JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 2004, p. 1214–1219 Vol. 42, No. 3
0095-1137/04/$08.00+0 DOI: 10.1128/JCM.42.3.1214–1219.2004

Development of a Real-Time PCR Assay for Detection of *Plasmodium falciparum*, *Plasmodium vivax*, and *Plasmodium ovale* for Routine Clinical Diagnosis

F. Perandin,[1] N. Manca,[1]* A. Calderaro,[2] G. Piccolo,[2] L. Galati,[2] L. Ricci,[3] M. C. Medici,[2] M. C. Arcangeletti,[2] G. Snounou,[4] G. Dettori,[2] and C. Chezzi[2]

Department of Laboratory Diagnosis, Section of Microbiology, University of Brescia, Spedali Civili, 25123 Brescia,[1] Section of Microbiology, Department of Pathology and Laboratory Medicine, University of Parma, 43100 Parma,[2] and Arcispedale S. Maria Nuova, Centrale Operativa, 42100 Reggio Emilia,[3] Italy, and Unité de Parasitologie Biomédicale and CNRS URA 2581, Institut Pasteur, 75724 Paris Cedex 15, France[4]

Received 2 July 2003/Returned for modification 7 September 2003/Accepted 24 November 2003

A TaqMan-based real-time PCR qualitative assay for the detection of three species of malaria parasites—*Plasmodium falciparum*, *P. ovale*, and *P. vivax*—was devised and evaluated using 122 whole-blood samples from patients who had traveled to areas where malaria is endemic and who presented with malaria-like symptoms and fever. The assay was compared to conventional microscopy and to an established nested-PCR assay. The specificity of the new assay was confirmed by sequencing the PCR products from all the positive samples and by the lack of cross-reactivity with *Toxoplasma gondii* and *Leishmania infantum* DNA. Real-time PCR assay showed a detection limit (analytical sensitivity) of 0.7, 4, and 1.5 parasites/μl for *P. falciparum*, *P. vivax*, and *P. ovale*, respectively. Real-time PCR, like nested PCR, brought to light errors in the species identification by microscopic examination and revealed the presence of mixed infections (*P. falciparum* plus *P. ovale*). Real-time PCR can yield results within 2 h, does not require post-PCR processing, reduces sample handling, and minimizes the risks of contamination. The assay can therefore be easily implemented in routine diagnostic malaria tests. Future studies are warranted to investigate the clinical value of this technique.

Mortality from malaria is higher today than it was 30 years ago (18). Malaria is a reemerging disease that is spreading to areas where it had been eradicated, such as Eastern Europe and Central Asia.

The recent increase in population movement to and from areas of endemicity through tourism, as well as migration due to wars and socioeconomic factors, has resulted in higher numbers of imported malaria cases, where a parallel increase of mortality, from 3.8 to 20% (6, 20), has been mostly ascribed to late or incorrect diagnosis.

Sensitive routine laboratory techniques for rapid and accurate malaria diagnosis are therefore desirable: first for diagnosis on admission, so as to initiate adequate treatment (14), thus avoiding unwarranted administration of antimalarial drugs, and second during the follow-up period in order to assess treatment efficacy.

For the last 100 years malaria has been diagnosed by microscopic examination of Giemsa-stained thick and thin blood films (2, 26). However, it is well documented that microscopy has limitations: it is time-consuming, and misdiagnosis of the infecting species is common if the microscopist lacks experience and/or when the parasitemia is low (6, 12, 22, 24).

Alternative techniques for laboratory diagnosis of malaria have been developed for use both in areas where malaria is endemic and in areas where it is not. Serological diagnostic methods and new rapid diagnostic tests (RDT) for antigen detection include Parasight-F (Becton Dickinson) (1), ICT Malaria Pf/Pv (ICT Diagnostic) (25), and OptiMAL (Flow Inc.) (15). The advantages offered by these methods, such as the fact that a result can be obtained within half an hour by nonskilled technicians, are tempered by three limitations (reviewed by Moody [13]). RDT methods do not offer improved sensitivity over microscopy; the sensitivity decreases as parasitemias fall below 100 parasites/μl (11). False positives are observed, particularly after treatment, as the parasite antigens detected can remain in the circulation following parasite clearance. Finally, current RDT are either specific to *Plasmodium falciparum* or they cannot distinguish between the parasite species present.

Several PCR assays for malaria diagnosis have also been developed, most often based on genus- or species-specific sequences of the parasites' 18S subunit rRNA gene, single-stranded rRNA (3, 7, 21, 22, 23). PCR-based assays have various advantages over microscopy and RDT: they are highly specific and are capable of high sensitivity (6, 20, 23, 27), and as few as five parasites per microliter of blood can be detected (13). They are thus ideal for revealing the presence of mixed infections overlooked by conventional methods (20, 22, 24).

However, current PCR-based assays often require multiple PCR assays to be performed on each sample and an analysis of the different PCR products and, moreover, do not provide quantification of the parasitemia.

Real-time PCR—a new methodology that employs fluorescent labels to enable the continuous monitoring of amplicon (PCR product) formation throughout the reaction—has recently been adapted to detect all four human malaria parasites indiscriminately and screen large numbers of samples (8),

* Corresponding author. Mailing address: Department of Laboratory Diagnosis, Section of Microbiology, University of Brescia, Spedali Civili, Piazzale Spedali Civili 1, 25123 Brescia, Italy. Phone: 39-030.3995652. Fax: 39-030.395258. E-mail: manca@med.unibs.it.

TABLE 1. Primers for nested PCR of 18S rRNA gene in malaria parasites

Species	Primer	Sequence (5′–3′)	Size of PCR product
Plasmodium sp.	rPLU5	CTTGTTGTTGCCTTAAACTTC	1.2 kb
	rPLU6	TTAAAATTGTTGCAGTTAAAACG	
P. falciparum	rFAL1	TTAAACTGGTTTGGGAAAACCAAATATATT	205 bp
	rFAL2	ACACAATGAACTCAATCATGACTACCCGTC	
P. vivax	rVIV1	CGCTTCTAGCTTAATCCACATAACTGATAC	120 bp
	rVIV2	ACTTCCAAGCCGAAGCAAAGAAAGTCCTTA	
P. ovale	rOVA1	ATCTCTTTTGCTATTTTTTAGTATTGGAGA	800 bp
	rOVA2	GGAAAAGGACACATTAATTGTATCCTAATG	
P. malariae	rMAL1	ATAACATAGTTGTACGTTAAGAATAACCGC	144 bp
	rMAL2	AAAATTCCCATGCATAAAAAATTATACAAA	

while another real-time PCR-based method allows quantitative detection of *P. falciparum* DNA in blood samples (4).

In this study a TaqMan-based real-time PCR qualitative assay for the rapid detection and identification in clinical specimens of three malaria parasites (*P. falciparum*, *P. vivax*, and *P. ovale*) is described and evaluated using whole-blood samples obtained from patients admitted with suspected malaria. Results from the real-time PCR assay were compared to conventional microscopy methods and to an established nested PCR assay (23, 24).

MATERIALS AND METHODS

Blood samples. For this retrospective study, we tested 122 whole-blood samples collected at the Section of Microbiology of Parma Hospital and at the Arcispedale of Reggio Emilia from patients returning from areas where malaria is endemic and who presented with fever associated with symptoms that the admitting physicians on duty considered indicative of a malaria infection (e.g., headache, abdominal pain, and diarrhea). The admission and clinical management of the patients were undertaken independently of this study. Five milliliters of blood was drawn into sterile tubes with EDTA, and thin blood films were prepared at the time of sample collection. Aliquots (500 μl) of whole blood were stored at 4°C or −20°C and used subsequently to purify templates for the PCR assays.

Microscopy. The blood smears were stained with 1% Giemsa stain in phosphate-buffered saline (pH 7.0) (26) and examined under the microscope at a magnification of ×1,000 for the presence of malaria parasites. Blood films were defined as negative if no parasites were observed in 300 oil immersion fields (magnification, ×1,000) on thin blood film by an experienced microscopist. Parasite concentration was calculated as described previously for areas of nonendemicity (13) by determining the number of parasitized red blood cells (not individual parasites) seen in 10,000 red blood cells and expressing the number of parasitized cells as a percentage (13). The approximate level of parasitemia (number of parasites per microliter) was calculated; it was assumed that 1 μl of blood contains 5×10^6 erythrocytes when the patient's baseline erythrocyte count was not available (13).

Preparation of DNA template. DNA was extracted from 200 μl of the blood samples using the High Pure PCR template preparation kit (Roche, Indianapolis, Ind.) according to the manufacturer's instructions.

Nested PCR. Purified DNA templates were used for amplification in a DNA thermal cycler (GeneAmp 2400 PE) using a genus-specific primer set (Table 1) as previously described (23, 24). In order to assess which of the parasite species was present in each sample, four separate amplification reactions were performed for the detection of each species (Table 1) (23, 24). All the oligonucleotides were synthesized by MWG Biotech S.r.l. (Ebersberg, Germany).

The PCR reagent mixture contained the following: a 250 nM concentration of each oligonucleotide primer, 125 μM deoxynucleoside triphosphate (Boehringer-Roche), 10× PCR buffer (500 mM KCl, 100 mM Tris-HCl [pH 8.3], 20 mM $MgCl_2$), and *Taq* DNA polymerase (2 U/100 μl; Boehringer-Roche). For each PCR tube, 95 μl of PCR reagent mixture and 5 μl of DNA were used. The reactions were subjected to 25 cycles (nest-1) under the following conditions: initial denaturation at 95°C for 5 min, annealing at 58°C for 2 min, extension at 72°C for 2 min, denaturation at 94°C for 1 min, final annealing at 58°C for 2 min, and final extension at 72°C for 5 min. Nest-2 was performed as nest-1, except that 30 cycles were performed.

Each experiment included four positive controls (consisting of *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae* genomic DNA, respectively, from positive blood samples) and one negative control (consisting of sterile double-distilled water).

Real-time PCR. Primers (forward and reverse) and TaqMan fluorescence-labeled probes for real-time PCR were designed using Primer Express software (Applied Biosystems, Foster City, Calif.) to specifically amplify the *P. falciparum*, *P. vivax*, and *P. ovale* 18S rRNA gene, in accordance with the sequences quoted by Snounou et al. (23). The primers and/or probes of real-time PCR partly or entirely overlapped with the species-specific nested-PCR oligonucleotides. The forward and reverse primers (FAL-F–FAL-R, VIV-F–VIV-R, and OVA-F–OVA-R) and probes (FAL probe, VIV probe, and OVA probe) employed are shown in Table 2.

Purified DNA templates were amplified in an Applied Biosystems 7700 analytical PCR system (SDS version 1.7) with a species-specific primer set and with the corresponding probe, synthesized by Applied Biosystems. Briefly, a 50-μl PCR mixture was performed using 5 μl of template, 25 μl of TaqMan 2× (Applied Biosystems) universal PCR master mix, a 300 nM concentration of each parasite species-specific primer set, and a 200 nM concentration of each corresponding probe. Amplification and detection were performed under the follow-

TABLE 2. Primers and probes selected for TaqMan PCR of 18S rRNA gene of malaria parasites

Species	Primer or probe	Sequence	Nucleotide positions[a]
P. falciparum	FAL-F	5′-CTTTTGAGAGGTTTTGTTACTTTGAGTAA	793–821
	FAL-R	5′-TATTCCATGCTGTAGTATTCAAACACAA	864–891
	FAL probe (FAM)	5′-TGTTCATAACAGACGGGTAGTCATGATTGAGTTCA	829–863
P. vivax	VIV-F	5′-ACGCTTCTAGCTTAATCCACATAACT	665–690
	VIV-R	5′-ATTTACTCAAAGTAACAAGGACTTCCAAGC	777–806
	VIV probe (TET)	5′-TTCGTATCGACTTTGTGCGCATTTTGC	695–722
P. ovale	OVA-F	5′-TTTTGAAGAATACATTAGGATACAATTAATG	1515–1545
	OVA-R	5′-CATCGTTCCTCTAAGAAGCTTTACAAT	1617–1643
	OVA probe (VIC)	5′-CCTTTTCCCTATTCTACTTAATTCGCAATTCATG	1549–1582

[a] The nucleotide positions are those reported in GenBank.

ing conditions: 2 min at 50°C to achieve optimal AmpErase uracil-*N*-glycosylase activity, 10 min at 95°C to activate the AmpliTaq Gold DNA polymerase, and 45 cycles of 15 s at 95°C and 1 min at 60°C. Each experiment included one reaction mixture without DNA as a negative control, and each specimen was run in duplicate.

During amplification, the ABI Prism sequence detector monitored real-time PCR amplification by quantitatively analyzing fluorescence emissions. The reporter dye signal was measured against the internal reference dye signal to normalize for non-PCR-related fluorescence well-to-well fluctuations. The threshold cycle represented the refraction cycle number at which a positive amplification reaction was measured and was set at 10 times the standard deviation of the mean baseline emission calculated for PCR cycles 3 to 15.

(i) Analytical sensitivity. To establish the minimum number of parasites detectable by the *Plasmodium* TaqMan assay (detection limit), blood samples from four patients infected, respectively, with *P. falciparum* (two patients), *P. ovale* (one patient), and *P. vivax* (one patient) were collected, and parasitemia was calculated using patients' baseline erythrocyte counts. The infected blood samples were diluted with uninfected erythrocytes from healthy individuals with known baseline erythrocyte counts. Tenfold serial dilutions were made to obtain a final parasitemia of 0% (0 parasite/μl of blood) for each sample.

All DNA aliquots purified from the dilutions were treated in duplicate for real-time PCR assay (two for *P. falciparum*, one for *P. ovale*, and one for *P. vivax*) in parallel to nested PCR.

(ii) Analytical specificity. To estimate the analytical specificity of the *Plasmodium* TaqMan assay, we obtained DNA from in vitro culture samples from other protozoa, *Toxoplasma gondii* and *Leishmania infantum*.

(iii) Clinical sensitivity and specificity. The clinical sensitivity and specificity of the *Plasmodium* TaqMan assay for detecting and identifying malaria parasites were calculated on 122 whole-blood samples, using nested-PCR-based species identification as the "gold standard" because of its superior performance over microscopy (6, 12, 24, 27). Sensitivity was calculated as the (number of true positives)/(number of true positives + number of false negatives), and specificity was calculated as the (number of true negatives)/(number of true negatives + number of false positives).

Genomic sequence analysis. All PCR amplification products obtained by both nested and real-time PCR were sequenced with a Big Dye terminator sequencing kit (Applied Biosystems) on an ABI Prism 310 sequencer (Applied Biosystems) following the manufacturer's instructions. Alignment of the sequences obtained was performed using the following single-stranded rRNA sequences deposited in GenBank (*P. falciparum* C-type, accession number M19173; *P. vivax* A-type, accession number U03079; *P. malariae* A-type, accession number M54897; *P. ovale* A-type, accession number L48987).

RESULTS

Real-time PCR: analytical sensitivity and specificity. Typical displays (amplification plots) of the *Plasmodium* TaqMan assay provided by ABI Prism 7700 for *P. falciparum*, *P. ovale*, and *P. vivax* are shown in Fig. 1A, C, and E, respectively. Positive signals (cycle threshold [C_T] value, value indicative of the target gene amount at which fluorescence exceeds a preset threshold) were found for all dilutions, with a detection limit of 2.9 (not shown) and 0.7 parasite/μl for *P. falciparum*, 4 parasites/μl for *P. ovale*, and 1.5 parasites/μl for *P. vivax*. The mean C_T values for *P. falciparum* (four replicates) ranged from 35.5 (0.016% parasitemia) to 37.6 (0.00001% parasitemia), those for *P. ovale* (four replicates) ranged from 29.2 (0.14% parasitemia) to 39.1 (0.0001% parasitemia), and those for *P. vivax* (five replicates) ranged from 24.3 (0.3% parasitemia) to 36.8 (0.00003% parasitemia). The C_T values showed reproducible linearity over a 10,000-fold range. A significant correlation coefficient was found for the mean C_T values and value of parasitemia (*P. falciparum*, $R^2 = -0.952$; *P. ovale*, $R^2 = -0.952$; *P. vivax*, $R^2 = -0.969$) (Fig. 1B, D, and F).

No signal was detected by real-time PCR when DNA obtained from non-*Plasmodium* protozoa species (*T. gondii* and *L. infantum*) or from humans was used (data not shown).

Real-time PCR results compared to microscopy, nested PCR, and genomic sequence analysis: clinical sensitivity and specificity. The results of microscopy, real-time and nested PCR, and genomic sequence analysis are given in Table 3. Among the 122 subjects (50 Italians and 72 non-Italians) with a suspected clinical diagnosis of imported malaria, 61 (50%) were positive by microscopy. Of these, 43 (70%) were diagnosed with *P. falciparum* infection (parasitemia range, 20 to 10^6 parasites/μl), 11 were diagnosed with *P. vivax* infection (parasitemia range, 300 to 15,000 parasites/μl), 2 were diagnosed with *P. malariae* infection (parasitemia, 3,000 and 5,000 parasites/μl), 3 were diagnosed with *P. ovale* infection (parasitemia range, 360 to 25,000 parasites/μl), 1 was diagnosed with *P. falciparum* or *P. vivax* infection (parasitemia, 2,000 parasites/μl), and finally 1 was diagnosed with *Plasmodium* sp. infection (parasitemia, <500 parasites/μl). The remaining 61 samples were negative.

Real-time PCR and nested PCR yielded identical results. There was broad agreement with microscopy, except as follows: one *P. falciparum* infection diagnosed by microscopy was shown to be a *P. ovale* infection in the other tests; one *P. falciparum* infection diagnosed by microscopy was shown to be *P. falciparum* plus *P. ovale* infection in the other tests; one *P. falciparum* or *P. vivax* infection diagnosed by microscopy was shown to be a *P. ovale* infection in the other tests; one *Plasmodium* sp. infection diagnosed by microscopy was shown to be *P. falciparum* plus *P. ovale* plus *P. malariae* infection by nested PCR and *P. falciparum* plus *P. ovale* infection by real-time PCR; four *P. vivax* infections diagnosed by microscopy were shown to be *P. ovale* infections in the other tests; one *P. ovale* infection diagnosed by microscopy was shown to be a *P. vivax* infection in the other tests; and one negative sample was shown to harbor *P. falciparum*, by real-time PCR and nested PCR. The remaining 60 samples negative by microscopy were also negative in the other tests (Table 3). Two *P. malariae*-positive samples identified by microscopy were confirmed by nested PCR but tested negative by real-time PCR amplification where only the *P. falciparum*-, *P. vivax*-, and *P. ovale*-specific primers/probes were used; these samples were therefore excluded from further analysis. Genomic sequence analysis of both nested-PCR and real-time amplification products confirmed the presence of the same *Plasmodium* species identified by the two PCR-based methods (Table 3).

Using nested-PCR results as the gold standard, real-time PCR detected malaria parasites in all 60 positive samples (100% sensitivity), with no detection of *Plasmodium* in the remaining 60 negative blood samples (100% specificity).

DISCUSSION

The use of microscopy to detect malaria parasites in stained blood smears remains to date the most practical and reliable means for detecting parasites in blood samples (12, 26). This technique is cheap and easy to perform, allows quantification, and when performed by a skilled microscopist distinguishes among the different stages of the four *Plasmodium* species causing malaria in humans. However, it is well documented that microscopy has limitations: for example, its sensitivity decreases with the malaria parasite density in blood (5, 12). When parasitemia is low, during mixed infections, after drug

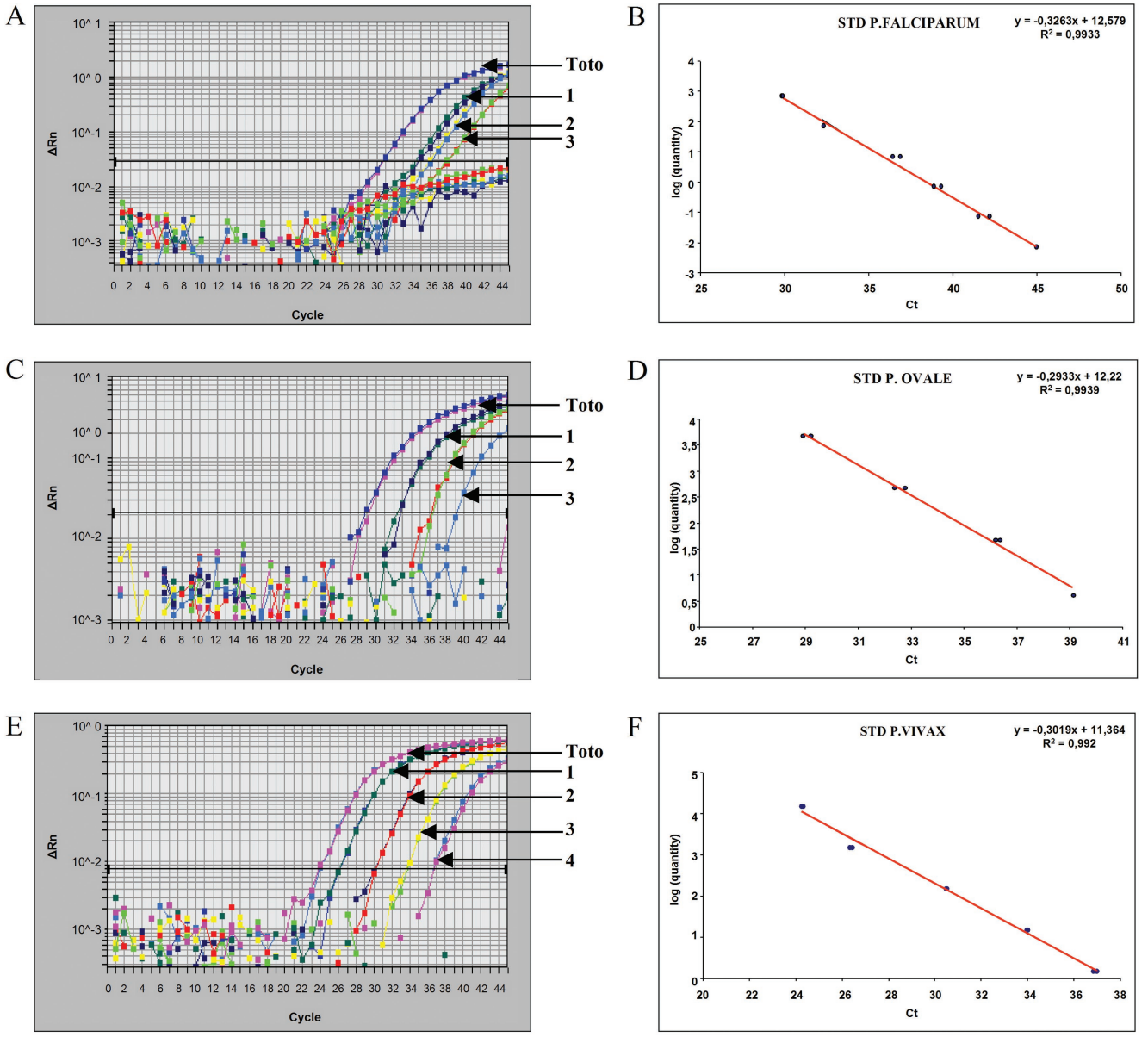

FIG. 1. Sensitivity of *P. falciparum*, *P. ovale*, and *P. vivax* real-time assay. (A) Amplification (as duplicate) of *P. falciparum* sample dilutions containing 704 (Toto), 70 (1), 7 (2), and 0.7 (3) parasites/μl. (B) Plot of mean C_T values from four replicates tested against the *P. falciparum* DNA inputs. The plot of C_T values and DNA input fits a linear function ($R^2 = 0.952$). (C) Amplification (as duplicate) of *P. ovale* sample dilutions containing 4620 (Toto), 462 (1), 46 (2), and 4 (3) parasites/μl. (D) Plot of mean C_T values from four replicates tested against the *P. ovale* DNA inputs. The plot of the C_T values and DNA input fits a linear function ($R^2 = 0.952$). (E) Amplification (as duplicate) of *P. vivax* sample dilutions containing 15,000 (Toto), 1,500 (1), 150 (2), 15 (3), and 1.5 (4) parasites/μl. (F) Plot of mean C_T values from five replicates tested against *P. vivax* DNA inputs. The plot of C_T values and DNA input fits a linear function ($R^2 = 0.969$).

treatment, or during the chronic phase of the infection, very long observation times and considerable expertise are required for correct diagnosis, and these are not always available at local medical centers in countries where the disease is not endemic (6).

In Italy, although for 80% of the cases less than 1 week elapses from the onset of malaria symptoms to microscopic diagnosis, the average time is 8.5 days, with a range of 1 to 28 days (19). As shown by various studies, PCR-based assays are more specific and sensitive than microscopy for detecting and identifying malaria parasites (10, 16, 17, 22, 23, 24, 27). It is vital to distinguish between the different *Plasmodium* species in order to administer the adequate antimalarial treatment. For instance, widespread resistance of *P. falciparum* to first-line and second-line antimalarial drugs such as chloroquine and pyrimethamine-sulfadoxine (Fansidar) in some countries necessitates the use of quinine or artemisin derivatives for rapid resolution of the parasitemia in vulnerable nonimmune patients (14). Several PCR assays based on the 18S rRNA genes have thus been developed for the diagnosis of malaria (7, 21,

TABLE 3. Detection of malaria parasites by various methods

Malaria-causing species[a]	Organism(s) detected by each method (no. of samples)			
	Microscopy	Real-time PCR	Nested PCR	Genomic sequence analysis[b]
P. falciparum	*P. falciparum* (43)	*P. falciparum* (41) *P. ovale* (1) *P. falciparum* + *P. ovale* (1)	*P. falciparum* (41) *P. ovale* (1) *P. falciparum* + *P. ovale* (1)	*P. falciparum* (41) *P. ovale* (1) *P. falciparum* + *P. ovale* (1)
Non-*P. falciparum*	*P. falciparum* or *P. vivax* (1) *Plasmodium* spp. (1) *P. vivax* (11) *P. malariae* (2) *P. ovale* (3)	*P. ovale* (1) *P. falciparum* + *P. ovale* (1) *P. vivax* (7) *P. ovale* (4) Negative (2) *P. ovale* (2) *P. vivax* (1)	*P. ovale* (1) *P. falciparum* + *P. ovale* + *P. malariae* (1) *P. vivax* (7) *P. ovale* (4) *P. malariae* (2) *P. ovale* (2) *P. vivax* (1)	*P. ovale* (1) *P. falciparum* + *P. ovale*[c] (1) *P. vivax* (7) *P. ovale* (4) *P. malariae*[d] (2) *P. ovale* (2) *P. vivax* (1)
Negative	Negative (61)	*P. falciparum* (1) Negative (60)	*P. falciparum* (1) Negative (60)	*P. falciparum* (1) Negative (60)
Total	122	122	122	122

[a] Malaria species are classified according to the diagnosis obtained from the blood slide examination.
[b] Sequences were obtained for all the PCR products resulting from both amplification methods, except for the two *P. malariae*-positive samples. The sequences were the same in all cases and confirmed the species diagnosis.
[c] Genomic sequence analysis was not carried out.
[d] Genomic sequence analysis was only performed for the nested PCR amplification product.

22, 23). These assays are, however, not adequate for use in a clinical setting, since the turnaround time from admission to diagnosis is incompatible with the urgency to initiate treatment. Recently developed RDT provide the necessary speed but perform poorly when parasite loads are low and are inadequate in cases where identification of the parasite species is desirable.

In this study we evaluated a real-time PCR qualitative assay for the detection of *P. falciparum*, *P. vivax*, and *P. ovale* in clinical specimens. Parasites were detected in 62 out of 122 blood samples collected on admission from patients with suspected malaria infection and who had traveled to areas where malaria is endemic.

All samples were tested by real-time PCR using the three species-specific primer sets and probes. The specificity of the assay and the absence of contamination were confirmed. The two *P. malariae* samples positive by microscopy and by nested PCR were negative by real-time PCR that detects only *P. falciparum*, *P. vivax*, and *P. ovale* species. In addition, genomic sequence analysis of the PCR products amplified by both methods confirmed specificity. We are now searching for a specific DNA target for the detection of *P. malariae*. As reported by Liu et al. (9), the rRNA gene of *P. malariae* has variant sequences; thus, sequencing of the gene from different isolates would be required to find universally conserved regions. In addition, real-time PCR requires the use of oligonucleotide sets with a low amount of CG, and this has also hampered the design of *P. malariae*-specific primers. Though the distribution of *P. malariae* is global and the infection can last for many years, infection due to *P. malariae* is relatively rarely observed in imported malaria.

The real-time PCR described here equaled the very high sensitivity of the established nested-PCR assays (13, 22) and showed a detection limit (analytical sensitivity) of 0.7, 4, and 1.5 parasites/μl for *P. falciparum*, *P. ovale*, and *P. vivax*, respectively. It is noteworthy that in our study where an experienced microscopist scored the stained blood smears, missed infections as well as misdiagnoses were common (9 out of 61, or 14.7%). Nonetheless, the clinical sensitivity of microscopy was high at 98% (61 of 62), even though the accuracy of identification was only 86% (53 of 61; the one *Plasmodium* sp. was not included). Malaria parasites were detected in one sample found negative by microscopy, two mixed infections (with *P. falciparum* plus *P. ovale*) were brought to light by both PCR assays, and finally in seven cases the correct species of parasite was misdiagnosed by microscopy. In the third of these groups, *P. ovale* was the species most frequently misdiagnosed (mistaken for *P. falciparum* once and for *P. vivax* four times). Thus, compared to the gold standard nested-PCR assay (23, 24), real-time PCR is highly sensitive (100%) and specific (100%) for the simultaneous detection of three *Plasmodium* species.

The main advantages of real-time PCR over nested-PCR assays are that (i) it is far less labor-intensive (only one PCR step, compared to at least two in nested PCR); (ii) it is performed in a closed system where post-PCR handling is not required (i.e., transfer of amplified template from the primary to the secondary amplification reaction and agarose gel electrophoresis for the detection of PCR products; this constitutes a major advantage since the risks of contamination are minimal); and finally (iii) the result of the assay can be obtained in only 2 h, versus a minimum of 8 h with nested PCR. The real-time PCR assay offers a practical and clinically acceptable alternative to rapid and accurate diagnosis of malaria infections in patients presenting with symptoms indicative of this disease.

To the best of our knowledge, two real-time PCR-based methods have already been described, one for quantitative detection of *P. falciparum* and the other for the qualitative detection of *Plasmodium* spp. in blood samples (4, 8). The real-time PCR developed in this study allows the simultaneous detection and quantification of *P. falciparum*, *P. vivax*, and *P. ovale*, the three parasite species responsible for most malaria admissions in areas of endemicity as well as areas of nonen-

demicity. It will be especially useful in countries where malaria is not endemic, where there is often a lack of microscopists experienced in malaria diagnosis. The assay is suitable for routine screening of *Plasmodium* sp. infections in clinical laboratories as an adjunct to other diagnostic methods such as blood film examination. The assay would be useful for in vivo drug efficacy studies, where it is important to detect resistant parasites before they reach high levels and result in a clinical recrudescent episode.

ACKNOWLEDGMENTS

This study was supported by the Ministry of University and Scientific Research FIL (60%) (Brescia, Italy) and by the Ministry of University and Scientific Research FIL (60%) (Parma, Italy).

REFERENCES

1. **Beadle, C., G. W. Long, W. R. Weiss, P. D. McElroy, S. M. Maret, A. J. Oloo, and S. L. Hoffman.** 1994. Diagnosis of malaria by detection of *P. falciparum* HRP-2 antigen with a rapid dipstick antigen-capture assay. Lancet **343:**564–568.
2. **Bruce-Chwatt, L. J.** 1987. From Laveran's discovery to DNA probes: new trends in diagnosis of malaria. Lancet **ii:**1509–1511.
3. **Gunderson, J. H., M. L. Sogin, G. Wollett, M. Hollingdale, V. F. De La Cruz, A. P. Waters, and T. F. McCutchan.** 1987. Structurally distinct, stage-specific ribosomes occur in *Plasmodium.* Science **238:**933–937.
4. **Hermsen, C. C., D. S. C. Telgt, E. H. P. Linders, L. A. T. F. van de Locht, W. M. C. Eling, E. J. B. M. Mensink, and R. W. Sauerwein.** 2001. Detection of *Plasmodium falciparum* malaria parasites *in vivo* by real-time quantitative PCR. Mol. Biochem. Parasitol. **118**: 247–251.
5. **Iqbal, J., A. Sher, P. R. Hira, and R. Al-Owaish.** 1999. Comparison of the OptiMAL test with PCR for diagnosis of malaria in immigrants. J. Clin. Microbiol. **37:**3644–3646.
6. **Kain, K. C., M. A. Harrington, S. Tennyson, and J. S. Keystone.** 1998. Imported malaria: prospective analysis of problems in diagnosis and management. Clin. Infect. Dis. **27:**142–149.
7. **Kawamoto, F., H. Miyake, O. Kaneko, M. Kimura, N. T. Dung, N. T. Dung, Q. Liu, M. Zhou, L. Duc Dao, S. Kawai, S. Isomura, and Y. Wataya.** 1996. Sequence variation in the 18S rRNA gene, a target for PCR-based malaria diagnosis, in *Plasmodium ovale* from Southern Vietnam. J. Clin. Microbiol. **34:**2287–2289.
8. **Lee, M. A., C. H. Tan, L. T. Aw, C. S. Tang, M. Singh, S. H. Lee, H. P. Chia, and E. P. Yap.** 2002. Real-time fluorescence-based PCR for detection of malaria parasites. J. Clin. Microbiol. **40:**4343–4345.
9. **Liu, Q., S. Zhu, S. Mizuno, M. Kimura, P. Liu, S. Isomura, X. Wang, and F. Kawamoto.** 1998. Sequence variation in small-subunit rRNA gene of *Plasmodium malariae* and prevalence of isolates with the variant sequence in Sichuan, China. J. Clin. Microbiol. **36:**3378–3381.
10. **Manca, N., I. Viani, F. Perandin, G. Piccolo, A. Calderaro, L. Galati, L. Ricci, G. Dettori, A. Turano, and C. Chezzi.** 2000. Search for malaria parasites by PCR and Southern blot in patients with imported malaria in Italy. New Microbiol. **23:**339–346.
11. **Mills, C. D., D. C. H. Burgess, H. J. Taylor, and K. C. Kain.** 1999. Evaluation of a rapid and inexpensive dipstick assay for the diagnosis of *Plasmodium falciparum* malaria. Bull. W. H. O. **77:**553–559.
12. **Milne, L. M., M. S. Kyi, P. L. Chiodini, and D. C. Warhurst.** 1994. Accuracy of routine laboratory diagnosis of malaria in the United Kingdom. J. Clin. Pathol. **47:**740–742.
13. **Moody, A.** 2002. Rapid diagnostic tests for malaria parasites. Clin. Microbiol. Rev. **15:**66–78.
14. **Morgan, U. M., and R. L. A. Thompson.** 1998. Molecular detection of parasitic protozoa. Parasitology **117:**S73–S85.
15. **Palmer, C. J., J. F. Lindo, W. I. Klaskala, J. A. Quesada, R. Kaminsky, M. K. Baum, and L. A. Ager.** 1998. Evaluation of the OptiMAL test for rapid diagnosis of *Plasmodium vivax* and *Plasmodium falciparum* malaria. J. Clin. Microbiol. **36:**203–206.
16. **Perandin, F., N. Manca, L. Galati, G. Piccolo, A. Calderaro, I. Viani, L. Ricci, G. Dettori, C. Chezzi, and A. Turano.** 2001. Usefulness of genus-specific PCR and Southern blot species-specific hybridization for the detection of imported malaria cases in Italy. New Microbiol. **24:**69–76.
17. **Perandin, F., N. Manca, G. Piccolo, A. Calderaro, L. Galati, L. Ricci, M. C. Medici, C. Arcangeletti, G. Dettori, C. Chezzi.** 2003. Identification of *Plasmodium falciparum*, *P. vivax*, *P. ovale* and *P. malariae* and detection of mixed infection in patients with imported malaria in Italy. New Microbiol. **26**: 91–100.
18. **Phillips, R. S.** 2001. Current status of malaria and potential for control. Clin. Microbiol. Rev. **14:**208–226.
19. **Romi, R., D. Boccolini, and G. Majori.** 1999. Malaria surveillance in Italy: 1997 analysis and 1998 provisional data. Eurosurveillance **4:**85–87.
20. **Rubio, J. M., A. Benito, P. J. Berzosa, J. Roche, M. Puente, M. Subirats, R. Lopez-Velez, L. M. Garcia, and J. Alvar.** 1999. Usefulness of seminested multiplex PCR in surveillance of imported malaria in Spain. J. Clin. Microbiol. **37:**3260–3264.
21. **Seesod, N., P. Nopparar, A. Hedrum, A. Holder, S. Thaithong, M. Uhlen, and J. Lundeburg.** 1997. An integrated system using immuno-magnetic separation, polymerase chain reaction, and colorimetric detection for diagnosis of *Plasmodium falciparum.* Am. J. Trop. Med. Hyg. **56:**322–328.
22. **Singh, B., A. Bobogare, J. Cox-Singh, G. Snounou, M. S. Abdullah, and H. A. Rahman.** 1999. A genus- and species-specific nested polymerase chain reaction malaria detection assay for epidemiologic studies. Am. J. Trop. Med. Hyg. **60:**687–692.
23. **Snounou, G., S. Viryakosol, X. P. Zhu, W. Jarra, L. Pinheiro, V. E. Rosario, and S. Thaithong.** 1993. High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction. Mol. Biochem. Parasitol. **61:**315–320.
24. **Snounou. G., S. Viriyakosol, W. Jarra, S. Thaithong, and K. N. Brown.** 1993. Identification of the four human malaria parasite species in field samples by the polymerase chain reaction and detection of high prevalence of mixed infections. Mol. Biochem. Parasitol. **58:**283–292.
25. **Tjitra, E., S. Suprianto, M. Dyer, B. J. Curie, and N. M. Anstey.** 1999. Field evaluation of the ICT Malaria Pf/Pv immunochromatographic test for detection of *Plasmodium falciparum* and *Plasmodium vivax* in patients with presumptive clinical diagnosis of malaria in eastern Indonesia. J. Clin. Microbiol. **37**: 2412–2417.
26. **Warhurst, D. C., and J. E. Williams.** 1996. Laboratory diagnosis of malaria: ACO broadsheet no. 148. J. Clin. Pathol. **49:**533–538.
27. **Zhong, K., and K. C. Kain.** 1999. Evaluation of a colorimetric PCR-based assay to diagnose *Plasmodium falciparum* malaria in travellers. J. Clin. Microbiol. **37:**339–341.