Genetic Polymorphisms Influence *Plasmodium ovale* PCR Detection Accuracy[▽]

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Detection of *Plasmodium ovale* by use of a nested PCR assay with a novel *Plasmodium ovale* primer set was superior to detection of *Plasmodium ovale* by real-time PCR assays. Nested PCR was also better at detecting *P. malariae*. The detection of *P. ovale* in many patients first admitted >2 months following their return to Italy indicated that *P. ovale* relapses are common.

Two of the four *Plasmodium* parasites that infect humans, *Plasmodium malariae* and *P. ovale*, are relatively poorly investigated. *Plasmodium ovale*, the last of the malaria parasites of humans to be described (22), is mainly found in West Africa, is infrequently reported in Asia and Oceania, and is yet to be described in the Americas (3). Hypnozoites occur in *P. ovale* infections and lead to relapses, although these are considered infrequent. *P. malariae*, on the other hand, is globally distributed, although its prevalence is considered low. *P. malariae* infections do not reach high parasite levels but are associated with chronic nephropathy and can persist for decades, although dormant liver forms (hypnozoites) are not thought to occur. Sensitive molecular techniques that allow the sensitive detection and the accurate identification of *Plasmodium* parasites, on the basis of amplification of their small-subunit rRNA (ssrRNA) gene, revealed that the prevalences of *P. malariae* and *P. ovale* infections were higher than previously thought (9, 14, 18, 21, 25). Sequence variations were, however, detected in these genes in *P. malariae* and, in particular, in *P. ovale*, in which two types were found (4, 8, 28, 30). This led to the design of novel oligonucleotide primer combinations to increase the accuracy of *P. ovale* detection (5, 10, 19).

We wished to conduct a comparative study of some of the molecular diagnostic approaches for the detection of these two species and to test a new set of primers designed to recognize all types of *P. ovale*. To this end, we used a set of 200 blood samples, collected on admission from patients presenting to Parma University Hospital (189 patients) and the Arcispedale di Reggio Emilia (11 patients) with symptoms consistent with

malaria between January 1999 and August 2005. The samples were processed as described previously (2). PCR analysis of a first set of 122 samples revealed that 62 were indeed positive for *Plasmodium*, and in 20 of these samples, non-*P. falciparum* species were found (13). The presence of *P. ovale* in a relatively high proportion of the samples (10/62) reflected the fact that many of the patients became infected following travel to a destination in West Africa. These observations made these samples suitable for the purposes of our study. We had noted in the course of the PCR analyses conducted with the samples described above and subsequent samples that for some patients, genus-specific primers revealed the presence of *Plasmodium*, but species identification could not be achieved with a set of species-specific primers (NP-1993 primers) that had been in use for the last 12 years (21). Some of these samples proved positive for *P. ovale* with a different set of primers (NP-2002 primers) designed to overcome the presence of polymorphisms in the ssrRNA gene of *P. ovale* that would affect detection efficiency (19). This provided an indication that some of the *P. ovale* strains in the samples collected were of the classic type, while others were of the variant type. In order to resolve this matter, primers rOVA3 and rOVA4, published previously and found to be specific for *P. ovale* (10), were used in the secondary reaction. The *P. ovale* samples described above were indeed found to be positive (amplified band of 434 bp); however, control DNAs purified from five patients infected with *P. vivax* alone were also positive (those from patients infected with *P. malariae* and *P. falciparum* proved negative). Sequence analysis confirmed that this primer set was not *P. ovale* specific under the conditions used (data not shown).

Comparison of the available *P. ovale* ssrRNA sequences (as found in the GenBank database in June 2006) confirmed that they fell into two distinct types (i.e., a classic type and a variant type), and these were aligned with primer sequences used in the 14 different *P. ovale*-specific PCR protocols (1, 5–7, 10–13, 15–17, 21, 23, 24, 27, 29) that we could identify from the literature. It was clear that some were suitable for the detec-

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TABLE 1. Oligonucleotides used for specific PCR-based detection of *P. ovale*

Name ^a	Sequence ^b	No. of sequences efficiently recognized ^c		Other ^d	Use ^e	Reference(s)
		Cl	Vr			
rOVA1	ATTTTGAAGAATACACTAGG	4	5	None	Hyb	26
rOVA1v	ATCTCTTTTGTCTATTTTGTATGGAGA	4	0	None	PCR	21
rOVA2	ATCTCCTTTACTTTTGTACTGGAGA	0	3	None	PCR	
rOVA2v	GGAAAAGGACACATTAATTGTATCCTAGTG	4	0	None	PCR	21
	GGAAAAGGACACTATAATGTATCCTAATA	0	5	Pv	PCR	
	GAAAATTCCTTTTCGGGGAATT	4	0	None	Hyb	6
O2	AAATTTCTTAGATTGCTTCCTTCAGT	4	5	None	Hyb	5
933	ATTTTGAAGAATATATTAGGAT	4	5	None	Hyb	7
DIG-11	TGCAAAATGTGTTCTTATT	4	5	None	Hyb	11
DIG-12	GACAATACACGTATCTG	4	5	None	Hyb	
DIG-13	AAAATCTCTTTTGTCTATT	4	3	None	Hyb	
DIG-14	TACTTTTGTATATAAGATG	4	5	None	Hyb	
PO-7	ATTGAACAAGTCAAACTCTGTTCC	0	2	None	PCR	24
PO-14	CATTTTGTCTCTTACGTATGTAC	4	5	None	PCR	
PO-15	GGTATAAGATGCTTAGGCAATAC	4	4	None	PCR	
PO-4R	CGCAATTCATGCTGTTTCTCTTTTGC	4	0	Pv	PCR	
OVR	GCATAAGGAATGCAAAGAACAG	4	5	None	PCR	16, 17
PoR2	TGAAGGAAGCAATCTAAGAAATTT	4	5	Pf, Pm, Pv	PCR	29
	CTGTTCTTTGCATTCCTT	4	5	None	PCR	12
rOVA3	CGGGGAAATTTCTTAGATTGC	4	5	Pv	PCR	10
rOVA4	GAGAAACAGCATGAATTGCG	4	4	Pv	PCR	
OVA-F	TTTTGAAGAATACATTAGGATACAATTAATG	4	0	None	PCR	13
OVA-R	CATCGTTCCTCTAAGAAGCTTTACAAT	4	5	Pf, Pm, Pv	PCR	
ova	CCTTTTCCCTATTCTACTTAATTCGCAATTCATG	4	5	None	Hyb	
Ovaprobe	AATAAGAAAATTCCTTTTCG	4	5	None	Hyb	15
PovaPRB	CTTTGCATTCTTATSCAAATGTGTTC	4	5	None	Hyb	27
PoF	CCCTATTCTTCTTAATTCGCA	4	0	None	PCR	1
PoR	AGTGGAGGAAAACATA	4	5	Pm	PCR	
	GACTAGGTTTGGATGAAAGTG	0	0	None	Hyb	23

^a Name used in original publication, if one had been assigned.

^b The sequences are all presented 5' to 3'. The oligonucleotides used for PCR amplification are provided as published previously, whereas those used as hybridization probes have been converted so that the sequence corresponds to the sense strand.

^c Four full-length ssrRNA sequences for the classic type (Cl) have been considered (GenBank accession numbers L48987, L48986, AB182489, and AB182490), while five full-length sequences for the ssrRNA variant type (Vr) have been considered (GenBank accession numbers AJ001527, X99790, AB182491, AB182492, and AB182493). The data in the column represent the total number of sequences predicted to be recognized efficiently, based on an analysis performed with the Amplify 3 (version 3.1.4) program (<http://engels.genetics.wisc.edu/amplify>) under the standard default settings. Boldface indicates that the oligonucleotide would not be predicted to hybridize to all the ssrRNA sequences known to date.

^d Prediction of hybridization with the ssrRNA genes of other *Plasmodium* species have been obtained with the Amplify 3 (version 3.1.4) program, under stringent conditions of >70% for stability and >80% for primability. The genes from the following species have been considered in this analysis: *P. falciparum* (GenBank accession numbers M19172 and M19173), *P. vivax* (GenBank accession numbers U03079, U03080, U07367, U07368, and U93095), and *P. malariae* (GenBank accession numbers M54987, AF487999, and AF488000). Boldface indicates that the oligonucleotide would be predicted to hybridize to or to be amplified from an ssrRNA sequence from one or more of the other *Plasmodium* species: *P. falciparum* (Pf), *P. malariae* (Pm), or *P. vivax* (Pv).

^e Hyb, the oligonucleotide is used as a probe to hybridize to an amplicon; PCR, the oligonucleotide is used as a primer for PCR amplification.

tion of both *P. ovale* ssrRNA types, whereas others were not (Table 1). Primers rOVA1 and rOVA2 span regions that differ between both types. Thus, oligonucleotide primers corresponding to the variant sequence were designed (primers rOVA1v and rOVA2v) and tested in the secondary *P. ovale*-specific reaction (with NP-2005 primers). By using a subset of 107 samples, detection of *P. ovale* (with both classic and variant ssrRNA types represented) and *P. malariae* was found to be more efficient when a nested PCR protocol was used than when two distinct real-time PCR (Rt-PCR) protocols were used (Table 2). The higher degree of accuracy of *P. ovale* detection by use of the new set of oligonucleotides (NP-2005 primers) compared to the accuracy of the previous protocols (with the NP-1993 and NP-2002 primers) was confirmed in a retrospective analysis of all samples found to be positive for this species (Table 3). The sensitivity of nested PCR amplifi-

cation was consistently found to be on the order to 5 to 50 parasites genomes per aliquot analyzed (data not shown).

The detection of *Plasmodium* parasites based on species-specific sequences in the ssrRNA gene (26) could be achieved with a high sensitivity through a nested PCR approach (21) and provided a significant improvement over microscopic examination of blood smears. The efficacy and the accuracy of detection rely on a lack of variation in the target sequences to which the amplification primers hybridize. To date, evidence of polymorphisms in the ssrRNA genes was obtained for *P. ovale* and *P. malariae* (4, 8, 28, 30), mainly isolates collected in Southeast Asia, but not for *P. falciparum* and *P. vivax*. This might reflect a different and possibly more ancient evolutionary pathway for *P. malariae* and *P. ovale*. From a practical point of view, polymorphisms in *P. ovale* gene regions coincident with the sequences of the primers used in the PCR protocols can ad-

TABLE 2. Detection of *P. malariae* and *P. ovale* by different PCR-based protocols

No. of samples by microscopy ^a	No. of samples detected by Rt-PCR with ^{b,c} :			No. of samples detected by nested PCR ^{c,d}
	2004R, <i>P. ovale</i>	2004R, <i>P. malariae</i>	2004P, <i>P. ovale</i>	
9 Pf	1	0	1	6 Pf 1 Po 1 Pf + Po 1 Pf + Pm + Po
12 Pv	6	1	5	5 Pv 1 Pm 6 Po
3 Pm	0	3	0	3 Pm
7 Po	7	0	3	7 Po
1 Pv or Po	1	0	0	1 Po
2 <i>Plasmodium</i> sp.	0	1	0	1 Pm 1 Pf + Pm + Po
73 negative	1	1	1	1 Pm 2 Po 70 negative
3 Pm and 7 or 8 Po	16 Po	6 Pm	10 Po	8 Pm and 20 Po

^a Diagnosis made on admission by microscopic examination of Giemsa-stained thin blood smears for the set of 107 patients included in the analyses. Pf, *P. falciparum*; Pv, *P. vivax*; Pm, *P. malariae*; Po, *P. ovale*.

^b The Rt-PCRs with primers 2004R (13) and 2004P (15) were performed as described previously.

^c Five microliters of purified template DNA was used for the amplification assays.

^d For nested PCR with primer NP-2002, following a primary reaction with the genus-specific primer set rPLU1-rPLU5, detection of *P. falciparum* (Pf), *P. vivax* (Pv), and *P. malariae* (Pm) was carried out with primers rFAL1-rFAL2, rVIV1-rVIV2, rOVA1-rPLU2, and rMAL1-rMAL2 (19); for nested PCR with primer NP-2005, the same primary reaction described for the nested PCR with primer NP-2002 was performed, but the detection of *P. ovale* (Po) was achieved by using the two pairs of *P. ovale*-specific primers: rOVA1-rOVA2 (20, 21) and rOVA1v-rOVA2v (rOVA1v, 5'-ATCTCCTTTACTTTTGTACTGGAGA-3'; rOVA2v, 5'-GGAAAAGGACACTATAATGTATCCTAATA-3').

TABLE 3. Characteristics of *P. ovale* samples and efficacy of detection by different PCR-based protocols

Age (yr)	Country of origin	Country visited	Latency (mo)	Parasitemia (%)	Microscopy result ^b	PCR result				
						Genus-specific PCR (NP-2002)	<i>P. ovale</i> , NP-1993 ^c	<i>P. ovale</i> , NP-2002	<i>P. ovale</i> , NP-2005	Other species, NP-2002 ^b
40	Italy	Senegal	5	—	—	+	+	+	+	—
4	Ghana	Ghana	NA ^e	<0.01	Pf	+	+	+	+	—
NA	Italy	Africa ^d	24	0.064	Pv	+	+	+	+	—
24	Mozambique	Mozambique	NA	0.2	Pv	+	+	+	+	—
NA	Italy	NA	NA	0.1	Pv	+	+	+	+	—
27	NA	NA	NA	<0.1	Pv	+	+	+	+	—
22	Italy	Ghana	NA	0.14	Pv	+	+	+	+	—
26	Cameroon	Cameroon	5	<0.01	Po	+	+	+	+	—
30	Cameroon	Cameroon	3	0.12	Po	+	+	+	+	—
35	Nigeria	Nigeria	0.1	0.24	Po	+	+	+	+	—
25	Burkina Faso	Burkina Faso	0.25	<0.01	<i>Plasmodium</i> spp.	+	+	+	+	Pf + Pm
10	Ivory Coast	Ivory Coast	2	0.5	Pf	+	+	+	+	Pf
26	Ivory Coast	Ivory Coast	NA	<0.1	Pv	+	+	+	+	—
29	NA	N/A	NA	<0.004	Pv or Po	+	+	+	+	—
19	Burkina Faso	Burkina Faso	NA	<0.001	Pf	+	+	+	+	Pf + Pm
42	Italy	Tanzania	NA	<0.1	Po	+	—	+	+	—
51	Ghana	Ghana	NA	0.3	Pv	+	—	+	+	—
63	Italy	Unknown	NA	0.2	Po	+	—	+	+	—
24	Cameroon	Cameroon	6	0.07	Po	+	—	+	+	—
23	Cameroon	Cameroon	NA	0.2	Po	+	—	+	+	—
30	Ivory Coast	Ivory Coast	NA	0.037	Po	+	—	—	+	—
29	Cameroon	Cameroon	5	—	—	+	—	—	+	—
3	Burkina Faso	Burkina Faso	NA	—	—	+	—	—	+	—

^a Period between last date in the country of endemicity and presentation to the hospital.

^b Pf, *P. falciparum*; Pm, *P. malariae*; Po, *P. ovale*; Pv, *P. vivax*.

^c NP-1993, the primary reaction was with genus-specific primer set rPLU5-PLU6, followed by separate secondary reactions, each with one of the following species-specific primer sets: rFAL1-FAL2, rVIV1-rVIV2, rOVA1-rOVA2, or rMAL1-rMAL2 (20, 21).

^d This patient had traveled to Panama and Costa Rica following his trip to Africa, but *P. ovale* is not known to occur in the Americas. He presented to hospital 6 months after his return from Latin America.

^e NA, not available.

versely affect the efficacy of detection of the variant types observed (Table 1). Thus, caution must be exercised when *P. ovale*- or *P. malariae*-specific oligonucleotides are selected from the literature, whether these were designed before or after the polymorphisms came to light (1, 13). Careful assessment of primer species specificity must be carried out experimentally, since minor variations in amplification conditions (which could, for example, result from the use of different thermocyclers or enzymes from different suppliers) might lead to a decrease in the specificity. This might explain why the rOVA3/rOVA4 combination designed to be specific for *P. ovale* (10) proved to be equally effective for the amplification of *P. vivax* genomic DNA. Taken together, these observations strongly advocate the development of standardized protocols for the detection of *Plasmodium* parasites and that these protocols be validated by reference laboratories before their adoption as an adjunct to routine diagnosis.

From a biological point of view, two aspects of the data on *P. ovale* are of interest. Parasites with the variant sequences are not confined to Southeast Asia, since the majority of the patients analyzed in this study had acquired the *P. ovale* infection in West Africa. For nine of the patients, the period between the date of return from the country of endemicity and the time of presentation at the hospital in Italy was known. Only two of these patients presented within a month of their return, whereas for all the others latency extended between 2 months and 2 years (Table 3). For these patients, the correct intake of chemoprophylaxis would not account for the suppression of the parasitemia for more than 2 months after their return to Italy. The most likely explanation is that the infections on admission in Italy were initiated from *P. ovale* hypnozoites, implying that relapses by this species might be more frequent than was previously thought.

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