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Two Techniques for Simultaneous Identification of *Plasmodium ovale curtisi* and *Plasmodium ovale wallikeri* by Use of the Small-Subunit rRNA Gene

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The primers traditionally used to detect *Plasmodium ovale* infections are known for not binding all *P. ovale* parasites within the small-subunit rRNA gene when used alone. We describe a simple, cost- and time-efficient multiplex nested PCR and a nested PCR using a novel set of primers for the simultaneous detection of *P. ovale curtisi* and *P. ovale wallikeri*.

Genes of the small-subunit (SSU) rRNA are highly conserved regions that not only allow the discrimination of different *Plasmodium* species but can also be used for the phylogenetic characterization of a wide range of different malaria parasites (11). For the diagnosis of human malaria parasites, a nested PCR technique (NP-1993 protocol) that binds the SSU rRNA gene was originally developed in the early 1990s (12). This technique soon became one of the most widely used and standardized PCR techniques for the detection and differentiation of human malaria parasites. The lower limits of detection were reported to be between a single parasite in 10 μl blood (0.000002% parasitemia) and six parasites in 1 μl blood (9, 11). Several modifications of the NP-1993 protocol followed (2, 8, 9). It soon became obvious that the NP-1993 protocol had some limitations for the diagnosis of *Plasmodium ovale* (primers rOVA1/rOVA2). Some patient samples that were positive for *P. ovale* by microscopy gave negative results with the nested PCR, and finally an updated protocol was described in 2002 (11). The primers for the Nest2 analysis of *P. ovale* were changed to genus-specific primer rPLU2 combined with rOVA1.

Until 2005, more than 14 different protocols had been published specifically for the diagnosis of *P. ovale*. On the basis of the availability of more specific diagnostic tools, *P. ovale* was divided into the classic and variant types. In 2005, the NP-2005 protocol using primers rOVA1v and rOVA2v to detect variant *P. ovale* parasites was presented (1).

Subsequent studies showed that the differences between the classic and variant types of *P. ovale* are not limited to the SSU rRNA gene. The *P. ovale* reticulocyte binding protein 2 gene (*porbp2*) and tryptophan-rich antigen gene (*potra*) are other examples of genetic differences. The perfect linkage between the two dimorphic forms of *P. ovale* finally led to the introduction of *P. ovale curtisi* (the former classic type) and *P. ovale wallikeri* (the former variant type) (13). Recent studies documented the sympatric distribution of *P. ovale curtisi* and *P. ovale wallikeri* in Africa and Asia and that they are morphologically indistinguishable (5, 7, 13).

The combination of NP-1993 primers rOVA1 and rOVA2 for the diagnosis of *P. ovale curtisi* and the NP-2005 primer rOVA1v and rOVA2v has previously been published but still requires two separate PCRs (5).

The principal aim of this study was to simplify the methodology for differentiating *P. ovale curtisi* and *wallikeri* (and thereby the methodology of adequate *P. ovale* molecular diagnosis) by developing a single PCR. We designed (i) new primers binding within the SSU rRNA gene that are highly specific for both species (*P. ovale curtisi* and *P. ovale wallikeri*) while not binding to other human *Plasmodium* species and (ii) a novel multiplex PCR protocol combining the primers of the NP-1993 and NP-2005 protocols.

Twenty-one *P. ovale* samples (11 *P. ovale curtisi* and 10 *P. ovale wallikeri*) were analyzed. Nineteen *P. ovale* samples had been collected at the Malaria Research Initiative Bandarban (MARIB) field site in Bangladesh under Institutional Review Board-approved protocols after obtaining written informed consent. These samples were identified to the species level (*P. ovale curtisi* and *P. ovale wallikeri*) by using nested PCR techniques (SSU rRNA and *potra* genes) and sequence analysis as described in detail previously (5). We also included two sequence-confirmed *P. ovale*-positive reference controls from Uganda provided by the Medical University of Vienna.

To confirm that the new techniques bind *P. ovale* only, we used 140 filter paper samples (95 *Plasmodium* positive and 45 *Plasmodium* negative) that had previously been described (4). All patient samples were collected under protocols approved by the ethical review committees of the International Centre for Diarrheal Disease Research, Bangladesh, and the Medical University of Vienna.

Standard nested and direct nested PCR techniques were used under conditions reported previously (3, 4). We used primers rPLU1/rPLU5 for the *Plasmodium* genus-specific Nest1 reaction and rPLU3/rPLU4 for the *Plasmodium*-specific Nest2 reaction. Whenever a sample was positive for *Plasmodium* sp. with genus-specific primers rPLU3/rPLU4, the following species-specific primers were used to classify it to the species level: rFAL1/rFAL2 for *P. falciparum*, rVIV1/rVIV2 for *P. vivax*, rMAL1/rMAL2 for *P. malariae*, and Pmk8/Pmkr9 for *P. knowlesi* (9, 10, 11).

For the diagnosis of *P. ovale*, we used the following standard-

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TABLE 1 Comparison of *P. ovale* diagnoses by different direct nested PCR protocols

Malaria parasite(s) detected[a]	Overall direct PCR results[b]	NP-2002 genus-specific PCR[c]	New primers rOVA1WC/ rOVA2WC[d]	Multiplex PCR[e]
None	45[f]	0	0	0
Pf	60	60	0	0
Pv	7	7	0	0
Pm	3	3	0	0
PoC	6	6	6	6
PoW	6	6	6	6
PoC + Pf	1	1	1	1
PoC + Pv	1	1	1	1
PoC + Pf + Pm	1	1	1	1
PoC + Pf + Pm + Pv	1	1	1	1
PoC + Pf + Pv	1	1	1	1
PoW + Pf	2	2	2	2
PoW + Pf + Pm	1	1	1	1
PoW + Pf + Pm + Pv	1	1	1	1
Pf + Pv	11	11	0	0
Pf + Pm	5	5	0	0
Pf + Pv + Pm	1	1	0	0
Pv + Pm	2	2	0	0
Pk	1	1	0	0

[a] Pf, *P. falciparum*; Pv, *P. vivax*; Pm, *P. malariae*; PoC, *P. ovale curtisi*; PoW, *P. ovale wallikeri*; Pk, *P. knowlesi*.
[b] Diagnosis made by direct PCR. Results of microscopy, standard nested PCR, and phylogenetic analyses of the *P. ovale* samples have been published previously (4, 5).
[c] Genus-specific PCR (NP-2002), primers rPLU3/rPLU4.
[d] Nested PCR with a new set of primers, rOVA1WC/rOVA2WC.
[e] Combination of two primer sets, rOVA1/rOVA2 and rOVA1v/rOVA2v.
[f] Each value is the number of samples tested. A total of 156 samples were tested.

TABLE 2 Summary of the results obtained with *P. ovale*-positive samples and different PCR protocols[a]

Organism (no. of samples)	PoR (NP-2002)	PoC (NP-1993)	PoW (NP-2005)	rOVA1WC/ rOVA2WC	Multiplex PCR
P. ovale wallikeri (7)	+[b]		+	+	+
P. ovale wallikeri (3)			+	+	+
P. ovale curtisi (11)	+	+		+	+

[a] PoR, rOVA1/rPLU2 (NP-2002); PoC, rOVA1/rOVA2 (NP-1993); PoW, rOVA1v/ rOVA2v (NP-2005); new primers, rOVA1WC/rOVA2WC; multiplex PCR, combination of rOVA1/rOVA2 and rOVA1v/rOVA2v primer sets.
[b] +, positive result.

ized commonly used protocols as reported previously (1, 9, 11): NP-1993 with rOVA1/rOVA2 (known to bind *P. ovale curtisi*); NP-2002 with rOVA1/rPLU2 (known to bind *P. ovale curtisi* and some *P. ovale wallikeri* strains); and NP-2005 with rOVA1v/ rOVA2v (known to bind *P. ovale wallikeri* only).

Primer design and conditions for PCR with rOVA1WC/ rOVA2WC. We chose all of the sequences of the *P. ovale* SSU rRNA gene (18 *P. ovale wallikeri* and 10 *P. ovale curtisi*) available in GenBank that had previously been used for the discrimination of the two species (5, 13). Alignment was performed by using ClustalX v.2.0.12, and primers that bind both *P. ovale curtisi* and *P. ovale wallikeri* were designed with the CLC MainWorkBench (CLC Bio, Aarhus, Denmark) (6). This resulted in *P. ovale*-specific primers rOVA1WC (5′-TGTAGTATTCAAACGCAGT-3′) and rOVA2WC (5′-TATGTACTTGTTAAGCCTTT-3′).

The Nest2 primers were tested with Nest1 products under the same conditions used for the identification of other human malaria parasites to the species level (with the exception of *P. knowlesi*) (3, 4, 11). A template volume of 2.5 μl was used in a 25-μl Nest2 reaction mixture (GoTaq PCR core system; Promega, Madison, WI) containing 125 μM each deoxynucleoside triphosphate, 2 mM $MgCl_2$, 1 U of GoTaq DNA polymerase, and 2.5 μl of each primer (10 μM). Cycling conditions with an Eppendorf Mastercycler personal (Eppendorf AG, Hamburg, Germany) were as follows: denaturation at 98°C for 4 min, followed by 25 cycles of annealing at 58°C for 2 min, extension at 72°C for 2 min, and denaturation at 94°C for 1 min, and a final extension at 72°C for 5 min. The primers were also tested with 35 cycles to obtain more PCR product.

Nest2 PCR products were visualized by gel electrophoresis with 2% agarose and Midori Green Advance (Biozym Scientific GmbH, Hessisch Oldendorf, Germany) staining.

Multiplex PCR. We combined the primers of the NP-1993 protocol, rOVA1/rOVA2, with the primers of the NP-2005 protocol, rOVA1v/rOVA2v (1, 12). The reaction mixture was identical to the one described above, with the exception that the amount of nuclease-free water was reduced by 5 μl and that the reaction mixture was prepared with 2.5 μl of each primer (10 μM). All further processing was identical to that performed when testing with primer pair rOVA1WC/rOVA2WC.

With direct nested PCRs, 111 out of 156 patient samples were positive for *Plasmodium* spp. (Table 1). Both, the *P. ovale* multiplex PCR and the new primer pair rOVA1WC/rOVA2WC gave positive test results for *P. ovale wallikeri* and *P. ovale curtisi* only and did not bind other human malaria parasites in 25 or 35 amplification cycles of either standard nested PCR or direct nested PCR. The only difference between 25 and 35 cycles was that the resulting bands in the gel electrophoresis were more intense with 35 cycles. Although microscopy is traditionally seen as the gold standard of malaria diagnosis, it does not allow a distinction between *P. ovale wallikeri* and *P. ovale curtisi*, which are genetically distinct but morphologically indistinguishable (13). With primers rOVA1/rOVA2 and rOVA1v/rOVA2v as the gold standard, both techniques had a sensitivity, specificity, and positive predictive value of 100% (Table 2).

The techniques described in this paper are not meant to allow the discrimination of *P. ovale curtisi* and *P. ovale wallikeri*. They allow the rapid, cost-efficient, sensitive, and highly specific identification of *P. ovale* for diagnostic purposes. Primers rOVA1WC/ rOVA2WC bind to a 662-bp sequence of *P. ovale curtisi* and a 659-bp sequence of *P. ovale wallikeri*, whereas the sets of primers used in the multiplex PCR bind a 787- to 789-bp sequence of *P. ovale curtisi* (primers rOVA1/rOVA2) and a 782-bp sequence of *P. ovale wallikeri* (primers rOVA1v/rOVA2v). We recommend that samples that give positive results with these techniques should, if necessary, be further analyzed with primers rOVA1/rOVA2 and rOVA1v/rOVA2v for the discrimination of *P. ovale curtisi* and *P. ovale wallikeri*, respectively.

The main advantage of using these techniques for the detection of *P. ovale* are that only one Nest2 PCR is needed for the detection of these parasites, making it is less time- and cost-intensive. Both techniques can be used with the same reaction mixture and under the same cycling conditions as used for other malaria parasites (with the exception of *P. knowlesi*) in a standard nested PCR and a direct nested PCR.

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