Recent Advances in Detection of \textit{Plasmodium ovale}: Implications of Separation into the Two Species \textit{Plasmodium ovale wallikeri} and \textit{Plasmodium ovale curtisi}

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Recent molecular studies indicate that \textit{Plasmodium ovale} malaria is caused by two closely related species of protozoan parasites, thereby imposing new challenges for detection and species differentiation. This minireview explores the potential value of innovative methods for the molecular diagnosis of malaria with a strong emphasis on the discrimination and genotyping of \textit{P. ovale wallikeri} and \textit{P. ovale curtisi} as well as tools for the simultaneous detection of \textit{P. ovale} sp. An update for the widely used NP-1993 to NP-2005 (SSU rRNA) protocols for all human malaria parasites is discussed.

According to the World Malaria Report 2012, approximately half of the world’s population is at risk of infection with malaria and 660,000 fatal cases were reported in 2011 (1). Malaria diagnosis is largely based on microscopy, which after more than 100 years is still considered to be the gold standard of malaria diagnosis. However, with the widespread availability of PCR, a new era in the detection of malaria is still considered to be the gold standard of malaria diagnosis. However, with the widespread availability of PCR, a new era in the detection of malaria is replacing microscopy as the method of choice for diagnosing malaria in various settings. In spite of a number of advantages (e.g., field deployment), the use of RDTs is limited where microscopy is not available. The recent availability of high-throughput sequencing technologies offers the potential for \textit{P. ovale} parasites are therefore urgently needed. This review focuses on the following:

1. Techniques used for the molecular phylogenetic analysis of \textit{Plasmodium} sp.
2. The most advanced and reliable techniques currently available for the diagnosis of \textit{P. ovale} malaria.

MOLECULAR PHYLOGENY OF \textit{PLASMODIUM} SPP. WITH THE MAIN FOCUS ON \textit{P. OVALE} SP.

Molecular phylogenetic studies of the genus \textit{Plasmodium} are mainly based on analyzing nuclear and mitochondrial genes. Mitochondrial genomes of different systematic groups differ in structure, size, and organization—e.g., \textit{Plasmodium falciparum} has a size of only 6 kb. The mitochondrial genome of malaria parasites is linear and shows tandem repeats.

Several studies using molecular tools for describing the phylogeny of the genus \textit{Plasmodium} have provided invaluable information not available previously. These studies suggest that most simian malaria parasites are in fact closely related to \textit{P. vivax}, al-
though their morphology may suggest otherwise (9). *P. simiovale* closely resembles *P. ovale* and received its name based on their morphological similarity, but genetic analysis has shown that *P. simiovale* is actually closely related to *P. vivax*. These studies have also shed light on the zoonotic potential of human malaria parasites. Duval et al. (10), for example, have confirmed the presence of *P. falciparum*, *P. ovale*, and *P. malariae* in African great apes.

The high capacity for differentiating malaria parasites has resulted in an increase of the number of parasite species considered to be “human” malaria parasites. *P. knowlesi* was first diagnosed in southeastern Asia in humans using molecular tools, and, based on its molecular dimorphisms, *P. ovale* was divided into two new species, namely, *P. ovale wallikeri* and *P. ovale curtisi* (3).

The following genes have been used for the molecular discrimination of *P. ovale curtisi* and *P. ovale wallikeri*.

**SSU rRNA.** The nuclear small subunit (SSU) rRNA genes are known to be highly conserved regions suitable not only for phylogenetic studies but also for the molecular detection of human malaria parasites (3, 5, 6, 11, 12, 13). The copy numbers of this gene were observed to be similar not only for phylogenetic studies but also for the molecular detection of human malaria parasites. *P. knowlesi* was first diagnosed in southeastern Asia in humans using molecular tools, and, based on its molecular dimorphisms, *P. ovale* was divided into two new species, namely, *P. ovale wallikeri* and *P. ovale curtisi* (3).

Several modifications of the NP-1993 protocol followed. In 1999, the group around Balbir Singh updated the NP-1993 protocol (19). Instead of the genus-specific nest 1 rPLU6 primer, they used the rPLU1 primer. The nest 1 product resulting from the use of primers rPLU1 and rPLU5 has a size of 1,670 bp and allowed the introduction of a genus-specific nest 2 PCR performed with primers rPLU3 and rPLU4 (240 bp). Instead of five PCRs, only two PCRs were needed to detect whether a blood sample is positive for malaria. Its limit of detection was reported as being 1 parasite/10 μl blood, 6 parasites/μl, and 1 to 10 parasite genomes per sample by different authors.

Further studies revealed that the NP-1993 protocol had some limitations in the detection of *P. ovale*. Some patient samples which were positive for *P. ovale* in microscopy gave negative results within the nested PCR, and so the protocol was updated in 2002 (20). The primers for the nest 2 species analysis of *P. ovale* were changed to a combination of a genus-specific primer, rPLU2, with rOVA1. NP-2002 involved genus-specific nest 2 primers rPLU2 and rPLU3.

By 2005, more than 14 different protocols had been published for the detection of *P. ovale* and it became evident that *P. ovale* needed to be divided into classic and variant types. In 2005, the group around Calderaro introduced the NP-2005 protocol. Instead of the use of the rPLU2 and rOVA1 primers, they recommended the use of two primer pairs: rOVA1v and rOVA2v for the detection of the variant type of *P. ovale* and rOVA1 and rOVA2 for *P. ovale curtisi* (18). A new set of primers (rOVA1v and rOVA2v) was designed which binds to both *P. ovale* species and are specific for the whole *P. ovale* complex. Within recent years, however, the number of species uploaded at GenBank (including those of rarer species) has increased, making specific primer design considerably easier.

**STANDARD NESTED PCR**

In 1993, a PCR protocol targeting the SSU rRNA gene was developed which soon became one of the most widely used and standardized techniques for the molecular detection of human malaria parasites (18). The NP-1993 protocol seemed to be perfectly suited for epidemiological studies. In NP-1993, species-specific rPLU5 and rPLU6 were used for the nest 1 PCR, amplifying a product of about 1,200 bp in size (18). For species determination, primers rFAL1 and rFAL2 for *P. falciparum* (206 bp), rVIV1 and rVIV2 for *P. vivax* (121 bp), rMAL1 and rMAL2 for *P. malariae* (145 bp), and rOVA1 and rOVA2 for *P. ovale* (787 bp) were used.

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Recently, two techniques have been reported for the simultaneous detection of the two *P. ovale* species. A new set of primers (rOVA1WC and rOVA2WC) was designed which binds to both *P. ovale wallikeri* and *P. ovale curtisi* while not binding to other human malaria parasites (22). Moreover, a multiplex PCR combing the NP-1993 primers (rOVA1 and rOVA2) with the NP-2005 primers (rOVA1v and rOVA2v) gave adequate results for the de-
can be differentiated (16). Although this technique has been devised to differentiate the two species of P. ovale, it was observed that the amplicon (74°C) (16). Within this real-time PCR melt curve, the peak melting temperature of the amplicon (73°C) was consistently 1 degree lower than that of the P. ovale Wallikeri amplicon (74°C) (16).


tution of P. ovale sp. (22). An updated NP-2013 protocol is provided in Table 1.

**OTHER MOLECULAR TECHNIQUES FOR THE DETECTION AND/OR DISCRIMINATION OF P. OVALE SPP.**

As discussed earlier, the non-protein-coding SSU rRNA genes show inter- and intraspecies-specific variations. Recently, a highly sensitive and specific real-time PCR assay that allows the differentiation of P. ovale curtisi and P. ovale Wallikeri was described (24, 25, 26).

The results of a study using an 18S SSU rRNA-hexaplex PCR suggest that the primers cover both species (27). In addition, the group around Bauffe et al. described a real-time TaqMan PCR (TaqPCR) using primer pair PoF and PoR and two probes (pPOC and pPOW) (13). The probes target at position 1,158 in the variant SSU rRNA sequences.

Oguike et al. have published a nested PCR method using the potra gene which allows discrimination of the two P. ovale species (16). In nest 1, primer pair PoTRAfwd3 and PoTRArev3 bind a 787-bp fragment. Using the internal primers PoTRAfwd5 and PoTRArev5, P. ovale Wallikeri and P. ovale curtisi (245 to 355 bp) can be differentiated (16). Although this technique has been shown to be specific for P. ovale sp., it was observed that the amplified fragment sizes also differ as a result of differences in the number of repeat units, limiting species discrimination: for P. ovale Wallikeri, 245, 299, and 335 bp, and for P. ovale curtisi, 299, 317, and 353 bp (28).

Recently, a specific semi-nested PCR method for the discrimination of the two P. ovale species was presented (nest 1; primers PoTRA-F and PoTRArev3). In the nest 2 reaction, the PoTRA-F primer is combined with primers specific for P. ovale Wallikeri (primer PowTRA-R; 389, 443, and 479 bp) and P. ovale curtisi (primer PocTRA-R; 443, 461, and 497 bp) (28). This technique allows not only the discrimination of the two P. ovale species but also the phylogenetic analysis of these parasites based on intraspecies-specific variations. Furthermore, the group around Tanomsing suggested that the number of potra size variations might exceed those evaluated (28).

Within the porbp2 gene, primers PoRBP2fwd and PoRBP2rev (nest 1) and primers PoRBP2TMfwd and PoRBP2TMrev (nest 2) flanking six single-nucleotide polymorphisms can be used for the specific amplification of the two P. ovale parasite species. In 5 positions, A and T residues in P. ovale curtisi are replaced by C and G residues in P. ovale Wallikeri. Within this real-time PCR melt curve, the peak melting temperature of the P. ovale curtisi amplicon (73°C) was consistently 1 degree lower than that of the P. ovale Wallikeri amplicon (74°C) (16).

**CONCLUSION**

Malaria diagnosis remains a challenge not just in resource-limited field settings but also in research and laboratory environments. In light of changing malaria epidemiology and the prospect of malaria elimination finally becoming a reality in several countries affected by malaria, highly sensitive and specific diagnostic tools may further gain importance in the near future. This particularly applies to the less common species P. ovale sp., P. malariae, and P. knowlesi, which may deserve more attention than they have been getting in recent years. We conclude the following:

- PCR-based techniques addressing all six human malaria species are recommended for epidemiological studies in southern and southeastern Asia and for malaria diagnosis in travelers returning from these regions whenever available.
- In other regions where P. knowlesi is not endemic (limited by the distribution of its intermediate hosts), epidemiological studies and returning travelers are recommended to be screened for the other 5 malaria species.
- If two separate PCRs for the detection of P. ovale curtisi and P. ovale Wallikeri are not available (e.g., due to cost and time constraints), P. ovale sp. should be detected using molecular

### Table 1: Recommendations of the NP-2013 protocol

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'–3')</th>
<th>Species/genus</th>
<th>bp</th>
<th>References</th>
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<tr>
<td>rPLU1</td>
<td>TCA AAG ATT AAG CCA TGC AAG TGA</td>
<td>Genus—nest 1&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>rPLU4</td>
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<td></td>
<td></td>
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<tr>
<td>rFAL1</td>
<td>TTA AAC TGG TTT GGG AAA ACC AAC TAT ATT</td>
<td>P. falciparum&lt;sup&gt;e&lt;/sup&gt;</td>
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<sup>a</sup>Cycling conditions: initial denaturation, 95°C for 5 min; 25 cycles for nest 1 and 30 cycles for nest 2 of 94°C for 1 min, 58°C for 1 min, and 72°C for 2 min; final extension, 72°C for 5 min (20, 22).

<sup>b</sup>Cycling conditions: initial denaturation, 95°C for 5 min; 30 cycles of 94°C for 1 min, 64°C for 1 min, and 72°C for 2 min; final extension, 72°C for 5 min (20).

<sup>c</sup>Cycling conditions: initial denaturation, 95°C for 5 min; 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min; final extension, 72°C for 5 min (23).

<sup>d</sup>Detection of P. ovale Wallikeri and P. ovale curtisi after positive results for P. ovale sp. (rOVA1wC/rOVA2wC) or as multiplex PCR for the detection of P. ovale sp. (22).
techniques validated to detect both species. Positive *P. ovale* samples should be further discriminated to the species level whenever possible.

- The role that the two *P. ovale* species are playing in the clinical presentation needs to be better defined to improve understanding of the role of species differentiation for malaria treatment.

**REFERENCES**

Mag. Dr. Hans-Peter Fuehrer fills a postdoctoral position at the Institute of Parasitology—University of Veterinary Medicine Vienna focusing on mosquito-borne zoonotic diseases. After studying zoology and parasitology, he finished his doctorate at the laboratories of Prof. Harald Noedl at the Medical University of Vienna in 2012 working on the molecular phylogeny and distribution of *Plasmodium ovale* in southern Asia. Furthermore, he was head of the laboratory at the Malaria Research Initiative Bandarban in rural Bangladesh. His main interests are *Plasmodium ovale*, neglected pathogens, and rodent-borne and vector-borne diseases.