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

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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 problem in Brazil, with > 2 million cases diagnosed 1992–1997.1 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 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.2

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.3 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: Hemocentro de Manaos, Amazonas; Hemocentro 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 Aggeu 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 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 Brazilian 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 leishmaniasis (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-AGTTACGTTAATAG-3′), GJ2 (5′-ACACCTTCATCC-AACACCTA-3′) and HR842 (5′-CTAAAACCTCCTTGT-GTTAG-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 assess the detection limit of the PCR assays. Amplification was done in a DNA thermal cycler (Perkin-Elmer Model 4800; Norwalk, CT).

Three PCR approaches were compared: simple PCR, PCR with digoxigenin labeling (PCR-DIG), and heminested PCR. In the simple PCR, GJ1 and GJ2 were used as primers. Because the gene for the small-subunit (SSU) rRNA is not highly repeated in *Plasmodium*, PCR-DIG and heminested PCR were also devised to increase the sensitivity of the assay. In the PCR-DIG, the primers GJ1 and GJ2 were also used, but the amplicon was labeled by incorporation of DIG-11-dUTP. The products of amplification were separated by gel electrophoresis and vacuum blotted onto a nylon membrane. The membrane was treated with anti-digoxigenin antibody Fab fragments conjugated to alkaline phosphatase, and chemiluminescence was developed according to the instructions of the supplier (Boehringer-Mannheim Biochemicals, Mannheim, Germany). The results were documented by short exposure to x-rays. In the heminested PCR, GJ1 and HR842 were used in the first PCR, and GJ1 and a single internal primer (GJ2) were used in the second PCR. The conditions for the simple PCR and PCR-DIG are as described below for the first PCR of the heminested PCR. The primers GJ1 and HR842 yield a 820-bp amplicon, whereas the primers GJ1 and GJ2 yield a 220-bp product.

In the first PCR, 25 μL of sample was amplified in a 50-μL volume containing 50 mM KCl, 10 mM Tris HCl, pH 8.3, 2.5 mM MgCl₂, 200 μM of each dNTP (Perkin-Elmer), 20 pmol of primers, and 2.5 U of Taq DNA polymerase (Perkin-Elmer). The amplification was performed for 30 cycles at 94°C for 1 min (denaturation), 50°C for 45 sec (annealing), and 72°C for 45 sec (extension). One microliter of the first PCR solution was amplified with 20 pmol of primers under the same conditions described above. Ten microliters of the PCR products were separated on 1.5% agarose gel and the bands visualized under ultraviolet light after ethidium bromide staining.

**Statistical analysis and calculation of the indexes of performance of the tests.** Statistical analysis was performed by Epi Info software (CDC, Atlanta, GA). Sensitivity of the PCR tests was assessed with microscopic detection as the gold standard. Specificity was calculated on the basis of the results of people without malaria who were not parasitemic and who were living in malaria-free regions. Sensitivity, specificity, and kappa indexes were calculated as described. The sample size used for the calculation of these indexes was not necessarily the same because some diagnostic tests were not performed in all selected patients.

**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
F I G U R E 1. Detection limits of polymerase chain reaction (PCR)-based assays. Panel A) Simple PCR; Panel B) PCR with digoxigenin labeling (PCR-DIG); and Panel C) Hemi-nested PCR. The PCR products resulted from samples containing the following amounts of *Plasmodium* genomic DNA: 100 ng (lane 1); 10 ng (lane 2); 1 ng (lane 3); 100 pg (lane 4); 10 pg (lane 5); 1 pg (lane 6); 100 fg (lane 7); 10 fg (lane 8); 1 fg (lane 9); 0.1 fg (lane 10); and 0.01 fg (lane 10). For simplicity, only 10 and 7 lanes are shown in Panels A and B, respectively. The products amplified with the primers GJ1 and GJ2 (213 basepairs [bp]) and GJ1 and HR842 (746 bp) are indicated by arrows. Lane C− = negative control.

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; hemi-nested 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 hemi-nested PCR assays, respectively. On the other hand, 10.0, 3.5, 5.6, and 15.4% were negative by micro-
TABLE 1

<table>
<thead>
<tr>
<th>Diagnostic test</th>
<th>Microscopical examination of blood smears</th>
<th>Hemi-nested PCR</th>
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</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>Hemi-nested PCR</td>
<td>37.8%</td>
<td>45.8%</td>
</tr>
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402, 400, 340, and 402 individuals were simultaneously tested by microscopical examination and QBC analysis, simple PCR, PCR-digoxigenin labeling (PCR-DIG), and hemi-nested PCR, respectively.

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 microscopical 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 (χ² = 42.06, P < 0.001) (Table 2). 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 the most important cause of severe disease, a method allowing detection of both species is preferable, considering the morbidity 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.2 Thus, parasite densities of 4–40 parasites/μL blood (1/200 to 1/2,000 parasites per leukocytes) are rarely detected,11 and in busy, routine examination, the sensitivity is 10-fold lower.2 In addition, the number of false-negative findings in oligoparasiticemic patients and false-positive findings due to artifacts is not negligible.12 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.13–16 The main concerns of this test are cost and the need for special equipment (centrifuge, fluorescence microscope, and capillary tubes).16 These features are clearly undesirable for fieldwork conditions.16–19 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.20 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.17,18,21

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.21 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.16,21–24 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.25 In this context, OptiMAL presents some advantages as it differentiates falciparum from nonfal-
Plasmodium falciparum malaria, and it is able to monitor the results of patient antimalarial treatment. The relatively high cost of immunochromatographic tests mandates careful consideration of the role of such tools in malaria control programs. These tests cost approximately US$1.20 in developing countries.21

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).2,7,26,27 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 hybridization2,27 or radioisotope handling,2,7,28,29 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.22,30,31

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,32 and the other uses either genus- or species-specific primers for the nest 2 amplification.33 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. The DNA quantity detected by the heminested PCR (0.01 pg) is equivalent to 50% of a simple sample processing. However, the detection limit experiments were performed with purified DNA, and the DNA purity of the processed blood samples was expected to be inferior. Thus, the performance of the tests were extensively evaluated in 402 people living within or outside of malaria-endemic areas and grouped as parasitemic or not according to conventional microscopy.

Specificity was calculated by assessing 63 people without parasitemia living in areas in which malaria transmission was absent, thus allowing us to exclude malaria. The PCR-based methods were shown to be more specific than the QBC test. The heminested PCR showed a sensitivity of 97.4%, which is more sensitive than QBC, simple PCR, and PCR-DIG. The PCR-DIG and QBC assays were more sensitive than the simple PCR. There was no significant difference between the QBC assay and the PCR-DIG. Although the specificity of the QBC test was lower than the PCR-based methods, the sensitivity was high enough to be used alternatively or together with microscopy, which would be particularly interesting in certain regions where it is impracticable to perform PCR.

The Brazilian Plasmodium strains have not been well characterized, particularly with respect to polymorphisms at the SSU RNA level. Thus, although the primers used targeted conserved regions for P. falciparum and P. vivax,34 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.34 Primers can be designed for preferential detection of a single species. In this context, Roy and others35 and Barker,36 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 others37 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 microscopical 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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