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We present a seminested PCR method that specifically discriminates between *Plasmodium ovale curtisi* and *P. ovale wallikeri* with high sensitivity. The test is based on species-specific amplification of a size-polyorphic fragment of the tryptophan-rich antigen gene, *potra*, which also permits discrimination of intraspecific sequence variants at this locus.
TABLE 1 Sizes of the sequenced postra fragments amplified using the different primer pairs

<table>
<thead>
<tr>
<th>Sample(s)</th>
<th>P. ovale subspecies</th>
<th>Potra fwd5 + Potra rev5 (bp)</th>
<th>PoTRA-F + Pocta-R (bp)</th>
<th>PoTRA-F + PocTRA-R (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>POW1 or POW2a</td>
<td>wallikeri</td>
<td>245</td>
<td>389</td>
<td></td>
</tr>
<tr>
<td>11 (Africa)</td>
<td>wallikeri</td>
<td>245</td>
<td>389</td>
<td></td>
</tr>
<tr>
<td>T7, T9, T11, T19b</td>
<td>wallikeri</td>
<td>299</td>
<td>443</td>
<td></td>
</tr>
<tr>
<td>T22 (+ P. vivax)</td>
<td>wallikeri</td>
<td>299</td>
<td>443</td>
<td></td>
</tr>
<tr>
<td>T12 (+ P. falciparum)</td>
<td>wallikeri</td>
<td>299</td>
<td>443</td>
<td></td>
</tr>
<tr>
<td>VP, TVZ1c</td>
<td>wallikeri</td>
<td>335</td>
<td>479</td>
<td></td>
</tr>
<tr>
<td>POCI4d</td>
<td>curtisi</td>
<td>299</td>
<td>443</td>
<td></td>
</tr>
<tr>
<td>VN, T14d</td>
<td>curtisi</td>
<td>299</td>
<td>443</td>
<td></td>
</tr>
<tr>
<td>POC2d</td>
<td>curtisi</td>
<td>317</td>
<td>461</td>
<td></td>
</tr>
<tr>
<td>T13e</td>
<td>curtisi</td>
<td>353</td>
<td>497</td>
<td></td>
</tr>
</tbody>
</table>

a POW1 and POW2 sequences were previously obtained (GenBank accession no. HM594180 and HM594181, respectively).
b GenBank accession no. for T19 is KF018430.
c GenBank accession no. for TVZ1 is KF018431.
d POCI and POC2 sequences were previously obtained (GenBank accession no. HM594182 and HM594183, respectively).

P. ovale wallikeri (TVZ1)-infected blood samples (with respect to annealing temperature as well as Mg2+ and oligonucleotide concentrations). The fragments obtained for each species were then cloned into the pCR 2.1 vector (Invitrogen, USA), and each plasmid was purified from the bacterial clones. These standard plasmids were used to optimize the conditions for the secondary amplification reactions (with respect to annealing temperature as well as Mg2+ and oligonucleotide concentrations) and to derive the limit of detection of the seminested PCR protocol. The concentration of each standard plasmid stock solution was determined by using the optical density of the solution at 260 nm. The copy number of each standard plasmid per μl was calculated as the mass of the plasmid standard (g/μl) divided by the calculated mass of each molecule (number of bp × 660 g/6.027 × 1023). A serial dilution series, in which there were 1, 2, 5, 10, 102, 103, 104, or 105 copies per μl, was then obtained, and 1 μl of each dilution was tested five times.

All reactions were carried out in a total volume of 20 μl in the presence of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 75 or 125 μM for each deoxyribonucleotide triphosphate, and 0.4 units of Taq polymerase (Invitrogen, USA). The primary amplification was carried out at a final concentration of 2 mM MgCl2 and 75 nM for each primer, with an annealing temperature of 56°C, whereas the secondary amplification was carried out using an annealing temperature of 60°C, with a final concentration of 3 mM for MgCl2 and 125 nM for each primer. One microliter of template was used to initiate both the primary and secondary amplification reactions. The cycling parameters consisted of an initial denaturation step at 95°C for 5 min, annealing for 1 min, and then extension at 72°C for 1 min, followed by a denaturation step at 94°C for 1 min. After a given number of cycles (25 cycles for the primary amplification and 30 cycles for the secondary amplification), a final extension step at 72°C was carried out before storage of the product at 4°C. Ten microliters of the secondary reaction products was electrophoresed on a 2% agarose gel, and the bands were then visualized by UV transillumination, following staining with ethidium bromide.

The limit of detection, as based on the template with a known number of plasmid molecules, was five copies, for which all five duplicates gave a positive result. The specificity of the reaction was confirmed by using high concentrations of genomic DNA (equivalent to 104 parasite genomes) from P. falciparum, P. vivax, P. malariae, or human DNA as templates alone (all reactions proved negative) or mixed with one or other of the standard plasmid templates that demonstrated that sensitivity was not affected. No detectable fragments could be amplified when plasmid DNA carrying the potra fragment from one P. ovale species was used as a template for the secondary amplification reaction, in which the primer pair specific for the potra of the other P. ovale species was used. The sensitivity and specificity of the protocol was then assessed by using genomic DNA purified from clinical blood samples containing P. ovale curtisi and P. ovale wallikeri that had been enumerated accurately (416 parasites/μl blood and 1,152 parasites/μl blood). These genomic DNAs were then serially diluted and assayed. The seminested protocol was able to consistently detect a parasitemia equivalent to 2 to 10 parasites/μl blood. The specificity and the consistency of the sensitivity were again confirmed by adding excess P. falciparum, P. vivax, P. malariae, or human genomic DNAs to the serially diluted DNA. Finally, genomic DNA templates from 30 patients infected with P. falciparum (n = 10), P. vivax (n = 10), or P. malariae (n = 10) were also tested and proved negative.

The seminested PCR protocol was then applied to DNA purified from 17 clinical blood samples: 7 samples infected with P. ovale curtisi (two of these were mixed infections with P. falcipa-
and 10 samples infected with *P. ovale wallikeri* (two of these were mixed infections, one with *P. falciparum* and the other with *P. vivax*). The species present in these samples had been previously established by analysis of the ssrRNA genes and the mitochondrial locus *pocytb* (9). The *potra*-based protocol presented here correctly identified the species present in each sample. Moreover, the isolates from each species could be classed according to the amplified fragment size. Three distinct allelic *potra* variants were sequenced for each species, and the predicted amino acid sequences were aligned (Fig. 1). Subsequent to this, two potentially new size variants were amplified (Fig. 2) from a *P. ovale curtisi* sample recently collected from a patient who had acquired the infection in Africa (the exact country had not been recorded).

Thus, we present a sensitive seminested PCR protocol that not only allows discrimination between *P. ovale curtisi* and *P. ovale wallikeri* with high specificity but also provides a simple means to identify genotypic variants within each of these species. We are aware that nested PCR protocols are associated with two disadvantages. The first is the additional cost and labor of carrying out an additional PCR. The second is the substantially increased risk of contamination inherent to the transfer of the PCR product from the first to the second reaction; in our experience, this risk can be substantially reduced by the allocation of a distinct laboratory space for this transfer. We feel that in this particular case, the use of a seminested PCR protocol can be justified. The limit of detection of nested PCR-based protocols is often higher than that of methods based on a single amplification step, an important consideration for *P. ovale* infections where parasite burdens are often quite low. Moreover, the nested PCR format is less sensitive to inhibitors present in the initial template. Finally, discrimination of allelic variants by size is most practically carried out following gel electrophoresis, a step that will negate any advantage of methods where the amplified product remains in closed tubes. Ultimately, the protocol presented here is intended for fundamental investigations on the two *P. ovale* species and not for implementation in a routine laboratory, as there is no evidence that the clinical course or the treatment required varies between *P. ovale wallikeri* and *P. ovale curtisi* infections.

As more samples are analyzed, it is likely that the number of *potra* size variants that occur would exceed those observed to date (five for *potra* and three for *powtra*). In conclusion, the *potra* genes could now serve as targets for molecular identification and as genetic markers suitable for a broad range of investigations of the epidemiology and biology of *P. ovale curtisi* and *P. ovale wallikeri*, similar to those carried out for *P. falciparum* and *P. vivax* (19–21).

**Nucleotide sequence accession numbers.** The *potra* gene sequences for samples T19, TVZ1, T13, and T14 were deposited in GenBank under accession numbers KF018430 to KF018433, respectively.

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**REFERENCES**


