested PCR. Analysis of these data suggests that several people may have been misdiagnosed by microscopy. This assumption is supported by the finding that there is an association between positive results by PCR and malaria history. The group of blood donors (part of Group 3) is particularly important because it concerns blood transfusion–mediated transmission of malaria. *Plasmodium* DNA was detected in 24, 17, and 14% by heminested PCR, PCR-DIG, and simple PCR, respectively. It is likely that these people had low-level infections that were undetectable through conventional microscopy or the QBC test. Thus, heminested PCR would be of great help in screening oligoparasitemic people, particularly in regional reference blood banks. This practice would diminish the number of malaria cases transmitted via transfusion.

The marginal cost of reagents for a single in-house PCR-based test, such as those described here, is < US$1.0, with the thermostable DNA polymerase being by far the most expensive component. This calculation does not take into account any additional costs arising from patents held on the PCR process. In practice, the results would be available at the end of a working day. The cost of reagents and consumable supplies per test was estimated as US$0.06 for the Giemsa staining method and US$0.83 for the QBC test.

Because PCR-based methods are amenable to automation, our long-term goal is to transfer the methodology to reference blood banks of endemic zones so that screening for *Plasmodium*-contaminated blood could be undertaken. The
ability to process many samples at once suggests that this technology could also be developed for large-scale, field-based epidemiologic surveys and for monitoring treatment.

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Reprint requests: Haiana Chariker Schindler, Centro de Pesquisas Aggeu Magalhães, Fiocruz, Av. Moraes Rego s/n, 50670-420, Cidade Universitária, Recife-PE, Brazil, Telephone: 55-81-2714000, Fax: 55-81-4531911.

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