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REVIEW ARTICLE

Two sympatric types of *Plasmodium ovale* and discrimination by molecular methods

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Abstract *Plasmodium ovale* is widely distributed in tropical countries, whereas it has not been reported in the Americas. It is not a problem globally because it is rarely detected by microscopy owing to low parasite density, which is a feature of clinical ovale malaria. *P.o. curtisi* and *P.o. wallikeri* are widespread in both Africa and Asia, and were known to be sympatric in many African countries and in southeast Asian countries. Small subunit ribosomal RNA (*SSUrRNA*) gene, cytochrome *b* (*cytb*) gene, and merozoite surface protein-1 (*msp-1*) gene were initially studied for molecular discrimination of *P.o. curtisi* and *P.o. wallikeri* using polymerase chain reaction (PCR) and DNA sequencing. DNA sequences of other genes from *P. ovale* in Southeast Asia and the southwestern Pacific regions were also targeted to differentiate the two sympatric types. In terms of clinical manifestations, *P.o. wallikeri* tended to produce higher parasitemia levels and more severe symptoms. To date, there have been a few studies that used the quantitative PCR method for discrimination of the two distinct *P. ovale* types. Conventional PCR with consequent DNA sequencing is the common method used to differentiate these two types. It is necessary to identify these two types because relapse periodicity, drug susceptibility, and mosquito species preference need to be studied to reduce ovale malaria. In this article, an easier method of molecular-level discrimination of *P.o. curtisi* and *P.o. wallikeri* is proposed.

Introduction

Malaria diagnosis is largely based on microscopy. After the widespread availability of polymerase chain reaction (PCR), based on PCR results, the number of species of human malaria parasites has increased from four to six species including *Plasmodium knowlesi* and the newly recognized species *Plasmodium ovale curtisi* (former classic type) and *Plasmodium ovale wallikeri* (former variant type).[1—3]

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P. ovale was the last human malaria parasite to be described. Stephens[4] observed some erythrocytes that were oval and have fimbriated edges in the blood of an East African malaria patient. In 1922, he named the parasite *P. ovale* because of the oval shape of some of the infected erythrocytes and wrote a full description of the forms in the blood in his publication.[4]

P. ovale is widely distributed in tropical countries, whereas it has not been reported in the Americas. It is not a problem globally because it is rarely detected by microscopy owing to low parasite density, which is a feature of clinical ovale malaria.[3] In addition, because of its morphological resemblance and its tertian periodicity, it can easily be mistaken for *Plasmodium vivax* in microscopic analysis.[1]

The *SSUrRNA* gene of *P. ovale* can be amplified by PCR to differentiate it from other malaria parasite species.[5] However, some of the *P. ovale* species identified microscopically cannot be detected by the PCR method because of the sequence variation of the target gene. Besides *SSUrRNA* gene, the sequence variations of other genes—specifically, the mitochondrial locus cytochrome *b* and the genes encoding cysteine protease and the ookinete surface antigens of *P. ovale*—facilitate the classification of this parasite into classic and variant types.[6] These two types of *P. ovale* have been found to coexist in Thailand, Lao PDR (People's Democratic Republic), Myanmar, and Vietnam.[7–10]

By analyzing the 3′ half of the *P. ovale SSUrRNA* genes, Li et al[11] suggested that *P. ovale* might be separated into two types or subspecies, Nigerian I/CDC and LS (London School of Hygiene and Tropical Medicine). Later, the presence of LS-type or variant-type *P. ovale* was confirmed in Vietnam and Africa.[12,13] All variant type isolates shared the same mutations at block 9 in the *SSUrRNA* gene. Nigerian I/CDC was the classic type and sequence analyses of the *SSUrRNA* gene, gene encoding ookinete surface antigen,[14] and cysteine protease gene all confirmed the occurrence of two different sequences of parasites grouped as variant type and classic type.[6]

Significant findings of classic and variant types in *P. ovale*

Four significant findings of the two variants of *P. ovale* have been found. Both the classic and variant types of parasites occurred in sympatry, and these two variants exist in the same endemic region. Microscopically, morphologies were not different between these two types. Both types were present in Asia and Africa. Higher parasitemia levels and higher proportions of single-species infection were common with the variant type in contrast to the classic *P. ovale* infections acquired in the same region.[6]

Two sympatric types—*P.o. curtisi* and *P.o. wallikeri*

Sutherland et al[3] concluded that these *P. ovale* forms are two distinct nonrecombining globally sympatric species. The investigators of that study proposed to name the classic type as *P.o. curtisi* and the variant type as *P.o. wallikeri*, in honor of Christopher F. Curtis (1939–2008) and David Walliker (1940–2007), respectively. Their study showed the following lines of evidence in support of the idea that the two *P. ovale* forms are distinct species. *P.o. curtisi* and *P.o. wallikeri* are widespread in both Africa and Asia and were known to be sympatric in many African countries and in southeast Asian countries. Twelve single-nucleotide polymorphisms (SNPs) are found in the *cytb* gene, which presents as a complete dimorphism between the classic form and the variant *P. ovale*.[3] Within-species polymorphism for a gene that is highly conserved among members of the genus *Plasmodium* pointed out the possibility of two distinct species. *In vitro* culture is not currently available for *P. ovale* parasites. Although experimental chimpanzee models for *P.o. curtisi* were established, there was no animal model for *P.o. wallikeri*. These lines of evidence favor the conclusion that the two *P. ovale* types are actually two distinct species.[3] Su[15] made a comment on the proposal by Sutherland et al[3] that it is necessary to estimate the transmission rates, frequencies of natural recombination, and genetic distances of the parasites, using more parasite samples.[15] The report by Su[15] opened up a new field of investigation. However, confirmation by molecular methods for the two sympatric *P. ovale* types is the important part of this issue. As it seems to be still too early to conclude that *P.o. curtisi* and *P.o. wallikeri* are two different species at this moment, it is necessary to wait and consider the factors mentioned by Su.[15]

Geographical distribution of *P.o. curtisi* and *P.o. wallikeri*

Until recently, there was little interest on the prevalence and geographic distribution of *P. ovale*. Win et al[16] showed that *P. ovale* was a widespread human pathogen in Southeast Asia. There have been many reports on the presence of *P. ovale* throughout the world.[12,17–25]

Regarding two distinct types of *P. ovale*, Win et al[6] studied the molecular differentiation of the variant and classic types of *P. ovale*. Patients with single infections were selected for molecular analysis. The isolates were obtained during their field surveys in Myanmar and Indonesia including variant isolates, ST243 (Rakhine State) and MC53 (Tanintharyi Division), both from Myanmar, and M474 (Flores Island, eastern Indonesia) and the three classic isolates of M3 (Shan State), M4 (Bago Division), and T134 (Mon State) from Myanmar.[6] In this study, the terminology of classic and variant types was used instead of *P.o. curtisi* and *P.o. wallikeri* because it was published before the two types were systematically named.

Although there was high prevalence of malaria in Bangladesh and other southern Asian countries, there remains shortage of data about the less common human malaria parasite, *P. ovale*. In the Chittagong Hill Tracts of Bangladesh, *P.o. curtisi* and *P.o. wallikeri* occur sympatrically, and patients have presented with mild symptoms or asymptomatic, characteristic of ovale malaria. The authors proposed that their study was the first evidence of sympatric *P.o. curtisi* and *P.o. wallikeri* in southern Asia within a relatively confined study area.[26]

From the diverse endemic areas of Thailand, blood samples with single infection of *P. ovale* were collected from 10 symptomatic malaria patients, and molecular analysis was undertaken to discriminate *P.o. curtisi* and *P.o. wallikeri*. The results revealed the coexistence of *P.o. curtisi* and *P.o. walliker* equally.[27]

The study in African countries observed that both *P.o. curtisi* and *P.o. wallikeri* were sympatric in Congo-Brazzaville, districts of Uganda, and Bioko Island, Equatorial Guinea. In Uganda and Equatorial Guinea, infections with *P. ovale* spp. are more common than expected, and both types contributed to ovale malaria in six sites.[28]

A 2015 report indicated that the first evidence of sympatric distribution of *P.o. curtisi and P.o. wallikeri* has been noted in India. The report included two cases of mono infection of *P. ovale*, and a fatal case of cerebral malaria due to mixed infection with *P. vivax*, *P. falciparum* and *P. ovale*.[29] The mixed infection was confirmed by PCR method described by Snounou et al[30] using *P. vivax*, *P. falciparum*, and *P. ovale* specific primer sets from the *SSUrRNA* gene of each species. The two cases of mono infection were caused by *P.o. wallikeri*, and the fatal case with mixed infection was caused by *P.o. curtisi*. The presence of *P.o. curtisi* and *P.o. wallikeri* was confirmed by DNA sequence analysis.[29]

Molecular discrimination of *P.o. curtisi* and *P.o. wallikeri*

Fuehrer et al[1] described that the three genes—*SSUrRNA* gene, *pocytb* gene, and *pomsp*-1 gene—have been used for molecular discrimination of *P.o. curtisi* and *P.o. wallikeri* However, their study indicated that dimorphisms have also been observed in *cox1*, *porbp2*, *podhfr-ts*, *pog3p*, *pocysp*, *potra*, *ldh*, and *gene* encoding ookinete surface antigens.[1]

The six major studies for genetic level discrimination between *P.o. curtisi* and *P.o. wallikeri* were the studies by Win et al,[6] Sutherland et al,[3] Oguike et al,[28] Fuehrer et al,[26] Putaporntip et al,[27] and Tanomsing et al.[31]

Win et al[6] proved that *P. ovale* had classical and variant types by studying the DNA sequence of the *SSUrRNA* gene. The two types of *SSUrRNA* genes from Nigerian I/CDC and LS strains could be sequenced, and these two types suggested representation of two variants. The sequence data of the *SSUrRNA gene*, gene encoding cysteine protease and ookinete surface protein, and *cytb* genes of *P. ovale* isolates from Myanmar and Indonesia were studied. The sequences of these genes were compared with available sequence data from Gene Bank. The information obtained supported the presence of *P. ovale* into at least two types. This study started to provide information on the presence of two distinct types of *P. ovale*.[6]

The more comprehensive study by Sutherland et al[3] revealed that there are two nonrecombining sympatric forms of *P. ovale*, and these two forms occur globally. However, the variation of the *SSUrRNA* gene between *P.o. curtisi* and *P.o. wallikeri* in their study is different from that in the study of Win et al, whereas the *pocytb* gene variations in the two studies were identical. The gene encoding cysteine protease and ookinete surface protein were not included in their study. A remarkable study was undertaken in which six genetic loci were investigated by DNA sequence analysis. *SSUrRNA* gene, *pocytb*, *pog3p*, *porbp2*, *potra*, and *podhfr-ts* genes were included in 51 isolates from two research institutions. In their study, there were 25-bp differences in nucleotide sequences between two sympatric types in the case of the *SSUrRNA* gene.[3] The study by Fuehrer et al[26] indicated 26-bp differences, and that there were only two inconsistencies in the two studies. In the study by Sutherland et al,[3] *P.o. curtisi* has AAT at the nucleotide position (NP) 282 to 284 and in the study of Fuehrer et al, TAT in the same position was noted in that type,[26] whereas *P.o. wallikeri* has no nucleotide at these three positions. Another discrepancy is that Sutherland et al[3] showed no nucleotide difference at NP 918, whereas Fuerher et al[26] showed a change from C to T at that NP between *P.o. curtisi* and *P.o. wallikeri* (Table 1).

Oguike et al[28] used *potra*, *porbp2*, and *pog3p* genes for discrimination of *P.o. curtisi* and *P.o. wallikeri* in their study. They have undertaken nested PCR with Potra 5 amplification followed by Potra 3 amplification. DNA sequence analysis showed four different sequence types—*P.o. curtisi*, *P.o. curtisi* + 6, *P.o. wallikeri* 1, and *P.o. wallikeri* 2. The sizes of the PCR products were 317 bp, 335 bp, 245 bp, and 245 bp, respectively. There were nucleotide sequence differences between these haplotypes even when the PCR product sizes were similar. For *porbp2* sequence, quantitative PCR (qPCR) was undertaken to differentiate *P.o. wallikeri* and *P.o. curtisi*, and final confirmation was done with *pog3p* gene sequence analysis.[28]

Putaporntip et al[27] studied two genes (*pomsp-1* and *pocytb*) in their study on *P. ovale* isolates in Thailand. All five *pomsp-1* sequences of *P.o. curtisi* contained 5181 bp, whereas two types of length of open reading frames (ORF) were observed in *P.o. wallikeri*. These two ORFs were 5016 bp and 5043 bp in size. In total, seven distinct *pomsp-1* nucleotide sequences were identified among 10 Thai isolates. The *pomsp-1* sequences of *P.o. wallikeri* were structurally differed from those of *P.o. curtisi* in terms of the number of repeat regions and several nucleotide differences. Most of the amino acid differences between PcMSP-1 and PwMSP-1 sequences occurred outside interspecies conserved domains of this protein. The nucleotide diversity at synonymous amino acid sites exceeds that of nonsynonymous sites in nonrepeat regions.[27] Because of the seven distinct sequence patterns, the *pomsp-1* gene cannot be applied to discriminate the two types.

The *potra*-based protocol was presented by Tanomsing et al,[31] and their study indicated that the genotypes of each type could be classified according to the amplified fragment size. Three distinct allelic *potra* variants were found, and the predicted amino acid sequences were aligned. Subsequently, two potentially new size variants were identified from a *P.o. curtisi* sample recently collected from a patient who had acquired the infection in Africa. If more samples are analyzed, the number of *potra* size variants may increase. The study may be valuable for identifying genotypes among *P.o. curtisi* and *P.o. wallikeri*.[31] However, it was observed that for the discrimination of *P.o. wallikeri* and *P.o. curtisi*, *SSUrRNA* gene and *pocytb* gene were more practicable and reproducible than *potra* gene.

Table 1 Difference between DNA sequences in *SSU rRNA* gene of *Plasmodium ovale curtisi* and *Plasmodium ovale wallikeri* from two studies

Nucleotide position	2 0 1	2 0 2	2 0 5	2 0 6	2 0 7	2 3 4	2 3 6	2 4 4	2 8 7	2 8 5	6 7 2	6 8 4	6 8 7	6 9 4	7 1 1	7 3 5	7 7 9	7 8 2	7 8 3	7 8 4	7 8 7	7 8 9	7 9 3	9 0 1	9 1 8
Nucleotide in *P.o. curtisi*	A	T	C	C	G	A	G	T	G	G	A	C	A	A	T	G	T	G	T	A	T	T	A	T	G	C
Nucleotide in *P.o. wallikeri* (Fuehrer et al, 2012)	T	G	T	T	A	G	A	C	T	A	G	G	T	G	C	C	C	A	—	—	—	C	T	C	A	T
Nucleotide in *P.o. curtisi*	A	T	C	C	G	A	G	T	G	G	A	C	A	A	T	G	T	G	A	A	T	T	A	T	G	C
Nucleotide in *P.o. wallikeri* (Sutherland et al, 2010)	T	G	T	T	A	G	A	C	T	A	G	G	T	G	C	C	C	A	—	—	—	C	T	C	A	C

The yellow highlighted nucleotides are different in two studies. Fuehrer et al[26] have shown 26 single-nucleotide polymorphisms (SNPs) between two species, whereas Sutherland et al[3] indicated only 25 SNPs between two species showing no change at NP 918.

Incubation period of ovale malaria

Imported *P. ovale* presentations start months after travelers have come back from their foreign trips. The average time between the return to their countries and clinical presentation is 82 days for *P. ovale*. For *P. falciparum*, it is 17.5 days. Other studies varied in incubation period with *P. ovale* infections in travelers and from 2 to 53 months after arrival. Late occurrence of *P. ovale* attacks may be attributable to the existence of latent hypnozoite stages in the liver, and diagnosis is difficult because of low parasitemia and mild symptoms in the endemic zone where the patient has stayed for an extended period.[32] Although incubation periods for *P.o. curtisi* and *P.o. wallikeri* were not compared in previous studies, Rojo-Marcos et al[33] have shown the interquartile range (IQR) of time from arrival to onset of symptoms (in days) of imported cases of two species to Spain. For *P.o. curtisi* it was 94.5 days, whereas for *P.o. wallikeri* it was 9.5 days.[33]

Difference of clinical findings in *P.o. curtisi* and *P.o. wallikeri* infections

In Spain, 37 imported infection cases were reported, of which *P.o. curtisi* infections were 21 and *P.o. wallikeri* infections were 14, which were confirmed by molecular methods. These cases were studied for clinical manifestations. Severe thrombocytopenia was common in *P.o. wallikeri* infection in contrast to *P.o. curtisi* infection. Hemolysis was more remarkable in *P.o. wallikeri* infection. *P.o. wallikeri* infection had a shorter incubation period, with 10 times shorter as mentioned above. Hypoalbuminemia and higher maximum temperature were the characteristics of *P.o. wallikeri* infection. Although further studies were necessary to confirm the result, these clinical findings were consistent with the observation of Win et al[6], who noted that parasites with variant-type sequences tended to produce higher parasitemia levels.[33]

One of the severe complications of malaria is acute respiratory distress syndrome (ARDS). This potentially grave complication has also been reported in *P. falciparum* infection and has been intensively studied. This complication is reported in malaria caused by *P. vivax*, *Plasmodium malariae*, *P. knowlesi*, and *P. ovale*. Because of the much lower morbidity and its limited geographical distribution, the complications of *P. ovale* have been overshadowed by other human malaria parasites such as *P. falciparum* and *P. vivax* in the field of medicine. Two cases of *P. ovale* infection acquired from the same location ended with different outcomes, ARDS complication and acute renal failure.[34]

Reasons for sympatry between two types of *P. ovale*

Sutherland et al[3] proposed the three mechanisms keeping *P.o. curtisi* and *P.o. wallikeri* apart. First, regional, ecological, or seasonal differences in their distribution are the factors maintaining a physical barrier between the two types. Second, the two types have mutually exclusive mosquito specificity or human host red blood cell specificity. A third mechanism could be mating incompatibility between the gametocytes of *P.o. curtisi* and *P.o. wallikeri* or the production of noninfective sporozoites after cross-fertilization events.[3]

Oguike et al[28] further highlights and supports the information that there was sympatry between the proposed species, *P.o. curtisi* and *P.o. wallikeri*, by Sutherland et al. The use of conventional and qPCR indicated that these methods could discriminate between the two parasite types from field samples. These two types of *P. ovale* contain their distinct genetic identity through unknown biological mechanisms. The multigenic dimorphism of these two parasites across a broad geographical range including the Republic of Congo, Equatorial Guinea, and three regions of Uganda in this study adds to the possibility of *P.o. curtisi* and *P.o. wallikeri* as separate species.[28]

The population prevalence of *P. ovale* spp. was observed to be between 1% and 6%, in Uganda and Equatorial Guinea. The prevalence increased to between 6% and 8% of malaria infections when it was considered alone or in combination with other *Plasmodium* spp. There were cryptic ovale infections, in the form of both latent liver hypnozoites and blood-stage infection below the level of PCR detection. If those cases that could not be diagnosed were included, the actual prevalence of *P. ovale* spp. infection in the study areas is higher than was observed. The contribution of these two types of *P. ovale* to human malaria infection has therefore been grossly overlooked.[28]

It is theoretically possible that blood group polymorphisms in the host restrict the two parasites to separate human population groups. There may be potential

Table 2 Recommendation: proposed primers, restriction enzymes, and restriction sites for PCR-RFLP of *SSU rRNA* gene and *pocytb* genes to discriminate *Plasmodium ovale curtisi* and *Plasmodium ovale wallikeri*

Gene	Primers	PCR product	Restriction enzymes	Restriction sites
SSU rRNA	For 5′-TGTACTTGTTAAGCCTTTAAG-3′	717 bp (*P.o. curtisi*)	*Eco*RI	GˆAATTC
	Rev 5′-AGCCAAAATAAGAAAAAGGAA-3′	714 bp (*P.o. wallikeri*)	689 bp (*P.o. curtisi*)	
	(this review)		714 bp (*P.o. wallikeri*)	
pocytb	For 5′-CTTACATTTACATGGTAGAC-3′	357 bp	*Nde*I	CÂTATG
	Rev 5′-GCCATTTTGAATTGTATAATAG-3′		357 bp (*P.o. curtisi*)	
	(Putaporntip, et al.[27])		183 bp, 174 bp (*P.o. wallikeri*)	

PCR-RFLP = polymerase chain reaction-restriction fragment length polymorphism.

difference between *P.o. curtisi* and *P.o. wallikeri* in host erythrocyte preference. The two parasite types may differ in recognition crucial protein molecules for the mating process for the explanation of sympatry between the two types.[28] The example is the ookinete proteins as shown by Tachibana et al.[14]

These assumptions have not yet been supported by strong experimental data. Therefore, on the information of two sympatric types of *P. ovale*, it is important to focus on important biological, epidemiological, or clinical differences between the two species such as relapse periodicity,[35,36] drug susceptibility, and mosquito species preference. Proper understanding of these features can ensure that malaria elimination strategies are able to reduce or stop the transmission of *P.o. curtisi* and *P.o. wallikeri*.[28]

Conclusions and further recommendations

Although there were multigenic diversities between *P.o. curtisi* and *P.o. wallikeri*, the diversity between these genes were not remarkable, and the SNPs were not accumulated in the continuous nucleotide sequences. It has been difficult to design the PCR primers for the conventional method of PCR. Although qPCR can easily distinguish between two distinct species of *P. ovale*, this method is relatively sophisticated and expensive that there have been only a few research studies that used this method to discriminate between *P.o. curtisi* and *P.o. wallikeri* up to now. Moreover, the SNP sites cannot be known via qPCR alone, and it can give the information that SNPs are present. For the conventional PCR method, single-step PCR or nested PCR (including seminested PCR) together with consequent DNA sequencing to investigate SNPs are a common and easier way to discriminate between these two sympatric types especially for research purposes or these can be used together with qPCR.

To discriminate *P.o. curtisi* and *P.o. wallikeri*, a recommendation is proposed in this review to use the PCR-restriction fragment length polymorphism (RFLP) method for the *SSUrRNA* gene, and confirmation is done using the PCR-RFLP method for the *pocytb* gene. For the *SSU rRNA* gene, from the NCBI (National Center for Biotechnology Information) downloaded sequence for *P.o. curtisi* and *P.o. wallikeri*,[26] the two primers areas were selected (Table 2). The PCR product size will be 717 bp in *P.o. curtisi* and 714 bp in *P.o. wallikeri* (because there is a 3-bp TTA deletion in *P.o. wallikeri*). After restriction enzyme digestion in the case of *P.o. curtisi*, 689 bp will come out, which will be lower than the 700-bp marker); in the case of *P.o. wallikeri*, 714 bp will appear above the 700-bp marker of the 100-bp ladder. For the sensitivity, nested PCR can be used with rPLU1 and rPLU5 genus-specific primers.[5] In this case, there is SNP in *P.o. curtisi* and *P.o. wallikeri*, nucleotide G to A at NP 901. This DNA sequence SNP G to A can be observed in two studies,[3,26] and this will be conserved between *P.o. curtisi* and *P.o. wallikeri*.

Regarding confirmation, PCR-RFLP analysis of the *pocytb* gene will be used. SNP at NP 756 is changed from nucleotide C in *P.o. curtisi* to nucleotide T in *P.o. wallikeri*, resulting in the appearance of a restriction site (*Nde*I) with consequently different RFLP pattern (Table 2). This confirmation will be also conserved because Win et al[6] and Sutherland et al[3] have shown the presence of this SNP out of 12 SNPs between *P.o. curtisi* and *P.o. wallikeri* in their studies.

We would like to conclude that by reviewing the molecular level data of *P.o. curtisi* and *P.o. wallikeri*, the easier and reproducible method to discriminate *P.o. curtisi* and *P.o. wallikeri* can be proposed.

Conflicts of interest

The authors declare no conflicts of interest.

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