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PCR as a Confirmatory Technique for Laboratory Diagnosis of Malaria

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We compared a nested PCR assay and microscopic examination of Giemsa-stained blood films for detection and identification of *Plasmodium* spp. in blood specimens. PCR was more sensitive than microscopy and capable of identifying malaria parasites at the species level when microscopy was equivocal.

Malaria in humans is mainly caused by infection with four *Plasmodium* species (*Plasmodium falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*). Malaria affects 300 to 500 million people annually worldwide and accounts for over 1 million deaths, mainly in African children (2, 5, 6, 7, 9, 13). Over the past decade, refugee migration, immigration, and international travel have increased significantly worldwide, contributing to an increase of malaria cases in the United States in persons returning from areas where malaria is endemic (1, 2, 5, 7, 9–13). In 2003, 767 cases of malaria among U.S. civilians and 306 among foreign nationals were reported to the Centers for Disease Control and Prevention (CDC) (2).

Effective treatment of malaria requires precise laboratory diagnosis. *P. falciparum*, which can be fatal, must be identified promptly and differentiated from the other *Plasmodium* species that cause malaria. In addition, treatment of *P. vivax* and *P. ovale* infections with primaquine to eliminate persistent liver stage is based on results from laboratory examination. Microscopic detection and identification of *Plasmodium* spp. in Giemsa-stained thick and thin blood smears continues to be the gold standard for the laboratory diagnosis of malaria (2, 6, 8, 9–12, 15). PCR is an attractive addition to microscopy for confirmatory identification of *Plasmodium* spp. in clinical specimens. Numerous PCR assays have been developed for the laboratory diagnosis of malaria, including conventional and real-time PCR techniques, that allow the differentiation of all four species of *Plasmodium* (4, 6, 8, 9–12, 14, 15).

In the present study, we compared PCR to microscopy using a modification of the technique originally described by Snounou et al. with primers targeting the *Plasmodium* spp. 18S rRNA genes (14). A total of 174 sets of stained blood smears and EDTA blood samples received at the CDC for routine or confirmatory malaria diagnosis were included in the study. Of this total, 136 specimens were received as routine submissions from state health departments, hospitals, or laboratories overseas between 1998 and 2003. An additional 38 specimens were submitted to CDC from 2002 to 2004 to confirm *Plasmodium* spp. as a follow-up to consultation provided by the CDC telediagnostic parasitology service (the DPDx project at www.dpd.cdc.gov/dpdx) (3). The digital images in these 38 cases contained parasites, with inadequate morphological features, to permit the final identification of species. In all instances, thick and thin stained blood smears (stained with Wright's, Wright's-Giemsa, or Giemsa stains, depending on the practices of the submitting institution) were analyzed at time of arrival at CDC for *Plasmodium* spp. detection and identification. DNA was extracted from 200 μl of EDTA blood with the QIAamp blood kit (QIAGEN, Inc., Chatsworth, CA) according to the manufacturer's instructions and stored at 4°C until PCR could be completed. Nested PCR was performed with primers described by Snounou et al. (14) (Table 1) with changes in the cycling parameters (Table 2) by using a GeneAmp 9700 PCR thermal cycler (Applied Biosystems, Foster City, CA). Known positive and negative samples from previous malaria diagnosed or uninfected individuals were used as controls. Samples that generated discrepant results were recoded and tested twice by nested PCR on different days. Discrepant results were resolved in favor of PCR if consensus was achieved in two of three samples; otherwise, they were resolved in favor of micros-

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

Species	Primer	Sequence (5′–3′)	Size (bp) of PCR product
Plasmodium sp.	rPLU5	CCTGTTGTTGCCTTAAACTTC	1,100
	rPLU6	TTAAAATTGTTGCAGTTAAAACG	
P. falciparum	rFAL1	TTAAACTGGTTTGGGAAAACC AAATATATT	205
	rFAL2	ACACAATGAACTCAATCATGA CTACCCGTC	
P. vivax	rVIV1	CGCTTCTAGCTTAATCCACAT AACTGATAC	120
	rVIV2	ACTTCCAAGCCGAAGCAAAGA AAGTCCTTA	
P. ovale	rOVA1	ATCTCTTTTGCTATTTTTTAG TATTGGAGA	800
	rOVA2	GGAAAAGGACACATTAATTGT ATCCTAGTG	
P. malariae	rMAL1	ATAACATAGTTGTACGTTAAG AATAACCGC	144
	rMAL2	AAAATTCCCATGCATAAAAAA TTATACAAA	

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TABLE 2. Cycling conditions of first- and nested-step PCR reactions

Reaction[a]	Cycling conditions
First step (*P. falciparum*, *P. vivax*, and *P. malariae*)	Denaturation at 94°C for 1 min, annealing at 60°C for 2 min, and extension at 72°C for 2 min (30 cycles)
First step (*P. ovale*)	Denaturation at 94°C for 30 s, annealing at 45°C for 30 s, and extension at 72°C for 1 min 30 s (30 cycles)
Nested step (*P. falciparum*, *P. vivax*, and *P. malariae*)	Denaturation at 94°C for 1 min, annealing at 55°C for 2 min, and extension at 72°C for 2 min (30 cycles)
Nested step (*P. ovale*)	Denaturation at 94°C for 30 s, annealing at 45°C for 30 s, and extension at 72°C for 1 min 30 s (45 cycles)

[a] AmpliTaq Gold PCR Master Mix (Applied Biosystems, Foster City, CA) was used in the first and nested PCR steps. Reactions were performed in a final volume of 25 μl containing 2.5 U of AmpliTaq Gold (present in the AmpliTaq Gold PCR Master Mix), with 0.4 μM concentrations of each primer.

copy. Sensitivity was calculated as the number of positive results divided by the sum of positives and false negatives multiplied by 100.

Results from the microscopic examination of stained blood films are presented in Table 3. The *Plasmodium* species present could not be determined for 17 (9.7%) of the total samples analyzed ($n = 174$) and in 16 (42.1%) of the 38 specimens submitted as telediagnosis cases. One mixed infection of *P. falciparum* and *P. ovale* was identified by microscopic analysis. *Plasmodium* DNA was detected in 59.8% (104 of 174) of the EDTA blood samples examined by using nested PCR (Table 3) and in 55.7% of stained smears by microscopy (97 of 174). Identification at the species level was achieved by nested PCR for all 104 specimens compared to 82.5% of the 97 specimens determined to be positive by microscopy. Five mixed infections were identified by using nested PCR; only one of these was detected by using microscopy. All discrepant results for the presence or absence of parasites were resolved in favor of PCR; nested PCR was able to detect *Plasmodium* DNA in a total of seven specimens that were determined to be negative by microscopy. Therefore, the sensitivities of microscopy and nested PCR were 93.3 and 100%, respectively ($P = 0.01$, Fisher exact test). Mixed infections were detected in five cases in which only one was resolved by microscopy. Based on the reproducibility of PCR results, incorrect speciation of *P. vivax* and *P. ovale* using microscopy was found in three samples. *P. ovale* was identified by microscopy in two specimens that later were identified as *P. vivax* using PCR. Similarly, one specimen that was identified as *P. vivax* by microscopy was identified as *P. ovale* by PCR.

In our study, this nested PCR was more sensitive compared to microscopy, allowing the detection of *Plasmodium* in cases with low parasitemias, as well as mixed infections of malaria. In all instances, specimens that were PCR positive and microscopy negative were collected from symptomatic patients with a history of travel to malaria areas of endemicity. Nested PCR was also able to detect one mixed infection with *P. falciparum* and *P. ovale* that was missed by microscopy, as well as detect three from submitted specimens from telediagnosis cases. PCR also better distinguished between *P. vivax* and *P. ovale*. We found that 2.2% of samples were incorrectly identified as *P. vivax* or *P. ovale* by microscopy.

Inconsistency in microscopy-based laboratory diagnosis is increasing in the United States due to the lack of fiscal and personnel investments needed to maintain and improve the laboratory identification of *Plasmodium* spp. The nested PCR used here allowed reliable detection of all four species of *Plasmodium*. We conclude that this nested PCR is valuable as a confirmatory test and implementation should be considered by reference laboratories and worldwide with adequate laboratory infrastructure to perform molecular procedures. Molecular techniques are a costly procedure, including the cost of labor and access to reagents, compared to the examination of blood smears. This represents a true impairment for its implementation in reference laboratories located in poor regions of the world, where malaria is endemic.

TABLE 3. Comparison of microscopy and PCR results

Submission type and test	No. of positive identifications						No. of negatives	Total
	P. falciparum	*P. vivax*	*P. ovale*	*P. malariae*	*Plasmodium* spp.[a]	*P. falciparum* and *P. ovale*		
All submissions								
Microscopy	40	26	10	3	17	1	77	174
PCR	50	31	14	4	0	5	70	174
Routine submissions								
Microscopy	34	21	5	2	1	0	73	136
PCR	37	23	5	3	0	1	67	136
Telediagnosis submissions								
Microscopy	6	5	5	1	16	1	4	38
PCR	13	8	9	1	0	4	3	38

[a] That is, samples in which a species identification could not be determined by examination of stained blood smears.

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