Recent advances in the diagnosis of *Plasmodium ovale*. Implications of the separation into the two species *P. o. wallikeri* and *P. o. curtisi* for diagnosis.

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Running title: Recent advances in the molecular diagnosis of *Plasmodium ovale*.

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Abstract:
Recent molecular studies indicate that ovale malaria is caused by two closely related species of protozoan parasites thereby imposing new challenges for diagnosis 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 *P. ovale wallikeri* and *P. o. curtisi* as well as tools for the simultaneous detection of *P. ovale* sp. An update for the widely used NP-1993 – NP-2005 (SSU rRNA) protocols for all human malaria parasites is discussed.
Introduction

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 polymerase chain reaction (PCR) a new era in the diagnosis of Plasmodium sp. has begun. Largely based on PCR results the number of human malaria parasites has increased from four to six species within only 10 years, namely: Plasmodium falciparum, P. vivax, P. malariae, P. knowlesi and the newly recognized species P. ovale curtisi (former classic type) and P. ovale wallikeri (former variant type) (2, 3). However, much of the research effort has gone into only two of these: P. falciparum and P. vivax.

There remains a major lack of information on the less common Plasmodium species. Until very recently the distribution of P. ovale was thought to be limited to sub-Saharan Africa, the Philippines, Papua New Guinea and some Indonesian islands (4). With its morphological resemblance and its tertian periodicity it can easily be mistaken for P. vivax or P. malariae in microscopic analysis. Within the past decade P. ovale was recognized to be endemic in most malaria-endemic countries in South- and South-East Asia and both classic and variant forms have been observed in Asia. Molecular phylogenetic analyses suggest that P. ovale actually comprises two species: P. ovale curtisi and P. ovale wallikeri. In the meantime several studies have supported this concept (3, 5). Both species are known to occur sympatric in Africa and Asia and even simultaneous infections with both parasites have been reported (6). Both species are indistinguishable by microscopy but seem to differ in their duration of latency (7).
In 2004 *P. knowlesi* was recognized as a “human” pathogen based on observations in Kapit division of Malaysian Borneo (2). This parasite has a daily (quotidian) life cycle, can reach high levels of parasitemia and is potentially fatal. Its distribution is limited to Southeast and parts of South Asia by the distribution of its intermediate (long-tailed macaques) and final host (mosquitoes of the *Anopheles leucosphyrus*-group) (8). In microscopy it can easily be mistaken for *P. malariae*.

Throughout the past decades rapid diagnostic tests (RDTs) have replaced 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 to settings where microscopy is not available or practicable. Most RDTs currently on the market provide little information on species or parasite density.

The limitations of microscopy, RDTs, but also non-specific PCRs impose certain limitations on diagnosis and species differentiation. Accurate, highly sensitive and specific molecular diagnostic tools capable of differentiating all six human malaria parasites are therefore urgently needed. This review will focus on:

a) Techniques used for the molecular phylogenetic analysis of *Plasmodium* sp.

b) The most advanced and reliable techniques currently available for the diagnosis of ovale malaria.

**Molecular phylogeny of Plasmodium spp. with the main focus on P. ovale sp.**
Molecular phylogenetic studies of the genus *Plasmodium* are mainly based on analyzing nuclear and mitochondrial genes. Mitochondrial genomes of different systematic groups vary in structure, size and organization – e.g. *Plasmodium falciparum* with its 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 *Plasmodium* have provided invaluable information not available previously. These studies suggest that most simian malaria parasites are in fact closely related to *P. vivax*, although 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 being considered to be “human” malaria parasites. *P. knowlesi* was first diagnosed in South-East 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).

Following genes have been used for the molecular discrimination of *P. ovale curtisi* and *P. o. wallikeri*:

**Small subunit ribosomal RNA (SSU rRNA).** The nuclear SSU rRNA genes are known to be highly conserved regions suitable not only for phylogenetic studies but also for the molecular diagnosis of human malaria parasites (3, 5, 6, 11, 12, 13). The copy numbers of this gene vary from 4-8. Two and three stage-specific types (A-, S- and O) have been described in malaria parasites. On the basis of partial sequences of the SSU rRNA *P. o.*
*curtisi* and *P. o. wallikeri* vary in at least 26 loci (6). Furthermore *P. o. wallikeri* showed intraspecific variability. Although several intra-species-specific variations have been described this gene is the one most commonly used for PCR analysis. Primers and probes have to be designed accordingly to exclude wrong negative results (see below).

**Cytochrome b (cytb).** A strict sequence conservation of this mitochondrial gene has been observed in *P. falciparum* and *P. vivax*. However divergences between *P. o. curtisi* and *P. o. wallikeri* have been described by Win et al. (2004) (5). They reported a divergence of 12 bps in a 1035 bp partial sequence. These results have been confirmed by several studies of which one analyzed and compared 357 bp partial cytb sequences of *P. ovale* clearly discriminating two mitochondrial cytb genotypes (3, 11, 14, 15).

**Merozoite surface protein-1 (MSP-1):** A low level of sequence diversity between *PocMSP-1* and *PowMSP-1* has been reported by Putapornpit et al. (2013) (15). Three imperfect repeated segments in the former and one in the latter have been observed. Differences between *P.o. curtisi* and *P. o. wallikeri* have been found in the interspecies variable domains of MSP-1. The complete MSP-1 genes vary in size: 5,181 bp for *P. o. curtisi* and 5,016-5,043 bp for *P. o. wallikeri*. Furthermore intraspecies-specific variations within the PoMSP-1 sequences were observed. The split of *PocMSP-1* and *PowMSP-1* seems to be relatively recent compared to other human malaria species (15).

Dimorphisms have also been observed in genes encoding *cytochrome c oxidase 1 (cox1)*; at least 12 loci; *lactate dehydrogenase (ldh)*, ookinete surface antigens, *P. ovale reticulate binding protein (porbp2)*, glyceraldehyde-3-phosphatase (pog3p), dihydrofolate reductase-thymidylate synthase (podhfr-ts), cystein proteinase (pocysp) and *P. ovale tryptophan rich antigen (potra)* (3, 5, 6, 11, 12, 13, 14, 16, 17).
Specific diagnostic tools for malaria diagnostics

In the past decades numerous protocols for PCR diagnosis of malaria have been published. Several of these cover the predominant species *P. falciparum* and *P. vivax* only, others also include *P. malariae* and *P. ovale*. However, only a few protocols address *P. knowlesi* and even less discriminate between *P. o. wallikeri* and *P. o. curtisi*. It also remains unclear in how far *P. ovale* primers bind both ovale species and are specific for the whole ovale-complex. However, within the last years the number of uploaded sequences at GenBank has increased (including those for rarer species) 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 diagnosis 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 reaction amplifying a product of about 1,200 bp in size (18). For species determination the 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.

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 primer rPLU6 they used the primer rPLU1. The Nest 1 product resulting from the use of the primers rPLU1 and rPLU5 has a size of 1,670 bp, and allowed for the introduction of a
genus-specific Nest 2 PCR with the 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 1-10 parasite genomes per sample by different authors.

Further studies revealed that the NP-1993 protocol had some limitations in the diagnosis
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 genus-specific
primer rPLU2 combined with rOVA1. NP-2002 involved the genus-specific Nest2
primers rPLU2 and rPLU3.

Until 2005 more than 14 different protocols were published for the diagnosis of *P. ovale*
and it became evident that *P. ovale* needs to be divided into the classic and the variant
type. 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 diagnosis of the variant type of *P. ovale* and rOVA1 and
rOVA2 (NP-1993 primers) for the determination of the classic form of *P. ovale* (21).

Several studies have shown that these primers can be used for the diagnosis and
separation of the newly recognized species *P. o. wallikeri* and *P. o. curtisi* – even in
mixed infections with both ovale species (6, 22). However, the NP-2002 ovale PCR
rOVA1/rPLU2 turned out to lack the sensitivity required for the detection of *P. o.
wallikeri*.

Recently two techniques have been reported for the simultaneous detection of both ovale
species. A new set of primers (rOVA1WC/rOVA2WC) was designed which binds both *P.
o. wallikeri and P. o. curtisi while not binding to other human malaria parasites (22). Moreover a multiplex PCR combining the NP-1993 primers (rOVA1/rOVA2) with the NP-2005 primers (rOVA1v/rOVA2v) gave adequate results for the diagnosis of P. ovale sp. (22). An updated NP-2013 protocol is provided in Tab. 1.

Other molecular techniques for the detection and/or discrimination of P. ovale sp.

As discussed earlier the non-protein coding SSU rRNA genes show inter- and intra-species-specific variations. Recently a highly sensitive and specific real-time PCR assay has been described allowing for the differentiation of P. o. curtisi and P. o. wallikeri (24, 25, 26).

A study using an 18S SSU rRNA-hexaplex-PCR suggests that the primers cover both species (27). In addition the group around Bauffe et al. described a real-time TaqMan PCR (TqPCR) using the primers PoF + PoR and two probes (pPOC and pPOW). 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 the discrimination of both ovale species (16). In Nest 1 PoTRAfwd3-PoTRArev3 primers bind a 787 bp fragment. Using the intern primers PoTRAfwd5-PoTRArev5 P. o. wallikeri and P. o. curtisi (245-355 bp) can be differentiated (16). Although this technique has shown to be specific for P. ovale sp. it was observed that the amplified fragment size also varies as a result of differences in the number of repeat units limiting species discrimination: P. o. wallikeri (245, 299 and 335 bp) and P. o. curtisi (299, 317 and 353 bp) (28). Recently a specific semi-nested PCR method for the discrimination of both ovale species was presented (Nest1: PoTRA-F/PoTRArev3). In the Nest 2 reaction the
PoTRA-F primer is combined with primers specific for *P. o. wallikeri* (PowTRA-R; 389, 443 and 479 bp) and *P. o. curtisi* (PocTRA-R; 443, 461 and 497 bp) (28). This technique not only allows for the discrimination of both ovale species but also the phylogenetic analysis of these parasites based on intra-species-specific variations. Furthermore the group around Tanomsing suggested that the number of *potra* size variations might exceed those evaluated (28).

Within the *porbp2* gene the primers PoRBP2fwd-PoRBP2rev (Nest 1) and PoRBP2TMfwd-PoRBP2TMrev (Nest 2) flanking six single-nucleotide polymorphisms can be used for the specification of the two ovale parasite species. In 5 positions A and T residues in *P. o. curtisi* are replaced by C and G residues in *P. o. wallikeri*. Within this rtpcr melt curve the peak melting temperature of the *P. o. curtisi* amplicon (73°C) was consistently one degree lower than *P. o. wallikeri* (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 that:
- PCR-based techniques addressing all six human malaria species are recommended for epidemiological studies in South and South-East Asia and for the malaria diagnosis in returning travellers from these regions whenever available.

- In other regions where *P. knowlesi* is not endemic (limited by its intermediate hosts’ distribution) epidemiological studies and returning travellers are recommended to be screened for the other 5 malaria species.

- If two separate PCRs for the diagnosis of *P. o. curtisi* and *P. o. wallikeri* are not available (e.g. due to cost and time constrains) *P. ovale* sp. should be diagnosed using molecular techniques validated to detect both species. Positive ovale samples should further be discriminated to species level whenever possible.

- The role the two *P. ovale* species are playing for the clinical presentation needs to be better defined as to better understand the role of species differentiation for malaria treatment.
References:


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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<td>rPLU1</td>
<td>TCA AAG ATT AAG CCA TGC AAG TGA</td>
<td>Genus – Nest1*</td>
<td>~1670</td>
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<td>rPLU3</td>
<td>TTT TTA TAA GGA TAA CTA CGG AAA AGC</td>
<td>Genus – Nest 2b</td>
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<td>rFAL1</td>
<td>TTA AAC TGG TTT GGG AAA ACC AAA TAT ATT</td>
<td>P. falciparum a</td>
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<td>Snounou and Singh (2002)</td>
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<td>rMAL1</td>
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<td>rVIV1</td>
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<td>rOVA1WC</td>
<td>TGT AGT ATT CAA ACG CAG T</td>
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<td>Fuehrer et al. (2012)</td>
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<td>rOVA2WC</td>
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<td>rOVA1</td>
<td>ATC TCT TTT GCT ATT TTT TAG TAT TGG AGA</td>
<td>P. o. curtisi a,d</td>
<td>787-789</td>
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* Cycling conditions: Initial denaturation: 95°C for 5 min; (94°C for 1 min; 58°C for 1 min; 72°C for 2 min) 25 cycles for Nest 1

and 30 cycles for Nest 2; Final Extension: 72°C for 5 min (20, 22)
b Cycling conditions: Initial denaturation: 95°C for 5 min; (94°C for 1 min; 64°C for 1 min; 72°C for 2 min) 30 cycles; Final Extension: 72°C for 5 min (20)

c Cycling conditions: Initial denaturation: 95°C for 5 min; (94°C for 1 min; 50°C for 1 min; 72°C for 1 min) 35 cycles; Final Extension: 72°C for 5 min (23)

d Diagnosis of *P. o. wallikeri* and *P. o. curtisi* after positive results at *P. ovale* sp. (rOVA1WC/rOVA2WC) or as multiplex PCR for the diagnosis of *P. ovale* sp. (22)
Hans-Peter Fuehrer MSc. PhD fills a PostDoc 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 PhD 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 South Asia. Furthermore he was the head of the laboratory at the Malaria Research Initiative Bandarban in rural Bangladesh. His main interests are Plasmodium ovale, neglected pathogens, rodent-borne and vector-borne diseases.