Semi-Nested, Multiplex Polymerase Chain Reaction for Detection of Human Malaria Parasites and Evidence of Plasmodium vivax Infection in Equatorial Guinea

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Abstract. A semi-nested, multiplex polymerase chain reaction (PCR) based on the amplification of the sequences of the 18S small subunit ribosomal RNA (ssrRNA) gene was tested in a field trial in Equatorial Guinea (a hyperendemic focus of malaria in west central Africa). The method uses a primary PCR amplification reaction with a universal reverse primer and two forward primers specific for the genus Plasmodium and to mammals (the mammalian-specific primer was included as a positive control to distinguish uninfected cases from inhibition of the PCR). The second amplification is carried out with the same Plasmodium genus-specific forward primer and four specific reverse primers for each human Plasmodium species. The PCR amplified products are differentiated by fragment size after electrophoresis on a 2% agarose gel. Four villages from three regions of the island of Bioko (Equatorial Guinea) and two suspected Plasmodium vivax-P. ovale infections from the hospital of Malabo were tested by microscopy and PCR. The PCR method showed greater sensitivity and specificity than microscopic examination and confirmed the existence of a focus of P. vivax infections in Equatorial Guinea suspected by microscopic examination. It also provided evidence of several mixed infections, mainly P. falciparum and P. malariae, the two predominant species causing malaria in Equatorial Guinea.

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Malaria has a major place among the endemic tropical diseases. More than half of the world’s population in approximately 100 countries is exposed to malaria. In Africa, 1.5–2.7 million people die of malaria each year.1 In sub-Saharan Africa, malaria is considered the leading cause of mortality, with 80% of the population having disease episodes and 90% being parasite carriers.1 Resistance of Plasmodium falciparum to drugs, of Anopheles to insecticides and, now, war and socioeconomic factors have limited the possibilities for disease control by two main objectives: rapid diagnosis (clinical and parasitologic) and correct treatment of all cases.

Equatorial Guinea is a small country located on the west coast of central Africa, with two islands, Bioko (3°43’N, 8°43’E), and Annobon, situated in the southern hemisphere (next to Republic of Sao Tomé and Príncipe) and a mainland region (Rio Muni) situated between Cameroon and Gabon. After Equatorial Guinea became an independent country in 1968, some studies on the malaria situation in this country were been reported. Studies of morbidity in Bioko and Annobon in 1992 reported hyperendemicity in both islands.2,3 In vivo studies of resistance in Bioko according to stratiﬁcation in central Africa concluded that Bioko should be classiﬁed as an area with frequent and high chloroquine resistance.4 These results were corroborated with in vitro studies of resistance to several antimalarial drugs.5,6

To overcome the limitations of the conventional microscopic diagnosis, several methods have been used. These include 1) the staining of parasite DNA and RNA with acridine orange (e.g., the quantitative buffy coat method in capillary tubes [QBC®]; Becton-Dickinson, Franklin Lakes, NJ) or staining of thick and thin blood ﬁlms with this dye;7–9 2) methods based on the detection of the activity of various enzymes (e.g., lactate dehydrogenase);10 3) a rapid antigen capture assay that detects circulating P. falciparum histidine-rich protein-2 (Parasight®F dipstick tests; Becton-Dickinson);11,12 4) serologic tests (immunofluorescence assay, ELISA, indirect hemagglutination assay) used mainly in nonimmune individuals or in epidemiologic studies; and 5) methods using radioactive or nonradioactive DNA probes for the detection of parasites, or a direct polymerase chain reaction (PCR).13–15

At present, various studies using the PCR have been reported for malaria diagnosis of the four species of Plasmodium that infect humans in different field conditions (blood sample collection, DNA template preparation and PCR assay).16–19 The present work, based on PCR methodology, is presented as a simpliﬁcation of the previously reported methods that are methodologically based on the use of a nested-PCR with four independent reactions for each of the human Plasmodium species in the second ampliﬁcation or in the use of a direct PCR only for detection of P. falciparum.16–19 The present method involves a semi-nested PCR ampliﬁcation with new primers based on the sequence of the small subunit ribosomal RNA (ssrRNA) gene. This PCR is a semi-nested multiplex-PCR using a single reaction for the second ampliﬁcation with a mixture of four speciﬁc primers for each human Plasmodium species and a universal Plasmodium primer.

The aims of the study were to 1) compare the PCR with the conventional microscopy in a ﬁeld study with samples from a hyperendemic area (Equatorial Guinea) and 2) conﬁrm by the PCR the suspected P. vivax infections observed by microscopy in some autochthonous populations in Equatorial Guinea. It has been previously reported that there is an interaction between the lack of Duffy glycoprotein (phenotype Fy(a−b−)) and resistance to infection by the malarial parasite P. vivax.20 Earlier studies proved that the majority of Blacks are of the phenotype Fy(a−b−), which is extremely rare in other racial groups, and indicates the resistance of the native populations of West Africa to P. vivax infection.21,22 In West Africa, some cases of P. vivax infections were reported but these were always linked to the white population.22 Five years ago in Equatorial Guinea, the team
of the Centro Hispano-Guineano de Enfermedades Tropicales of Malabo suspected the presence of \textit{P. vivax} infections in the hospital. The arrival of new molecular approaches, mainly the PCR, has made it possible to identify some foci where \textit{P. vivax} infections are maintained.

**MATERIALS AND METHODS**

**Study site and population studied.** Microscopy was performed at the Centro Hispano-Guineano de Enfermedades Tropicales, located on the island of Bioko in Equatorial Guinea. The PCR was performed at the Servicio de Parasitología, Instituto de Salud Carlos III in Madrid, Spain. The study was carried out in December 1996 during the dry season, and was approved by the Ethical Committee of the Health Ministry of Equatorial Guinea and the Ethical Committee of the Institut de Salud Carlos III.

One hundred fifty-nine children less than six years of age from four villages on the island of Bioko participated in the study: Bakake (19), Bososo (47), Sampaca (41), and Basupú (53). Parents or legal guardians of the children were informed of the nature of the study and all provided oral informed consent before allowing the children to participate. Two suspected \textit{P. vivax} or \textit{P. ovale} malaria infections from the General Hospital of Malabo (capital of the country) were also studied.

**Blood sample collection.** Blood samples were obtained during the malaria survey \textit{Control of Malaria Based on the Use of Deltamethrin-Impregnated Bed Nets}. Fingerprick blood samples were collected and thick and thin blood smears were prepared for microscopic observation. Parasitemia per microliter of blood was measured as parasite count = (white blood cell count \times parasites measured per 100 white blood cells)/100, in which the white blood cell count was 4,000. Thin and thick films were stained with 10% Giemsa and examined by four technicians in the Centro Hispano-Guineano de Enfermedades Tropicales and re-examined by technicians of Instituto de Salud Carlos III in Spain.

Fingerprick samples on filter paper were obtained for the PCR assay. Each filter paper specimen was stored in a plastic bag at room temperature and shipped to Madrid.

The field study was carried out to measure the test sensitivity, specificity, and efficiency of the PCR method by comparing the PCR and thin-thick smear (TS); in this study, thin-thick smears were used as the gold standard. All samples were retested using the PCR.

**Preparation of the DNA template.** The isolation of DNA from blood was carried out using the Chelex method. \textsuperscript{21} One milliliter of distilled water was added to a 3-mm\textsuperscript{2} area of a dried blood spot in a 1.5-mL tube and incubated for 30 min at room temperature. The samples were centrifuged for 3 min at 14,000 rpm, most of the supernatant was removed, and 150 µl of 5% Chelex\textsuperscript{®}-100 Resin (Bio-Rad Laboratories, Hercules, CA) was added to give a final volume of 200 µl. The samples were incubated for 30 min at 56°C, vortexed for 10 sec, boiled for 10 min, vortexed again for 10 sec, and centrifuged for 2–3 minutes at 14,000 rpm. The appropriate supernatant volume was used as the PCR template and the rest was stored at 4°C.

**Primer design and PCR assay for parasite detection.** Seven PCR primers were designed by comparison of published ssrRNA gene sequences obtained from the European Molecular Biology/Genbank (Table 1). A reverse primer (UNR) was designed that would hybridize universally with all \textit{Plasmodium} species and a wide range of vertebrates. There were two forward primers, one designed to hybridize with mammals (HUF), and the other to all \textit{Plasmodium} species (PLF). There were four other reverse primers, of which FAR hybridizes only with \textit{P. falciparum}, MAR only with \textit{P. malariae}, VIR only with \textit{P. vivax}, and OVR only with \textit{P. ovale}.

**Semi-nested multiplex PCR.** Detection and identification of malaria species were simultaneously performed using a sequence of two (semi-nested) PCRs, and the sizes of the products were estimated after electrophoresis on 2% agarose gels and staining with ethidium bromide.

The first reaction included primers UNR, HUF and PLF, which were expected to yield two products: a 231-basepair (bp) band from UNR and HUF (the positive control for each individual sample) and a 783–821-bp band (depending on the \textit{Plasmodium} species) from UNR and PLF that should...
detect the presence of any malaria species. However, this fragment appeared only when the parasitemia was greater than 3%; thus, detection of a malaria infection always requires the second reaction.

The second amplification is a multiplex PCR that is the Plasmodium species identification reaction. It incorporates the products of the first reaction along with those of primers PLF, MAR, FAR, OVR, and VIR. Infections with different human Plasmodium species yield products of different sizes. A band of 269 bp from PLF and MAR indicates a P. malariae infection, a band of 395 bp from PLF and FAR indicates a P. falciparum infection, a band of 436 bp from PLF and OVR indicates a P. ovale infection, and a band of 499 bp from PLF and VIR indicates a P. vivax infection. Mixed infections would be expected to show all the appropriate bands.

The PCR mixture for the first reaction contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.001% (w/v) gelatin, 1% glycerol, 200 μM of each dNTP, the PCR primers, 2.5 units of AmpliTaq DNA polymerase (Perkin-Elmer, Norwalk, CT), and 41.1 μl of template DNA in a final volume of 50 μl. The concentration of primers used were 25 pmoles for UNR and PLF and 1.25 pmoles for HUF.

In the multiplex PCR, the reaction mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl₂, 0.001% (w/v) gelatin, 1% glycerol, 200 μM of each dNTP, the PCR primers, one unit of AmpliTaq DNA polymerase, and template DNA in a final volume of 25 μl. The concentration of primers used was empirically determined: PLF 25 pmoles, FAR = 15 pmoles, MAR = 3.12 pmoles, OVR = 6.25 pmoles, and VIR = 2.5 pmoles. The amount of template used was 2 μl of the PCR product of the first reaction diluted to a final volume of 1 ml with water, and 2 μl was added to the PCR. A 2400 GeneAmp PCR system (Perkin-Elmer) thermal cycler was used for both reactions. Reactions conditions were denaturation at 94°C for 5 min, followed by (first reaction) 40 cycles at 94°C for 45 sec, 62°C for 45 sec, and 72°C for 60 sec or (multiplex PCR reaction) 35 cycles at 94°C for 20 sec, 62°C for 20 sec, and 72°C for 30 sec. The final cycle was followed by an extension time at 72°C for 10 min.

**Table 2**

<table>
<thead>
<tr>
<th>PCR</th>
<th>Positive</th>
<th>Negative</th>
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<td>126</td>
</tr>
<tr>
<td>PCR Negative</td>
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<td>33</td>
<td>33</td>
</tr>
<tr>
<td>Total</td>
<td>110</td>
<td>49</td>
<td>159</td>
</tr>
</tbody>
</table>

| Sensitivity | 100% |
| Specificity | 67.3% |
| PPV         | 87%  |
| NPV         | 100% |
| KC          | 79.8% |

* PPV = positive predictive value, NPV = negative predictive value; KC = Kappa coefficient.

**Table 3**

<table>
<thead>
<tr>
<th>Thin-thick blood smears</th>
<th>F</th>
<th>M</th>
<th>O</th>
<th>FM</th>
<th>FO</th>
<th>Totals</th>
</tr>
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<tbody>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
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<td>0</td>
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<td>0</td>
<td>0</td>
<td>56</td>
</tr>
<tr>
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<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>O</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
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<td>5</td>
<td>0</td>
<td>8</td>
<td>0</td>
<td>36</td>
</tr>
<tr>
<td>FMO</td>
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<td>0</td>
<td>2</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>FO</td>
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<td>0</td>
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<td>1</td>
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</tr>
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<td>0</td>
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<td>2</td>
</tr>
</tbody>
</table>

* F = Plasmodium falciparum; M = P. malariae; O = P. ovale; FM = P. falciparum + P. malariae; FMO = P. falciparum + P. malariae + P. ovale; FO = P. falciparum + P. ovale; FV = P. falciparum + P. vivax; MO = P. malariae + P. ovale.

**RESULTS**

**Prevalence, sensitivity and specificity.** The total prevalence in the four villages for both thin-thick smears and the PCR was 69.4% and 79.3%, respectively. The PCR detected 10% of the infected subjects that were not detected by the thin-thick-films. The prevalence of infections per village (PCR) in children less than six years of age was 47% in Bakake, 83% in Bososo, 85% in Sampaca, and 81% in Basupi. Based on the method of Fleiss, the concordance between the two methods by the Kappa coefficient was 0.79. The sensitivity of the PCR was 100% when compared with the thin-thick-films (gold standard) and the specificity was 67% (positive predictive value = 87%, negative predictive value = 100%) (Table 2). The thin-thick-films showed that parasitemias ranged from 40 to 27,200 parasites/μl.

**Species identification.** The identification of malaria species by the PCR was incomplete in a sample that was classified by microscopy as P. falciparum positive. This may have been due to inhibition in the PCR because no positive control (HUF and UNR fragments) was amplified in three repeated attempts. Microscopy detected 11 (7%) double infections: 10 P. falciparum plus P. malariae and one P. falciparum plus P. ovale, while the PCR detected 44 (29%) double infections: 36 (23%) P. falciparum plus P. malariae, three (2%) P. falciparum plus P. ovale, two (2%) P. falciparum plus P. vivax, and three (2%) P. malariae plus P. ovale. The PCR also detected two cases with three species of Plasmodium (P. falciparum, P. malariae, and P. ovale) in a single human sample (Table 3 and Figure 1). The PCRs were repeated twice for all samples and identical results were obtained.

Two possible P. vivax infections were detected by microscopy in two children less than five years of age from the hospital in Malabo. These were confirmed by the PCR method. In the field study, two other samples that were diagnosed by microscopy as P. falciparum infections were diagnosed by the PCR as mixed infections of P. falciparum and P. vivax. The two thin-thick smears from the hospital showed large and spreading trophozoites with a fragmented cytoplasm and large and often irregular gametocytes (male and female). The red blood cells were enlarged, irregular in shape, and contain Schuffner’s dots.

The 47 cases (96%) in which there were differences be-
between the microscopy and PCR results suggest an increase in the sensitivity of the PCR; in these cases, the PCR identified one or two additional malaria parasite species not observed microscopically. The PCR showed a result different from that of microscopy in only two (4%) cases, detecting a different malaria parasite species. Microscopic re-examination of these cases confirmed the PCR result; in one case, *P. ovale* was incorrectly identified as *P. malariae* and in the second, *P. ovale* was incorrectly identified as *P. falciparum*.

**DISCUSSION**

With the spread of parasite resistance to antimalarial drugs and the increasing difficulty in controlling malaria in some areas, mainly in African countries, it is important to diagnose malaria accurately and to treat it correctly. Microscopic observation of parasites stained with Giemsa in thin-thick smears is an inexpensive and simple method that is still used in areas with high malaria transmission and where the diagnosis of malaria is part of primary health care. The early diagnosis of a malaria attack is particularly important in young children (mainly from three months to three years of age) in whom severe untreated malaria infections can be life-threatening and where chemoprophylaxis in areas of malaria stable transmission is targeted to groups of risk (children less than five years of age and pregnant women). Mixed malaria infections in the same person can be cause of important alterations in the mode of provoked infection or pathogenicity. Several malaria infections from endemic countries are hypopotent or subpatent infections, with very low parasitemias. The problem of emigration, accompanied by the possibilities of tourists and professionals travelling to areas at risk for malaria, has increased the number of cases in areas in which malaria transmission was low or previously eradicated. In these cases, an accurate malaria diagnosis is very important so that a possible recrudescence after an incorrect treatment of infected individuals (e.g., blood donors) can be avoided.

The semi-nested multiplex-PCR described in this study was more sensitive than the traditional thick-thin film method. Validation of the method by direct and indirect methodology showed a higher sensitivity of 0.1 parasites/µl of blood, including mixed infections. This method is simple, reproducible, and has several advantages with respect to other methods used in field studies. It showed high sensitivity and sensibility and it can be performed quickly when many samples must be tested (for up to 10 tests per day, microscopy is a good method, but when a larger number of samples need to be tested, the PCR is the preferred method since it depends only on the number of wells in the thermal cycler and/or the number of thermal cyclers in the laboratory).

Also, the PCR does not require special training for interpretation of the results, in contrast to microscopy, in which specific training is needed for species differentiation, and it is not affected by the subjectivity of the observer.

The detection of *P. vivax* infections in Equatorial Guinea, a West African country with a black population without the Duffy receptor, opens new perspectives about the transmission of this parasite in this population. Two of the four *P. vivax* samples were single infections, hospital samples identified by microscopy and the PCR, and the other two samples, field trial samples identified by the PCR, were mixed infections (*P. falciparum* plus *P. vivax*). These *P. vivax* infections were detected in samples from Mulatto children. Since individuals with the Fy null allele do not produce either FyA or FyB antigens, it is possible that a focus exists composed of individuals who maintain the transmission of *P. vivax* in the population. Future studies on a possible correlation between the expressed Fy allele and infection by *P. vivax* may explain the infections detected in Equatorial Guinea. This possible association could, for example, be detected by carrying out a study of the Fy genotype and its mutations.25

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