Development of a PCR Assay Followed by Nonradioactive Hybridization Using Oligonucleotides Covalently Bound to CovaLink NH Microwells for Detection of Four *Plasmodium* Species in Blood Samples from Humans

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We developed and evaluated a PCR-based assay to detect four *Plasmodium* species in 79 blood samples from 56 travelers returning from areas where malaria is endemic. DNA amplification targeting a small region of the 18S rRNA gene was performed with *Plasmodium* genus-specific primers. The biotinylated PCR products were then identified by PCR-colorimetric CovaLink NH microwell plate hybridization (CMPH) using species-specific phosphorylated probes covalently bound to a pretreated polystyrene surface. The results from PCR-CMPH showed high specificity, and for 47 of the 56 patients (84%), microscopy and PCR-CMPH results were in agreement. Discordant results were reevaluated with microscopy examination, other molecular methods, and DNA sequencing. Except for one patient, discrepancies were resolved in favor of PCR-CMPH: three mixed infections were detected, four species identification errors were corrected, and two negative results were shown to be positive. Our results indicate that PCR-CMPH is a simple, rapid, and specific method for malaria diagnosis. It employs stable reagents and inexpensive equipment, making it suitable for routine epidemiological use.

Malaria is a potentially life-threatening infection causing up to 500 million clinical cases and 2.7 million deaths per year worldwide (11, 21). Of the four species of malaria parasites that infect humans in tropical and subtropical areas (*Plasmodium falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*), *P. falciparum* is associated with the majority of cases of infection in Africa. It is also a frequent cause of cases imported by travelers and is responsible for severe disease and high mortality. With *P. vivax* and *P. ovale*, hypnozoites are persistent stages in the liver cells and, once reactivated, may cause clinical relapse many months after the initial event (6). Moreover, most regions where malaria is endemic feature infections involving two or more of these species (23). In clinical practice, mixed *Plasmodium* species infections are often unrecognized or underestimated (14). Failure to detect mixed infections could potentially result in a misdiagnosis of *P. falciparum* with an important risk of severe disease. Rapid identification of the four malaria species that infect humans is thus essential for accurate diagnosis and successful treatment. Because of an increasing occurrence of drug-resistant parasites, it is all the more necessary to define more suitable therapy. In this context, a reliable test able to differentiate the various malaria species and to detect mixed infections would help to manage the disease. In both epidemiological and medical studies, light microscopy still represents the “gold standard” (7). The method is specific and sensitive but laborious and time-consuming in

cases of very low parasitemia. Moreover, the procedure requires well-trained personnel for the morphological differentiation of *Plasmodium* species, particularly when one species is numerically dominated by another in a mixed infection (11, 23). Therefore, alternative tools were developed to address these problems, such as immunochromatographic tests based on antigen detection (ParaSight F test, ICT Now Malaria, etc.). However, such tests have limitations. Recently, molecular biology techniques have been described. The 18S rRNA gene has been widely used as a DNA target for the differentiation of plasmodial species by nested PCR (19, 20), quantitative PCR assays (1, 3, 4, 18), or hybridization (9, 22). Other genus-specific genes have also been extensively investigated, such as the large-subunit rRNA or the circumsporozoite protein genes (8).

In this study, we have developed and evaluated a PCR followed by CovaLink NH microwell plate hybridization (CMPH) for qualitative detection and identification of four malaria species from human blood samples. First, the 18S rRNA gene was chosen as an amplification target in a PCR using *Plasmodium* genus-specific primers, one of which was biotinylated. A nonradioactive hybridization assay was then used for the detection of the biotinylated PCR product. The method employed four species-specific capture probes covalently immobilized onto polystyrene microwells by a phosphoramidate bond. The hybrid molecules were detected by a streptavidin-peroxidase complex and its chromogenic substrate (Fig. 1). We present a clinical retrospective study of this PCR-CMPH method using blood samples from 56 patients returning from areas where malaria is endemic. The results are compared with results from conventional diagnosis techniques and other molecular biology tools.

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FIG. 1. Principle of the colorimetric Covalink NH microwell plate hybridization. The 18S rRNA gene was chosen as an amplification target in a PCR using *Plasmodium* consensus primers, one of which was biotinylated. The species-specific capture probes are covalently immobilized onto polystyrene microwells by a phosphoramidate bond. The hybrid molecules were detected by a streptavidin-peroxidase complex and its chromogenic substrate.

MATERIALS AND METHODS

Patient sampling and conventional malaria diagnosis. A total of 79 blood samples from 56 travelers returning from areas where malaria is endemic (for some patients, several blood samples were collected during the first days of the hospitalization period) and presenting suggestive clinical features were collected in parasitology-mycology laboratories of hospitals in Nancy and Tourcoing (France). The specimens were kept at -80°C and were then investigated for the presence of *Plasmodium* species by using conventional light microscopy, the ICT Now Malaria test (Fumouze Diagnostics, France), STEVOR PCR (5), or nested PCR (19, 20).

Treatment of blood samples. Briefly, 500 μl of thawed whole blood was pretreated with 10 μl of a 10% saponin solution and vortexed. The pellets were recovered after 5 min of centrifugation at $8,000 \times g$ and resuspended in 25 μl of lysis buffer (40 mM Tris-HCl, pH 8.0, 80 mM EDTA, pH 8.0, 2% sodium dodecyl sulfate [SDS]) (19, 20). DNA was then extracted by using either a QIAamp DNA Mini kit (QIAGEN, France) or the MagNA Pure LC automated system (Roche Diagnostics, France) with the MagNA Pure LC Microbiology Kit M^{Grade} according to the manufacturer's instructions. Samples were kept at -80°C until use.

Target sequences, primers, and probes. First, one consensus primer set was selected to amplify the 18S rRNA gene of four *Plasmodium* species from human blood samples. The 5'-end-biotinylated sense primer (Biot-rPLU6 [5'-TTAAA ATTGTTCAGTTAAACG-3']; Proligo, France) corresponds to a common sequence of *P. falciparum*, *P. ovale*, *P. malariae*, and *P. vivax* previously described by Snounou et al. (19, 20). The antisense primer (PR3 [5'-GTTATTCATGCTGTAGTATTC-3']) was designed in another conserved domain obtained after an alignment of published sequences available in the GenBank database (Fig. 2). In contrast to the primer pair, sequences corresponding to the *P. falciparum*, *P. ovale*, and *P. vivax* probes were selected in variable domains according to a method described previously by Whitley et al. (22). The previous 18S rRNA sequence alignment allowed us to design a *P. malariae*-specific probe (Fig. 2).

PCR amplification. The assay was performed using a 50- μl reaction mixture volume containing 2 U of FastStart Taq polymerase (Roche Diagnostics, France); 5 μl of $10\times$ PCR buffer-MgCl₂ (20 mM); 20 pmol each primer; 200 μM dATP, dGTP, dTTP, and dCTP; and 5 μl of sample DNA.

The reaction mixture volume was adjusted to 50 μl with ultrapure water. PCR amplification was performed using an iCycler IQ system (Bio-Rad, France) under the following conditions: initial denaturation of DNA at 94°C for 3 min, followed by 30 cycles at 94°C for 1 min, 58°C for 40 s, and 72°C for 1 min. Those steps were followed by an additional 5-min extension step at 72°C . Five microliters of PCR product was electrophoresed in a 2% agarose gel in the presence of ethidium bromide and visualized under UV light. The expected *Plasmodium* genus PCR product size was 300 bp.

In order to address the discrepant results obtained between microscopy and PCR-CMPH hybridization, STEVOR and nested PCR were performed under the conditions described previously by Filisetti et al. and Snounou et al., respectively (5, 19, 20).

CovaLink NH microwell plate hybridization and colorimetric detection. (i) **Procedure for probe covalent binding to CovaLink NH strips.** Species-specific capture probes detecting *P. falciparum*, *P. vivax*, *P. ovale*, or *P. malariae* were phosphorylated at the 5' end and high-performance liquid chromatography purified (Proligo, France). Each probe was covalently bound to the polystyrene surface of CovaLink NH microwell strips (VWR International, France) as de-

FIG. 2. Alignment of sequences from the partial 18S rRNA genes of four *Plasmodium* species from human blood samples used in the study. PF, *P. falciparum*; PO, *P. ovale*; PV, *P. vivax*; PM, *P. malariae*. The sequence of the human 18S rRNA gene is also shown. Localizations of the sense and antisense primers (Biot-rPLU6 and PR3, respectively) are shown in gray shaded letters in the boxes. In these boxes, unshaded letters mark the sequence polymorphisms between the four *Plasmodium* species or the human sequence. Sequence data are derived from GenBank and represent parts of the sequences given under the accession numbers shown. Below the primer localization, sequence alignment identifies the region where the species-specific probes hybridize the target amplicons.

TABLE 1. Species-specific primers used for sequencing of some amplicons from disparate results between conventional techniques and PCR-CMPH^a

Primer (sequence)	5' Position	Expected size (bp)
PFS1 (5'-CATTTAACTGGTTTGGGAAAAC-3')	662	204
PFS2 (5'-AATGAATCAATCATGACTACC-3')	866	
POS1 (5'-GCATTCTTATCCAAAATGTG-3')	719	441
POS2 (5'-GAAATTCCAAAAGGAATTTTCT-3')	1162	
PVS1 (5'-TGCGCATTTTGTCTACTA-CGTGT-3')	617	450
PVS2 (5'-TTAACTCCGAAGAGAAAACCTCT-3')	1067	
PMS1 (5'-GTATATTATAACATAGTTGTAC-3')	679	518
PMS2 (5'-ACACTCATATATATGAATGTCTC-3')	1197	

^a 5'-end positions are related to sequences corresponding to GenBank accession numbers M19173 (*P. falciparum*), AJ001527 (*P. ovale*), U93235 (*P. vivax*), and AF488000 (*P. malariae*).

scribed previously by Rasmussen et al. (17). Briefly, each phosphorylated probe (concentrations between 25 and 75 pmol/well) was denatured for 10 min at 95°C and rapidly cooled on ice to prevent secondary structure formation. Cold 0.1 M 1-methylimidazole (1-Mi) (pH 7.0; Sigma, France) was added to a final concentration of 10 mM 1-Mi. The mixture was dispensed (75 µl/well) into CovaLink NH microwell strips standing on ice. Twenty-five microliters of freshly prepared 2 M 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC; Sigma, France) dissolved in 10 mM 1-Mi was then added to each well. Several final concentrations of EDC varying from 5 µM to 500 mM were tested. The sealed microwell plate was then incubated for 5 h at 50°C. After incubation, the solution was removed, and each well was washed three times with a washing solution (0.4 N NaOH containing 0.25% SDS) heated to 50°C. The microwells were then soaked for 5 min and washed again three times using the same heated washing solution. Finally, the plate was washed an additional six times at room temperature with Tris-EDTA (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). The coated strips filled with Tris-EDTA can be stored at 4°C for several months. Before being used, the coated strips were washed twice with TTSS buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20, 0.1% SDS).

(ii) **Hybridization assay.** A variable volume of ultrapure water was added into a tube containing 40 to 80 µl of *Plasmodium* genus PCR product, yielding a final volume of 100 µl. After denaturation for 10 min at 95°C followed by rapid cooling on ice, 670 µl of water and 770 µl of SSBP buffer (30 mM sodium citrate–1.91 M NaCl, pH 7.0, containing 2% sarkosyl and 1% polyvinylpyrrolidone-360K) were added. The contents of the tube were transferred into wells coated with specific probes for *P. falciparum*, *P. vivax*, *P. ovale*, or *P. malariae* (175 µl/well).

All samples were analyzed in duplicate. The sealed strips were left to incubate at 42°C for 1 h. The solution was then removed, and the wells were washed six times with 300 µl of TTSS buffer at 58°C. Finally, 180 µl of peroxidase-labeled streptavidin (Roche Diagnostics, France) diluted 1:500 with TTSS buffer was added to each well, and incubation was performed for 1 h in darkness at room temperature. After six washings with 300 µl of TTSS buffer, 200 µl of chromogenic substrate (3,3',5,5'-tetramethylbenzidine; Sigma) heated at 37°C was added, and the wells were incubated for 5 min in darkness. Coloration development was stopped with 30 µl of 1.6 M H₂SO₄, and the optical density for each well was determined by using a Behring Processor III spectrophotometer (Dade Behring, France) at a wavelength of 450 nm with 690 nm as a reference. The background noise corresponds to the mean absorbance measured in a coated well that did not receive the PCR product. An absorbance three times higher than the background noise (background noise + 3 standard deviations) is indicative of a positive result (9, 16). The signal obtained for 5'-end-biotinylated oligonucleotides with sequences complementary to the probes (400 fmol/well) was considered optimal and was therefore used as a positive control in every hybridization assay.

Sequencing of PCR products. Four original sets of *Plasmodium* species-specific primers were designed to sequence some PCR products showing discrepancies between microscopy and PCR-CMPH (Table 1). The amplicons were first purified by using a QIAquick PCR purification kit (QIAGEN, France). Five microliters of purified PCR products was electrophoresed in a 2% agarose gel in the presence of ethidium bromide and visualized under UV light in order to evaluate its concentrations using the quantified Smartladder as a reference (Eurogentec, Belgium). The two strands of amplified DNA were then sequenced by using the PCR primers (Table 1) and the BigDye Terminator v1.1. cycle sequencing kit according to the manufacturer's instructions (Applied Biosystems,

TABLE 2. Colorimetric detection of various concentrations of the *Plasmodium* genus PCR product according to the amount of 5'-end-phosphorylated probe (p-Pfal) used for covalent binding to each well^a

p-Pfal/well (pmol)	Signal (<i>A</i> ₄₅₀)	Background noise (<i>A</i> ₄₅₀)	S/N ratio
With 5 µl of PCR product ^b			
25	1.025	0.037	27.64
50	1.090	0.028	38.92
75	1.493	0.031	48.16
With 10 µl of PCR product ^b			
25	1.190	0.037	32.16
50	1.510	0.028	53.92
75	1.722	0.031	55.58

^a Values given are the mean absorbance values from experiments conducted in duplicate.

^b PCR product prepared from a human blood sample infected with *P. falciparum* (3% parasitemia).

France). Briefly, in a final volume of 10 µl, 1 µl of PCR product was added to 2 µl of mix, 1 µl of buffer, and 1.6 pmol of primer. Sequencing reactions were performed using a thermal iCycler IQ system (Bio-Rad, France) with a first step of denaturation at 96°C for 1 min, followed by 25 amplification cycles at 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. The resulting products were then purified by a succession of washing and centrifugation steps. Finally, the pellets were resuspended and analyzed using an automated sequencer (ABI Prism 3100 Genetic analyzer). Similarity searches were performed by using the Blastn program at the NCBI website (<http://www.ncbi.nlm.nih.gov/BLAST/>).

RESULTS

PCR specificity. With the primers set (Biot-rPLU6-PR3) specific for the *Plasmodium* genus, a 100% detection rate was obtained when parasitemia was higher than 0.01% (500 parasites/µl). A 300-bp amplicon was visualized by agarose gel electrophoresis, as expected from bioinformatics studies. For lower levels of parasitemia, the PCR product was not consistently observed. An additional specific band of a slightly higher molecular size was sometimes observed when both asexual and sexual sequences of the 18S rRNA genes were amplified, as described previously (15).

Optimization of CMPH. Optimization was performed by varying the EDC concentrations with serial 10-fold dilutions from 5 µM to 500 mM. For concentrations below 5 mM, the signal-to-noise (S/N) ratio fell proportionally to the EDC concentration decrease (data not shown). Therefore, the 50 mM concentration was considered optimal.

When the amount of probe went up from 25 pmol to 75 pmol, with 5 or 10 µl of the PCR product, the S/N ratio increase was not proportional, but in all cases, the signal remained in the acceptable range for positive detection. It was not thought to be worthwhile to increase the amount of probe beyond 50 pmol, as this would have made the development of a routine assay and epidemiological study too expensive (Table 2).

Hybridization and washing conditions were also optimized (data not shown). A hybridization temperature of 42°C for 1 h and washings at 58°C with TTSS buffer were considered optimal. These stringent washing conditions facilitate the detection of positive samples with minimal background noise of 0.030 ± 0.013 (calculated from 50 values) and without a loss of covalently bound material. For positive controls (5'-end-biotinyl-

TABLE 3. Comparison of results obtained by PCR-CMPH and light microscopy as a standard for malaria diagnosis

Finding	Species detected by:		No. of patients (no. of samples) ^a
	Microscopy	PCR-CMPH	
Results agree	<i>P. falciparum</i>	<i>P. falciparum</i>	26 (45)
	<i>P. malariae</i>	<i>P. malariae</i>	3 (3)
	<i>P. ovale</i>	<i>P. ovale</i>	8 (11)
	<i>P. vivax</i>	<i>P. vivax</i>	5 (6)
	Negative	Negative	5 (5)
Total agreement			47 (70)
Results disagree	<i>P. falciparum</i>	Negative	1 (1)
	<i>P. ovale</i>	<i>P. vivax</i>	2 (1)
	<i>P. ovale</i>	<i>P. vivax</i> + <i>P. falciparum</i>	1 (2)
	<i>P. vivax</i>	<i>P. ovale</i>	1 (1)
	<i>P. vivax</i>	<i>P. ovale</i> + <i>P. falciparum</i>	1 (1)
	<i>P. vivax</i>	<i>P. vivax</i> + <i>P. falciparum</i>	1 (1)
	Negative	<i>P. ovale</i>	1 (1)
	Negative	<i>P. falciparum</i>	1 (1)
Total disagreement			9 (9)
Total			56 (79)

^a For some patients, several blood samples were collected during the first days of the hospitalization period.

ated oligonucleotides complementary to the probes), the S/N ratio obtained was between 80 and 100.

Detection and species-specific identification of PCR products by CMPH. The results for the 79 blood samples tested with PCR-CMPH were compared to results obtained by conventional microscopy and molecular biology methods.

In order to evaluate the sensitivity of the assay, a human blood sample infected with *P. falciparum* (1.25×10^4 parasites/ μ l) was serially 10-fold diluted with uninfected human blood and tested by PCR-CMPH. The limit of PCR-CMPH detection from whole blood is approximately 10 parasites/ μ l, corresponding to a parasitemia level of 0.00025% and taking 5×10^6 erythrocytes/ μ l as a reference.

A comparison of results obtained by blood smear microscopy and PCR-CMPH showed complete concordance in the diagnosis of malaria for 47 of the 56 patients tested (84%) (Table 3).

Forty-two patients were found to be infected by only one *Plasmodium* species: 26 patients were infected by *P. falciparum*, 3 were infected by *P. malariae*, 5 were infected by *P. vivax*, and 8 were infected by *P. ovale*. Results were positive with their respective probes (S/N ratio ranging from 1.41 to 72.82) but negative with the three other probes. No cross-hybridization was observed with the four probes, confirming their theoretical specificity (depending on the few 18S sequences actually available in databases). In cases in which serial blood specimens were collected at the beginning of treatment, a decrease of the positive signal was noted with PCR-CMPH, corresponding to the decrease of parasitemia observed by microscopy (data not shown).

Five patients for whom the malaria diagnosis was excluded on the basis of microscopy and immunochromatographic test (ICT Now Malaria) were found to be noninfected when PCR-CMPH was used with the four probes.

Results disagreed for nine patients (Table 3). Two patients had negative microscopy results, but one of them, positive by

the ICT Now Malaria test, was identified as being positive for *P. ovale* by PCR-CMPH (S/N ratio of 25.25). *P. falciparum* was detected in the second negative sample identified by microscopy (S/N ratio of 13.48). Those PCR-CMPH results were confirmed by nested PCR and DNA sequencing, proving the presence of *P. ovale* in the first case and *P. falciparum* in the second case. For five patients, PCR-CMPH detected *P. ovale* or *P. vivax*, whereas light microscopy identified *P. vivax* or *P. ovale*, or vice versa. For these results, the evidence was supported by nested PCR and further examination of the blood films. For three patients, light microscopy identified the parasite as *P. ovale* or *P. vivax* only, whereas PCR-CMPH detected mixed infections (one of the former plus *P. falciparum*); the presence of *P. falciparum* was confirmed by sequencing. Finally, in one case, PCR-CMPH was negative although *P. falciparum* was found by microscopy, STEVOR PCR, and nested PCR.

Moreover, negative controls were tested with blood from patients that were addressed in the hospital for other pathologies (toxoplasmosis, invasive mycosis, etc.). No cross-reaction was observed for these samples with the four probes.

DISCUSSION

In the last few years, PCR-based assays for malaria have proven to be more specific and sensitive than microscopy and antigen capture rapid diagnostic tests, but in order to be used for routine diagnosis, they must be simple, rapid, and inexpensive. Nested PCR is largely employed in laboratories to detect and identify *Plasmodium* species, although it is considered to be laborious and technically cumbersome. Moreover, in nested PCR, post-PCR handling is required to transfer amplicons from the primary to the secondary amplification reaction. This constitutes a major risk of contamination, making that technique inaccurate for diagnostic purposes. Several studies have shown that such problems could be avoided by combining a single round of PCR with a specific identification by using hybridization (9, 22). Microtiter plate hybridization joined to colorimetric detection is particularly well adapted to routine use in clinical laboratories, as it can easily be handled and automated. A simple approach is to use a specific capture probe immobilized on a microwell surface. Immobilization can be obtained by passive adsorption, UV light, or covalent binding of base-modified oligonucleotides. A disadvantage is that capture probes are bound more or less efficiently to the microwell at multiples sites. The ideal immobilization of the probe onto the polystyrene surface is obtained by covalent binding at only one point preferably located at either the 3' or the 5' end (17).

In this study, we developed a PCR followed by nonradioactive hybridization using oligonucleotides covalently bound by their 5' end to CovaLink NH microwells for the detection and identification of four *Plasmodium* species detected in human blood samples. The microwell surface was evaluated in order to develop a simple, sensitive, and specific strategy with a genus-specific PCR to detect the presence of the *Plasmodium* spp. Next, in a rapid hybridization step, amplicons were identified by species-specific capture probes.

Previous studies have described the hybridization ability of an oligonucleotide covalently attached to CovaLink NH

microwells (2, 17). The microwell surface has secondary amine groups positioned at the end of a spacer arm covalently grafted onto the polystyrene. The oligonucleotide is covalently linked by its 5'-terminal phosphate group to the surface by means of a phosphoramidate bond (16). This method was applied recently to detect pathogenic bacteria by sandwich hybridization using a capture probe and a biotin-labeled oligonucleotide (10).

To perform this hybridization step, EDC is necessary to activate the phosphate group located at the 5' end of the probe. Rasmussen et al. (17) have previously shown that with an EDC concentration of 50 mM, 85% of the 5'-end-phosphorylated DNA molecules were bound exclusively by the 5' end, which is particularly suitable for hybridization. On the other hand, Lund et al. previously reported that carbodiimide may modify the bases of nucleic acids, causing a nonspecific attachment of DNA, and this can be deleterious to hybridization (12). Therefore, the EDC concentration was evaluated in order to obtain the optimal S/N ratio by reducing either the noncovalent binding or the nonexclusive attachment by the 5' end or by avoiding potential chemical changes in the bases of nucleotides. A 10-fold increase or decrease of the initial 50 mM concentration, as suggested previously by Rasmussen, did not show any significant change in the S/N ratio. The 50 mM concentration was therefore left unchanged.

In this assay, 79 blood samples were selected from 56 patients with suspected malaria infection who had traveled in areas where malaria is endemic. All samples were tested by PCR-CMPH and classical diagnosis techniques, and results were compared. For 47 out of 56 patients (84%), microscopy and PCR-CMPH were in agreement. For the remaining nine patients, PCR-CMPH corrected and completed the identification made by microscopy. Thus, three mixed infections were identified by PCR-CMPH, whereas classical diagnosis methods (microscopy and antigen detection) did not recognize them. It is generally considered that one species of parasite may influence the prevalence of another in mixed infections (13). This could explain why it is difficult to detect mixed infections by routine microscopy, particularly when only one blood sample is available. PCR-CMPH clearly offers an advantage in this situation.

In five cases, the microscopy results were proven to be wrong because of confusion between *P. vivax* and *P. ovale*. Such a mistake, resulting from morphological similarities, happens frequently even with experienced observers, especially when those observers lack information about the clinical and travel history of the patient.

Microscopy was negative for two blood samples, although PCR-CMPH detected a potentially fatal *P. falciparum* infection in one case and *P. ovale* with possible relapse in the other case. PCR-CMPH, with a sensitivity of about 10 parasites/ μ l, can detect parasitemia below the detection level of microscopy (the absolute detection limit of microscopy is about 50 parasites/ μ l) (7, 22).

In contrast, we encountered one case of *P. falciparum* infection detected by microscopy and confirmed by two PCR techniques (STEVR PCR and nested PCR), but PCR-CMPH labeled it as negative; the DNA was amplified but undetected by hybridization. The discrepancy was probably due to a sequence mismatch that was able to decrease probe affinity sig-

nificantly. This suggestion was made previously by Kimura et al. (9) and Whiley et al. (22), who used similar hybridization techniques for *P. vivax* strains in which such mismatches occurred. In our study, we have aligned the sequences available in the GenBank database in order to assess the specificity of the probes chosen by Whiley et al. and to design a specific *P. malaria* probe. The bioinformatics study was extended with TIGR (<http://www.tigr.org/index.shtml>) and Plasmo-DB (<http://plasmodb.org>) databases, which comprise a higher sequence number, exclusively concerning *P. falciparum* and *P. vivax*. The BlastN search made between the *P. falciparum* and *P. vivax* probe sequences and the corresponding 18S sequences have revealed variable homologies. This can significantly decrease probe affinity. Nevertheless, in spite of this theoretic variability, in our study, each probe was able to detect all the blood samples except one.

In conclusion, our results show that PCR-CMPH has advantages over microscopy in detecting mixed infections and correcting species identification errors. This can be of great value for successful medical treatment and also for epidemiological studies. Whereas a diagnosis of malaria is a matter of urgency, PCR-CMPH is not a "rapid test" and should be used to confirm and complete the microscopic examination.

Compared to hybridization techniques described previously by other authors such as Whiley et al., who used expensive streptavidin-coated microwells, it is easier, as in the PCR-CMPH test, to directly bind the probe onto the polystyrene surface (22). Concerning the labeling, only one of both consensus primers is biotinylated in the amplification, whereas in the latter technique, a second marker is necessary to detect the PCR product (digoxigenin-anti-digoxigenin peroxidase conjugate).

In the last few years, other new methodologies such as real-time PCR have been developed (1, 2, 4, 18). Real-time PCR employs fluorescent labels to monitor amplicon formation throughout the reaction. This technology was adapted to the qualitative and quantitative detection of all four malaria parasites that infect humans and is suitable for the screening of large numbers of samples (11). It offers a rapid and accurate alternative for the diagnosis of malaria. However, the high cost of this technique prevents it from being introduced into developing countries. Moreover, it needs to be handled by highly qualified personnel.

On the contrary, PCR-CMPH is technically simple and easy to handle. It uses stable reagents: precoated strips stored at 4°C can be employed for up to 10 months (2). It does not require electrophoresis with toxic reagents such as ethidium bromide and can be operated with simple enzyme-linked immunosorbent assay material. It is rapid and allows the simultaneous detection and differentiation of all *Plasmodium* species that infect humans within 6 h after receipt of clinical samples. Finally, PCR-CMPH could be automated, thus permitting the comparative analysis of many samples in parallel, and is adaptable for routine epidemiological use in areas where malaria is endemic.

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