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Detection of Four *Plasmodium* Species in Blood from Humans by 18S rRNA Gene Subunit-Based and Species-Specific Real-Time PCR Assays

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There have been reports of increasing numbers of cases of malaria among migrants and travelers. Although microscopic examination of blood smears remains the "gold standard" in diagnosis, this method suffers from insufficient sensitivity and requires considerable expertise. To improve diagnosis, a multiplex real-time PCR was developed. One set of generic primers targeting a highly conserved region of the 18S rRNA gene of the genus *Plasmodium* was designed; the primer set was polymorphic enough internally to design four species-specific probes for *P. falciparum*, *P. vivax*, *P. malarie*, and *P. ovale*. Real-time PCR with species-specific probes detected one plasmid copy of *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale* specifically. The same sensitivity was achieved for all species with real-time PCR with the 18S screening probe. Ninety-seven blood samples were investigated. For 66 of them (60 patients), microscopy and real-time PCR results were compared and had a crude agreement of 86% for the detection of plasmodia. Discordant results were reevaluated with clinical, molecular, and sequencing data to resolve them. All nine discordances between 18S screening PCR and microscopy were resolved in favor of the molecular method, as were eight of nine discordances at the species level for the species-specific PCR among the 31 samples positive by both methods. The other 31 blood samples were tested to monitor the antimalaria treatment in seven patients. The number of parasites measured by real-time PCR fell rapidly for six out of seven patients in parallel to parasitemia determined microscopically. This suggests a role of quantitative PCR for the monitoring of patients receiving antimalaria therapy.

The development of tourism and travel during the last decades caused an increase of malaria cases reported in migrants and travelers returning from malaria-endemic areas. In Switzerland, since the beginning of the 1980s, the number of reported cases per year has more than doubled, reaching 300 cases in 2000 (24). This could be underestimated, as in many developed countries, only 25 to 50% of the cases are reported to health authorities (31). The case fatality rate associated with imported malaria varies from 0.6% to 3.8% and could be reduced with prompt recognition of the disease. Unfortunately, delays averaging 1 week from presentation to treatment have been reported by some investigators (14).

Microscopic examination of thin and/or thick blood smears has been the mainstay of malaria diagnosis. It is an inexpensive, rapid, and relatively sensitive procedure, allowing the detection of 50 to 500 parasites/μl (thick versus thin smears) (22). It also enables identification of plasmodial species and quantification of parasites, both of which are important to assess disease severity and to prescribe adequate therapy. However, interpretation of smears requires considerable expertise, particularly at low-level parasitemia, potentially leading to false negative results or unreliable species determination. This dependence on the microscopist's expertise can be illustrated by the results of four quality controls of the United Kingdom National External Quality Assessment Scheme for Parasitology: among the 262 laboratories tested in 2002, the correct species identification varied from 63.7% to 95%. Finally, mixed infection, seen in migrants, can be missed by this conventional method (31).

Rapid diagnostic tests based on malarial antigen capture with immunochromatographic (ICT) strip technology and use of monoclonal antibodies have been developed to improve the timeliness, sensitivity, and objectivity of malaria diagnosis. These ICT tests, the P.f/P.v (Amrad, Sydney, Australia), the NOW ICT malaria test (Binax, Inc., Portland, Maine), and the OptiMal (Flow Inc., Portland, Oreg.), can potentially detect the four *Plasmodium* species that infect humans. A recent evaluation showed a poor performance by those tests in diagnosing non-falciparum malaria with a sensitivity of 44% for *P. vivax* by ICT P.f/P.v (27). As the second panmalarial antigen line of rapid diagnostic tests may be positive with all four *Plasmodium* species, these tests cannot be used to determine the cause of a mixed infection.

Molecular methods based on DNA amplification have been applied to malaria diagnosis since the late 1980s (6, 25, 36–38). Their value lies in their high sensitivity, detecting ≤5 parasites/μl. With nested or seminested PCR methods targeting the small-subunit 18S rRNA gene, all four species could be identified. PCR also showed increased sensitivity in comparison to microscopy for the diagnosis of mixed infection (32, 35). Unfortunately, these conventional PCR assays are technically demanding and time-consuming. Moreover, they are prone to carry over contamination during the manipulation of postamplification products, a problem already observed in the unique

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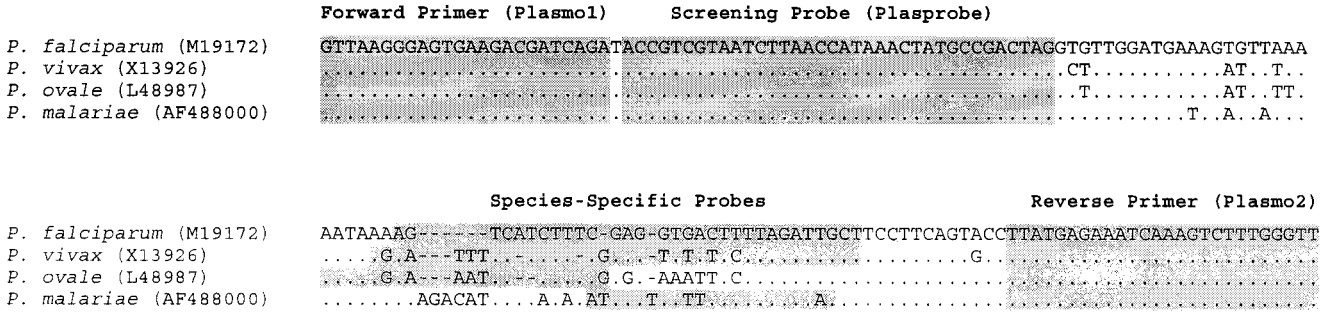

FIG. 1. Alignment of partial 18S rRNA genes.

study testing the use of PCR as a routine method for malaria diagnosis (23).

Real-time PCR methods are a recent development of established PCR methodologies. They use fluorescent labels for continuous monitoring of amplicon formation throughout the reaction. The advantages of these techniques are quantification of pathogens, reduced risk of contamination, and availability of results within 3 h (including sample preparation). Few studies assessed their application in parasitology (1, 2, 7, 19, 20, 26)

The goal of this work was to develop a quantitative multiplex real-time PCR for the simultaneous detection of *P. falciparum, P. vivax, P. malariae* and *P. ovale*, and its evaluation on blood samples, with microscopy used as the gold standard comparative method.

MATERIALS AND METHODS

Origin of the specimens. A total of 97 EDTA-treated blood samples were investigated by PCR: 66 samples collected from 60 travelers or migrants attending the University Hospital of Lausanne, Switzerland, with suspected malaria and whose blood was submitted for routine microscopic examination (thin smears Giemsa) to the laboratory of parasitology. They were prospectively collected from July 2002 to December 2003, stored at −20°C, and run blindly. Another 31 samples were collected from seven patients attending the University Hospital of Lausanne, Switzerland, with known *P. falciparum* malaria based on routine microscopic examination. At least four samples were taken per patient, at day 0 (before treatment), and then at days 1, 2, and 3 after beginning of treatment. For one patient, seven samples were examined (from day 0 to day 9). They were collected prospectively from July 2003 to December 2003, and all samples of each patient were run in parallel to estimate the level of *P. falciparum* parasitemia during treatment.

Conventional microscopy. Microscopy of thin Giemsa-stained smears was considered the reference method. The smears were read for at least 20 min by three technicians (first microscopy). Final species diagnosis was based on the majority agreement. Samples with discrepancy between microscopy results and those of the *Plasmodium* species-specific real-time PCR were blindly submitted to a second independent microscopist (second microscopy). Parasitemia was estimated by expressing the percentage of parasitized red blood cells seen among 5,000 red blood cells on stained thin blood films. To express the parasitemia as number of parasites present in 1 μl of blood, it was assumed that 1 μl of blood contained 5×10^6 red blood cells, so 1% parasitemia corresponds to 1 parasite/100 red blood cells or 50,000 parasites/μl of blood (22).

DNA extraction. DNA was extracted from 200 μl of thawed EDTA-blood with the MagNA Pure LC automated system (Roche, Basel, Switzerland), with the MagNA Pure LC DNA isolation kit I according to the manufacturer's instructions. At least four negative extraction controls were added to each plate.

Real-time PCR assays. Primer and probe sequences were selected from the small subunit of rRNA (18S) (Fig. 1). Sequence alignment of the block 9 region (29) allowed us to design a forward primer, Plasmo 1 (5′-GTTAAGGGAGTG AAGACGA TCAGA-3′), and a reverse primer, Plasmo 2 (5′-AACCCAAAGA CTTTGATTTC TCATAA-3′), to amplify a 157- to 165-bp segment of the four plasmodial 18S genes. For screening purposes, a *Plasmodium* 18S screening TaqMan probe was labeled with 5′FAM (6-carboxyfluorescein) and 3′TAMRA (6-carboxytetramethyl-rhodamine) as the reporter and quencher, respectively (Plasprobe: 5′-FAM-ACCGTCGTAA TCTTAACCAT AAACTATGCC GAC TAG-TAMRA-3′) was designed to detect all *Plasmodium* sp.-positive samples.

Four species-specific probes were selected to differentiate between the species: *P. falciparum* labeled with 5′FAM and 3′TAMRA (Falcprobe: 5′-FAM-AGCA ATCTAA AAGTCACCTC GAAAGATGAC T-TAMRA-3′), *P. vivax* labeled with 5′VIC (6-carboxyfluorescein) and 3′TAMRA (Vivprobe: 5′-VIC-AGCAA TCTAA GAATAAACTC CGAAGAGAAA ATTCT-TAMRA-3′), *P. malariae* (minor groove binder probe) labeled with 5′FAM (Malaprobe: 5′FAM-CTATCTAAAA GAAACACTCAT), and *P. ovale* (minor groove binder probe), labeled with 5′VIC (Ovaprobe: 5′VIC-CGAAAGGAATTTTCTTATT). They were designed to avoid intraspecies polymorph regions (GenBank accession numbers M19172 for *P. falciparum*, X13926 for *P. vivax*, AF488000 for *P. malariae*, and L48987 for *P. ovale*). Such intraspecies variations have already been described to cause false negative results in studies with microtiter plate hybridization of amplicons (16, 17, 21, 39). Moreover, in order to overcome the shortness of the nonpolymorphic region within *P. ovale* and *P. malariae* species, minor groove binder probes (Applied Biosystem, Rotkreuz, Switzerland) were used. All primers and probes were designed with Primer Express Software (Applied Biosystem) and came from Applied Biosystem.

Positive controls. For each species, plasmids containing the block 9 region of the 18S gene were constructed in order to facilitate sensitivity assessment.

DNA of *P. falciparum*, *P. malariae*, *P. vivax*, and two different *P. ovale* strains, P.o1, the usual strain, and the new strain P.o2, which shows sequence variation within the target region, as described by Kawamoto (16), were extracted from samples diagnosed and confirmed to the species level at the Swiss Tropical Institute of Basel (Switzerland); 5 μl of each of these DNAs were individually added to a reaction mixture containing 0.4 μM each of primers Plasmo1 and 2, 1 U of Ampli*Taq* DNA polymerase (Applied Biosystem)/reaction, 25 μl of buffer containing 20 mM Tris-HCl, 100 mM KCl, 3 mM $MgCl_2$, 0.02% gelatin, and 400 μM deoxynucleoside triphosphates (Microsynth, Balgach, Switzerland). PCR was performed in a GeneAMP PCR system 9700 (Applied Biosystem) with the following thermal profile: 10 min at 95°C, 1 min at 60°C, 1 min at 72°C, 45 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 45 s, and 1 cycle for 10 min at 72°C. PCR products were purified with the QIAquick PCR purification Kit (Qiagen) and cloned into the pGEM-T Easy vector (Promega, Wallisellen, Switzerland) according to the manufacturer's instructions. Isolation of recombinant plasmid DNA was performed with QIAprep Miniprep (Qiagen), and the presence of the correct insert was confirmed by sequencing.

The five plasmids were linearized with PstI, purified with the QIAquick PCR purification kit, and quantified spectrophotometrically (GeneQuant pro RNA/DNA calculator, Amersham Phamacia Biotech, Uppsala, Sweden). These positive controls were 10-fold diluted, stabilized by adding human genomic DNA (10 ng/μl; Roche, Basel, Switzerland), and stored at −20°C. The effect of human DNA on the real-time PCRs was studied by adding decreasing amounts of plasmodial DNA (10^5, 10^3, and 10^1 copies/reaction) to different concentrations (10, 100, and 200 ng/μl) of human DNA (Roche).

18S screening real-time PCR. The 18S screening real-time PCR is a monoplex PCR designed to detect *Plasmodium* sp.-positive samples. The reaction was performed in a final volume of 25 μl containing 5 μl of DNA, 200 nM Plasmo1, 200 nM Plasmo2, 50 nM 18S screening TaqMan probe (*Plas*probe), and 12.5 μl of TaqMan Universal Master Mix (Applied Biosystems). Amplification and detection of the amplified product were performed on an ABI Prism 7700 (Applied Biosystems). A negative extraction control as well as an inhibition control (spiked specimen) were included for each sample. The following conditions were used

TABLE 1. Sensitivity and reproducibility of the 18S screening real-time PCR and the *Plasmodium* species-specific real-time PCR when tested with a single target

Target	Probe	Mean C_t at dilution[a]:			
		10^3 copies	10^2 copies	10^1 copies	10^0 copies (% of isolates)
P. falciparum	Plasprobe	29.1 ± 1.0	33.1 ± 0.6	37 ± 0.7	40.3 ± 1.5 (100)
	Falcprobe	29.3 ± 0.5	32.7 ± 0.6	36.1 ± 1	38.9 ± 0.8 (66)
P. vivax	Plasprobe	28.3 ± 0.6	31.9 ± 0.7	36.7 ± 1.2	38.6 ± 0.3 (66)
	Vivprobe	30.2 ± 0.2	33.7 ± 0.4	37.6 ± 0.5	40.2 ± 0.4 (83)
P. malariae	Plasprobe	28.8 ± 0.5	32.8 ± 0.4	36.3 ± 0.2	39.1 ± 0.6 (83)
	Malaprobe	28.2 ± 0.5	32 ± 0.4	35 ± 0.4	38.4 ± 1.8 (50)
P. ovale	Plasprobe	29.6 ± 1.1	33.1 ± 1.4	36.8 ± 0.5	40.1 ± 1.4 (83)
	Ovaprobe	28.7 ± 0.9	32.4 ± 1.1	35.6 ± 1.5	39.3 ± 1.1 (66)
P. ovale (new)	Plasprobe	29.9 ± 2.2	33.2 ± 2.2	36.5 ± 1.6	40.0 ± 0.5 (66)
	Ovaprobe	29.8 ± 1.0	33.3 ± 1.0	36.9 ± 0.8	39.6 ± 1.1 (100)

[a] Mean values and standard deviations are based on two experiments, each done in triplicate.

for PCR: an initial step at 50°C for 2 min, 95C° for 10 min, and 45 cycles of 95°C for 15 s and 60°C for 1 min. The sample was considered positive by identifying the threshold cycle number (C_t) at which normalized reporter dye emission raised above background noise. If the fluorescent signal did not increase within 40 cycles (C_t 40), the sample was considered negative.

***Plasmodium* species-specific real-time PCR.** The *Plasmodium* species-specific real-time PCR is a multiplex PCR for the determination of the four different *Plasmodium* species. It was performed in two simultaneous separate reactions.

In the first reaction, primers Plasmo1 and Plasmo2 and the two TaqMan probes corresponding to *P. falciparum* and *P. vivax* were mixed. This PCR assay was performed in a final volume of 25 μl, containing 12.5 μl of TaqMan Universal Master Mix (Applied Biosystems), 200 nM each of Plasmo 1 and Plasmo 2 primers, 80 nM Falcprobe, and 80 nM Vivprobe

Similarly, in the second reaction, the same primers, Plasmo 1 and Plasmo 2, and the two probes corresponding to *P. malariae* and *P. ovale* were mixed. This PCR assay was also performed in a final volume of 25 μl, containing 12.5 μl of TaqMan Universal Master Mix (Applied Biosystems), 200 nM each of Plasmo 1 and Plasmo 2 primers, 80 nM Malaprobe, and 80 nM Ovaprobe. Exclusion of a plasmodial variant not detected by those four probes was obtained by running the 18S screening real-time PCR in parallel.

Each reaction of the monoplex and the multiplex PCRs was performed in triplicate in order to assess the reproducibility, and the same conditions were used for the amplification steps as well as for the interpretation of a positive versus negative result as described above.

Sensitivity of the real-time PCRs. The sensitivity of primer and probe sets was assessed on 10-fold serial dilutions (10,000 to 1 copy) of positive control DNAs, first in the 18S screening real-time PCR and then in the *Plasmodium* species-specific real-time PCR. The serial dilutions of DNAs were done in a solution containing 10 ng/μl human DNA (Roche, Rotkreuz, Switzerland) in order to test the efficiency of the amplification when the relative amount of human DNA increased. For the measure of reproducibility of the threshold cycle number (C_t) results, the mean value and standard deviations were calculated from triplicates, and assays were repeated twice. Furthermore, different concentrations of plasmid DNA from each species were mixed and tested by the *Plasmodium* species-specific real-time PCR to ascertain the ability of the assay to coamplify different DNA targets in order to detect mixed infections.

Specificity of the real-time PCRs. The specificity of the genus-conserved *Plas*probe was evaluated on DNA of other 18S-possessing eukaryotes, i.e., human genomic DNA (Roche, Rotkreuz, Switzerland), *Aspergillus* (IP 2279–94), *Toxoplasma* and *Pneumocystis* (extracted from clinical samples diagnosed at our Institute), and *Neospora* and *Leishmania* (provided by the Institute of Parasitology, University of Bern, Switzerland). The specificity of each species-specific probe was also tested with those pathogens and cross-reactivity within plasmodial species was assessed. For each DNA, a species-specific monoplex real-time PCR assay was performed in duplicate with 10 ng of DNA. A negative control (no template) and an inhibition control (spiked sample with a known amount of positive control DNA) were tested simultaneously for each organism.

Additional tests performed. Additional tests were only performed on the samples for which microscopy and real-time PCR gave different results. An immunochromatographic test (ICT Malaria P.f/P.v, AMRAD-ICT Diagnostic, Sydney, Australia, no longer available and now replaced by the NOW ICT malaria test) was first performed on all samples for which microscopy and real-time PCR gave different results. This test is known to represent a useful adjunct for the diagnosis of low-level parasitemia in returning travelers who have initiated presumptive antimalarial treatment and also when expert microscopy is not available. However it was not designed to distinguish between the different non-falciparum species (6, 27, 30). ICT was carried out as recommended by the manufacturer except that these analyses were done with retrospectively frozen (−20°C) EDTA blood samples. The reliability of ICT performed on frozen blood was assessed by comparing 10 positive and 10 negative samples before and after freeze-thawing. Freezing did not modify reading of ICT (results not shown).

Second, a nested PCR was performed as an adjunct for the diagnosis of malaria on samples for which microscopy and real-time PCR gave different results not settled by ICT. This validated method was used as it was considered the reference standard for low-level parasitemia because of its high sensitivity, high specificity, and ability to distinguish mixed infections (35). It was performed blindly by the Swiss Tropical Institute of Basel, Switzerland, with a method described previously.

Finally, sequencing of PCR products was performed on samples for which microscopy and multiplex PCR gave different results regarding the species. Sequencing was performed with a commercially available ABI Prism BigDye terminator cycle sequencing kit (Applied Biosystems) as indicated by the manufacturer. Products were electrophoresed on an ABI Prism 310 automated sequencer (Applied Biosystems) as described by the manufacturer.

Clinical data. Prophylaxis and treatment administration prior to sampling were investigated when discrepancies between test results were observed for a given patient.

Statistical analysis. The median of C_t values of the 18S screening real-time PCR with positive result of blood film examination was compared to the median of the C_t values of the 18S screening real-time PCR with negative result of blood film examination with the Mann-Whitney test (Graph Pad Prism 3.02).

RESULTS

Single target amplification. To determine the sensitivity and the specificity of the primers and the different probes, monoplex PCRs were first performed with the primer set Plasmo 1 and 2, with either the screening *Plas*probe or one of the four species-specific probes. All these different monoplex PCRs displayed the same level of sensitivity, detecting 1 to 10 copies/5 μl of the diluted positive controls, i.e., extracted DNA of each five plasmodial DNAs. The reproducibility of these results was determined by testing triplicates of 10-fold serial dilutions of positive controls in two independent experiments (Table 1). For all species, at a concentration of 1 copy per reaction on average, four of six, five of six, three of six, four of six and six of six of the replicates were positive for *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale 1*, and *P. ovale 2*, respectively, consistent with a stochastic distribution of positives. Changing the probe sets from a monoplex to a multiplex format did not modify this sensitivity.

TABLE 2. Sensitivity of the 18S screening monoplex real-time PCR versus microscopy for 66 EDTA-treated blood samples from 60 patients

Microscopy result	No. of samples (no. of patients) with PCR result:		Total no. of samples (no. of patients)
	Positive	Negative	
Positive	31 (28)	3 (3)	34 (31)
Negative	6 (6)	26 (23)	32 (29)
Total	37 (34)	29 (26)	66 (60)

When testing the *Plas*probe against other 18S eukaryotic DNA, positive results were observed for *Aspergillus*, *Toxoplasma*, *Neospora*, and *Pneumocystis*. This was explained by the hybridization of the *Plas*probe with a sequence conserved not only within the plasmodial genus but also within several homologous eukaryotes, but not with human DNA. The four species-specific probes displayed a high specificity and neither intraplasmodial cross-reaction nor other homologous eukaryotes detection was observed. Increasing the amount of human DNA in the reaction mixture did not affect the efficiency of amplification, as the slope of standard curve generated from dilution series of positive controls (10^5, 10^3, and 10^1 copies/amplification) remained unchanged (data not shown).

Multiple target amplification. To evaluate the ability of the *Plasmodium* species-specific real-time PCR to detect mixed plasmodial infection, mixtures of different plasmids in various relative concentrations were amplified in a single reaction. Copies of *P. malariae* (plasmids) (50,000 to 1,000 copies/μl) were diluted in water and, in parallel, diluted in a solution containing 50,000 copies/μl of *P. falciparum* plasmids. An attenuated amplification plot was observed for the species present at lower concentration (*P. malariae*), due to the common competition of primers. The detection limit of the minority species compared to the species present in majority did not vary with the total amount of target DNA but with the species mixture, and a better discrimination was achieved when the less-concentrated species had the smallest amplification product. For example, when mixing *P. falciparum* (159 bp) with *P. malariae* (165 bp), the first could be detected in a minority proportion of 1:999, whereas the second could only be discriminated from a 1:99 mixture. The minority species could yet be detected, even in the presence of a 100- to 1,000-fold excess of the species that was coamplified.

Analysis of the 97 EDTA-treated blood samples. Sixty-six EDTA-treated blood samples from 60 patients with suspected malaria were analyzed with the 18S screening real-time PCR for screening purpose and with *Plasmodium* species-specific real-time PCR for identification at the species level. Both PCRs were done blindly, and the results were then compared to those of microscopy (Tables 2 and 3).

18S screening real-time PCR (Table 2). Thirty-one out of 66 samples (47%) were positive by both the 18S screening real-time PCR and microscopy, whereas 26 samples were negative by both methods, giving a concordance rate of 86% (57 of 66) between microscopy and 18S screening PCR.

Six samples (six patients) were PCR positive and microscope negative on initial examination. The ICT test (five samples) or nested-PCR (one sample) confirmed the 18S screening PCR positive result for these samples (Table 3). Moreover, analysis of the patients records showed that three of these six samples were from patients already treated for a malaria infection, previously diagnosed either by microscopy (two samples) or by ICT test (one sample) (Table 3, patients 1, 2, and 3). Three other samples were obtained from travelers (patients 4, 5, and 6) with a recent history of chemoprophylaxis. For these various reasons, all six samples were considered true positive PCR results.

Three samples (three patients) were weakly positive by microscopy with a negative 18S screening real-time PCR result (Table 2). All three were found negative by the second microscopist as well as by nested PCR. In two of these samples only one trophozoite of undetermined species was found on initial microscopic examination. For the last one, the ICT result was in agreement with the first positive microscopy result whereas the second microscopy and molecular methods reported it as negative.

***Plasmodium* species-specific real-time PCR.** When the 37 samples positive with the 18S screening real-time PCR were further tested with the *Plasmodium* species-specific real-time PCR, *P. falciparum* was detected as a single agent in 28 of them (76%). *P. vivax* and *P. ovale* were detected in five (14%) and four (11%), respectively. No *P. malariae* and no mixed infection were detected by the *Plasmodium* species-specific real-time PCR.

Of the 31 samples that tested positive by both the screening real-time PCR and microscopy (Table 2), an agreement at the species level was found in 22 cases, giving a crude concordance rate of 71%. Nine samples from eight patients were discordant. Six out of these nine samples were considered mixed infections (*P. falciparum*, and *P. malariae*) on initial microscopic examination but not by the specific PCR (*P. falciparum* positive). In order to resolve these discrepant results, the samples were blindly retested by a second microscopist and by the nested

TABLE 3. Analysis of the six samples positive by 18S screening monoplex real-time PCR and negative by microscopy[a]

Patient no.	Chemoprophylaxis	Previous positive thin smears result	Days of antimalarial treatment	Species-specific real-time PCR result (C_t)	ICT P.f/P.v result	Nested PCR result
1	Unknown	Positive for *P. falciparum*	7	*P. falciparum* (39)	*P. falciparum*	ND
2	Unknown	Positive for *P. vivax*	3	*P. vivax* (37)	Non-*P. falciparum*	ND
3	Mefloquine	ND	1	*P. falciparum* (32)	*P. falciparum*	ND
4	Mefloquine	ND	0	*P. vivax* (38)	Negative	*P. vivax*
5	Mefloquine	ND	0	*P. falciparum* (36)	*P. falciparum*	ND
6	Chloroquine + proguanil	ND	0	*P. falciparum* (31)	*P. falciparum*	ND

[a] ND, not done.

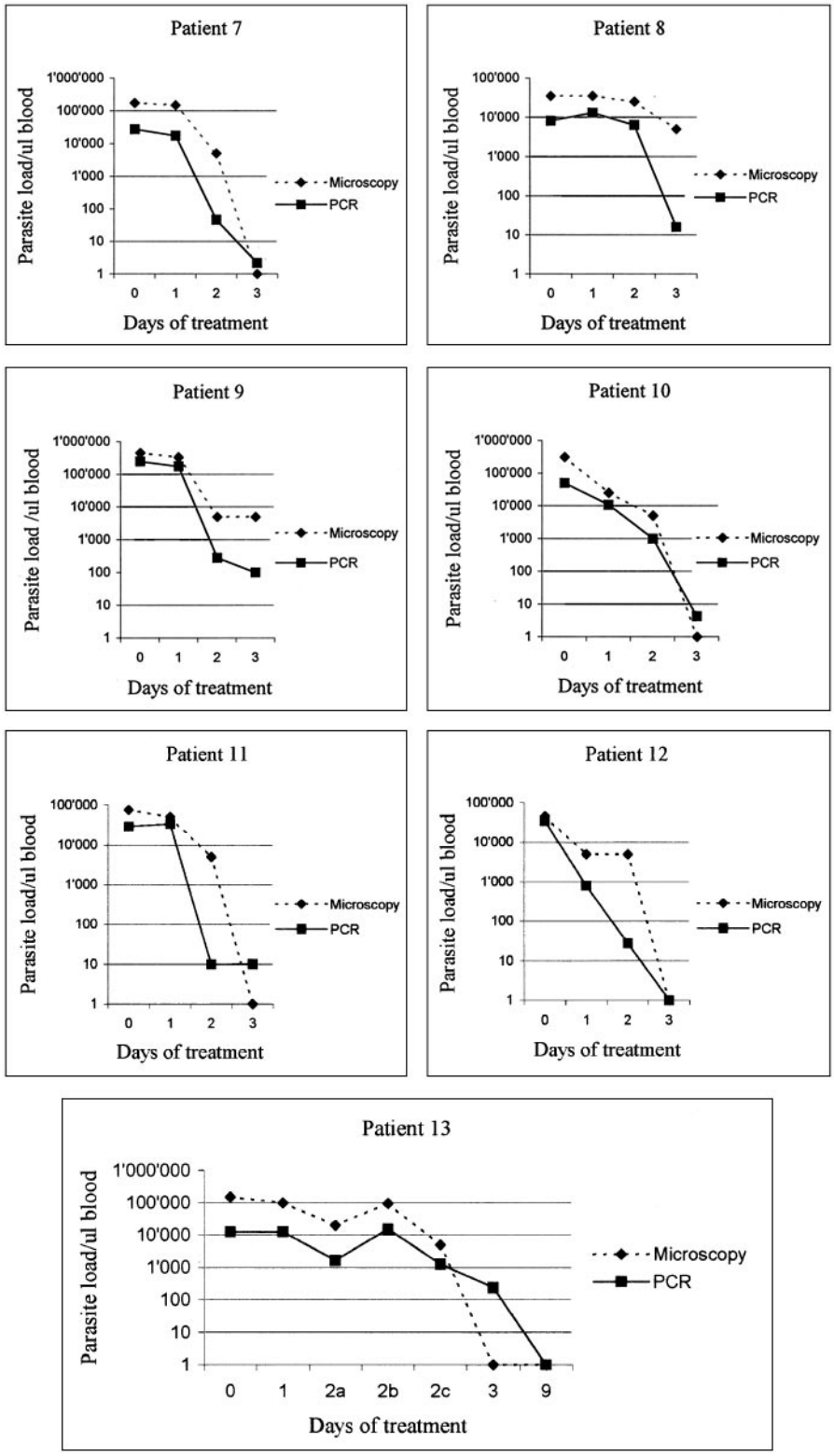

FIG. 2. Parasite load for seven patients receiving antimalaria treatment.

PCR method. Only one mixed infection was detected by both methods. The three other discordant single species identification were found positive for *P. malariae*, *P. vivax*, and *P. ovale* on initial microscopic examination, whereas they were found positive by real-time PCR for *P. ovale* for two specimens and *P. falciparum* for the last one. In order to investigate those differences further, these samples were tested by nested PCR and sequencing. Both techniques confirmed the species deter-

mined by the species-specific real-time PCR. Thus, eight of nine discordances at the species level were resolved in favor of the species-specific real-time PCR.

An attempt to quantify the parasite (*P. falciparum*) load by real-time PCR was made. It was based on the cycle threshold (C_t) value, which indicates the cycle where fluorescence detected from amplification of the target gene exceeded the preset threshold. When correlating the C_t value of the 18S screening real-time PCR with the quantitative result of blood film examination, all 31 samples positive by microscopy had C_t values between 20 to 40 (median 26), while the six samples negative by microscopy but positive by PCR exhibited C_t values between 30 and 40 (median, 36). The difference of the median C_t between positive and negative microscopy samples is statistically significant ($P = 0.0013$).

Thirty-one EDTA-treated blood samples from seven patients with known *P. falciparum* malaria were analyzed for the quantification of parasitemia during treatment (Fig. 2).

The number of parasites determined by microscopy and by species-specific real-time PCR was followed for seven patients (patients 7 to 13) receiving antimalarial treatment. At least four samples for each patient were taken, from day 0 (day of the diagnosis, before therapy) to day 3 of treatment. For six out of seven patients, the number of parasites determined by real-time PCR fell rapidly as did the parasitemia determined microscopically during the first 3 days of treatment. For one patient (patient 13), the parasitemia did not decrease as expected after beginning of treatment. Additional blood samples (from day 2) showed an important parasitic load despite the first drop, consistent with a sequestration-release mechanism (5). The values estimated by thin blood smears have a good correlation with the values calculated by real-time PCR, although consistently higher.

DISCUSSION

The difficulty in maintaining competent microscopists for malaria diagnosis, particularly in areas where the disease is not endemic, prompted the development of nonmicroscopic alternatives. To be clinically useful and to overcome diagnostic microscopy limitations, such a test should be rapid, easy to handle, and highly sensitive, detecting low levels of parasitemia, a situation that is frequently encountered in travelers returning from malaria-endemic areas (27). It should also reliably differentiate between *Plasmodium* species. This test should finally not be prone to subjective variations in interpretation.

Conventional PCR assays developed during the last decade could potentially provide all these advantages, but their nested or seminested format is not suitable for emergency diagnosis (8, 9). These techniques do not allow direct measurement of parasitemia, an important parameter for monitoring response to therapy (4, 13). The spread of drug resistance, already a problem in tropical areas, emphasizes the need for true quantitative methods to predict treatment failure (15). The present development of a multiplex real-time PCR with TaqMan methodology should offer simultaneous detection and differentiation of all four human *Plasmodium* species within 3 h of receipt of clinical samples as well as a quantitative measure of parasitemia.

The method used to extract DNA from blood samples influences the sensitivity of malaria detection by PCR (10). The most appropriate method for blood extraction remains to be established. The current study used the MagNA Pure LC (Roche) automate with the MagNA Pure LC DNA isolation kit I for its rapidity, its low risk of cross-contamination, and because it yields highly purified DNA. On 97 EDTA-treated blood samples, no PCR inhibition was observed, and all extraction controls tested negative.

Several reports have shown that the DNA-based amplification methods had higher sensitivity (as few as 1 parasite/μl of blood) than examination of thin blood smears, especially in cases of low parasitemia or mixed infections (3, 4, 23) and that they have the ability to differentiate *Plasmodium* species (31–33,35-37). Other reports have described quantitative real-time PCRs able to monitor the effectiveness of antimalarial therapy but without being able to distinguish between the four species (18, 34). But no report has yet described the use of a quantitative real-time technology, able to monitor treatment and detect the four *Plasmodium* species at the species level in a single experiment without nested PCR or a hybridization step.

The choice of the most studied gene for malaria diagnosis, the small subunit of the rRNA gene, made possible the selection of two primers which theoretically could amplify any of the analyzed human plasmodial sequences from GenBank. As the genome of plasmodial parasites, at least *P. falciparum*, possesses seven loci of this sequence (12), the performance of the amplification is enhanced. Combined with these two primers, the genus-conserved *Plas*probe was able to detect 1 copy per reaction of all four human *Plasmodium* species, and the same sensitivity was achieved with the four species-specific probes. This sensitivity is far much higher than the expected sensitivity that can be achieved by an experienced microscopist with the examination of the thick or thin blood film procedure.

This enhanced sensitivity compared to microscopic examination was confirmed with clinical specimens, where a screening PCR enabled the detection of parasites in six microscopy-negative samples. In agreement with clinical findings, all six cases appeared to be true malaria infection cases, and the positive PCR results were confirmed either by ICT or by a nested PCR with another target. For the three patients with posttreatment samples and the three patients under chemoprophylaxis, positive PCR blood specimens could be interpreted as the persistence of circulating DNA from gametocytes. Another hypothesis derived from a murine model would consider this DNA as coming from viable but drug-damaged parasites unable to initiate further infection (12, 13). One of these microscopy-negative, PCR-positive samples corresponded to the detection of an early infection, as the sample taken 4 days later from the same patient showed a positive blood smear. This case would have benefited from earlier therapy.

In our experiments, cross-reactivity of the screening PCR due to non-*Plasmodium* spp. was not observed in clinical specimens. This is probably because the population studied, i.e., returned travelers, was not at risk of carrying such pathogens. Nevertheless, any positive result with the screening PCR must undergo species identification with multiplex real-time PCR. In case of negativity of this second diagnostic step, sequencing of PCR products may distinguish between a plasmodial infection and another 18S eukaryote infection or contamination by an environmental nonpathogenic species. In the first situation,

mutations in the targeted sequence might explain the hybridization failure of one of the species-specific probe, a situation that led Kawamoto et al. (17) to the discovery of *P. malariae* and *P. ovale* polymorphisms in the same region of the 18S ribosomal gene. In the second situation, besides the species identification as determined by BLAST search in GenBank, differentiation between pathogenic or contaminant nonplasmodial eukaryote will take into account information such as exposition to a particular agent, clinical findings, quantity of starting template in the sample, and eventually other results by conventional methods.

Although microscopic examination of blood smears remains the gold standard, this method suffers from insufficient sensitivity, as discussed above, and also from insufficient specificity. It also relies on the microscopist's experience and requires considerable training to obtain and maintain the necessary skills. This was obvious with the three samples that were positive by microscopy but negative when tested by 18S screening real-time PCR result. For two samples, only one trophozoite of undetermined species was found during the initial microscopic examination but never reconfirmed by another test. One patient left the hospital with no additional malaria test and was considered as not having malaria. For another patient, the ICT result was first reported as positive and then the microscopy reported as positive for *P. falciparum* (one trophozoite). The second independent microscopist did not confirm this microscopy result. As this patient had previous positive results and was known to be under treatment, the microscopy might have been influenced by these previous results.

With only one set of primers for all PCR simplified the preparation of mixtures and allowed quantitative comparison between different plasmodial species. This set showed the same sensitivity for single species infection when used in monoplex with the screening Plasprobe or in multiplex (two separate reactions) with the four species-specific probes. A detection threshold of 1 to 10 per 1,000 was measured for the minority species in experimental simulation of mixed infection with positive controls mixtures. This limitation does not apply to the nested PCR method, which avoids primer competition by using species-specific primers and can thus be considered the reference standard for mixed-infection diagnosis. Among the 37 screening PCR-positive returned travelers, nested PCR detected only one *P. falciparum* and *P. ovale* mixed infection, a low prevalence consistent with the 3 to 5% generally found in that population (23, 31). The multiplex real-time PCR failed to detect this mixed infection. This might have been because the ratio between *P. falciparum* and *P. ovale* was over 10,000 to 1. Thus, the lack of mixed infection but one cannot provide firm support for the reliability of multiplex real-time PCR to detect both species together in a clinical setting. More work needs to be done in this area.

Several systems of real-time PCR have been developed. They are user friendly, rapid, and free of contamination. Moreover, these PCRs overcome the conventional PCR by allowing quantification of the targeted copies in the specimen (28). Blood samples from patients receiving treatment were monitored for at least 3 days. The efficacy of antimalarial therapy could be followed by determining the reduction of the parasite load for all but one patient, the second or third specimen being significantly less positive than the initial sample. The quantitative values estimated by thin blood smears were consistently higher than those calculated by real-time PCR, possibly due to the multiplication factor inherent to calculation of the number of parasites present in 1 μl of blood (11, 34).

DNA-based methods were also developed to overcome difficulties in determining the correct species identification faced by laboratories that may not keep appropriate microscopic experts (33). Changing patterns of accepted morphological appearances of malaria species, possibly due to drug pressure or strain variation, cannot be resolved merely by reference to an atlas of parasitology (22). Moreover, the information obtained by microscopy might, in some cases, be biased, especially when parasite levels are very low or when the parasites have lost their shape after the beginning of treatment or after chemoprophylaxis. This was obvious in our study with the "misidentified" mixed infections or even misidentified single-infection samples. Finally, when considering all samples tested, 34 single infections were diagnosed by both microscopy and multiplex real-time PCR. Sequencing of the four (12%) discordant results confirmed the PCR species identification of *P. vivax* or *P. ovale*. These species were frequently misdiagnosed by microscopy, indicating that the molecular method can make a decisive contribution in identification of such less common species.

Laboratory methods that require more than 1 h to provide a clear diagnosis of malaria are not considered rapid tests. Therefore, PCR cannot strictly be considered a rapid technique for the initial diagnosis of malaria at present, and currently such procedures have been established in our institution as a second-line diagnosis for patients with high clinical suspicion of malaria but a negative microscopy or when difficulty in species identification occurs. The decreasing delay in diagnosis by real-time PCR techniques as well as the need for more accurate and nonsubjective results will soon make their use more realistic for the detection of malaria parasites in routine laboratory practice. Even for monitoring treatment, although microscopy is suitable when the species is already known, the advent of automated PCR platforms as well as the disappearance of experienced microscopists will make molecular diagnossis more appealing at a reasonable cost, even or especially during nights and weekends.

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