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Sympatric Occurrence of *Plasmodium ovale curtisi* and *Plasmodium ovale wallikeri* in Archived Samples in Saharevo in the Eastern Foothill Area of Madagascar

Running title: *P. ovale* Subspecies in Saharevo, Madagascar

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Abstract: Introduction: Four *Plasmodium* species that infect humans coexist on the island of Madagascar. *P. falciparum* and *P. vivax* are predominant while *P. malariae* and *P. ovale* remain rare. Recently, two subspecies of *P. ovale* were discovered through molecular analysis: *P. o curtisi* and *P. o. wallikeri.* Thus, we undertook a study that aims to assess *P. o. curtisi* and *P. o. wallikeri* infection in archived blood samples using nested PCR. **Methods:** Whole blood samples collected from patients with suspected malaria in Saharevo (eastern foothill area) from 1996 to 2005 were analyzed. These samples were stored at −20 °C until use. Microscopy examination of these samples had already been performed previously. **Results:** Of the 557 examined samples, 438 malaria infections were confirmed using nested PCR [78.6%, 95%CI: 74.9–81.9%]. Among these malaria cases, twelve patients presented singularly with *P. ovale* [2.7%, 95%CI: 1.5–4.9%] including six *P. o. curtisi*, five *P. o. wallikeri*, and one co-infection of *P. o. curtisi* + *P. o. wallikeri*. **Conclusion:** This study provides the first molecular evidence of the sympatric occurrence of *P. o. curtisi* and *P. o. wallikeri* in Madagascar. Sequencing provided definitive genotyping, confirming the nested PCR findings. Therefore, further studies assessing the prevalence of the rare species such as *P. ovale* are needed to better understand the distribution of this species to guide malaria control strategies.

Keywords: *P. ovale curtisi*; *P. ovale wallikeri*; Nested-PCR; sympatric infection; Madagascar

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1. Introduction

Malaria remains a major public health issue on the island of Madagascar, where four out of the five *Plasmodium* species that infect humans coexist. *P. falciparum* and *P. vivax* are predominant while *P. malariae* and *P. ovale* remain rare [1]. Relatively little attention has been paid to *P. ovale* malaria infection, which shares the particularity with *P. vivax* to form latent-stage parasites in the liver, referred to as hypnozoïtes. This phenomenon is responsible for the later resurgence of parasites, with new malaria episodes occurring without recent events of exposure [2,3]. *P. ovale* has been reported in all subtropical continents, but is most commonly found in tropical Africa [4]. In Madagascar, *P. ovale* was already reported in 1947 following microscopy examination [5]; but in 1970 researchers still doubted the existence of this species [6]. Later, several studies based on microscopic diagnosis have shown that *P. ovale* is actually present in Madagascar [7–9]. Although *P. ovale* infections are generally considered benign, severe cases, including acute respiratory distress and splenic rupture, have been reported [10–13]. This highlights the clinical relevance of studying these rare malaria parasites and the potential burden they impose on patient outcomes and healthcare.

Recently, Sutherland et al. demonstrated using molecular analysis the existence of two subspecies of *P. ovale*: *P. o curtisi* and *P. o. wallikeri* [14]. Since no study had yet identified specifically the existence of *P. o. curtisi and P. o. wallikeri* in Madagascar, we undertook this study to determine whether these subspecies co-occur in archived isolates collected between 1996 and 2005 from the eastern foothill area, where *P. ovale* had previously been described using microscopy [8].

2. Methods

2.1. Study Site and Blood Sample Collection

Saharevo is located in the eastern foothills of Madagascar, 100 km east of Antananarivo (latitude 18°82' S, longitude 48°10′ E, altitude 900 m), where the village is fully set up at the boundary between the east coastal and the highlands areas (Figure 1). Also, thanks to its intermediate geographical location, Saharevo is characterized by a climate and biotope just at the interface between the more extreme faces observed in the highlands and on the eastern coast.

2.2. Nucleic Acid Isolation

DNA extraction from whole blood was performed using the QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. The incubation time with proteinase

K was 10 min at 56 °C and DNA was eluted twice from the column with 100 μL of PCR-grade H_20 to improve the yield of the extraction.

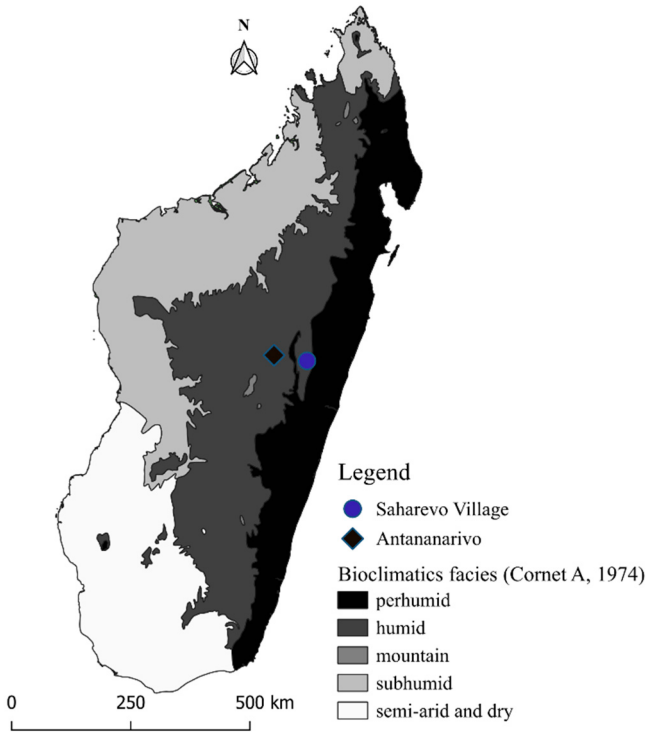

Figure 1: Geographical location of Saharevo village.

2.3. Parasite Detection Using Nested PCR

We used the methods described by Snounou et al. for detecting *P. falciparum*, *P. vivax* and *P. malariae* [15], and that of Fuehrer et al., slightly modified, for detecting *P. ovale* subspecies [16]. Briefly, regarding *P. ovale* detection and identification, molecular analysis was performed using nested PCR and small subunit rRNA (SSU rRNA) was the target gene. For the PCR1, three microliters of extracted DNA was added to 2.5 μL of buffer, 2.5 mM $MgCl_2$, 0.2 mM of dNTP, 0.4μM of primer pairs for PCR1 (RPLU1: 5′-TCAAAGATTAAGCCATGCAAGTGA-3′/RPLU5: 5′-CCTGTTGTTGCCTTAAACTTC-3′), and 0.25 μL of taq polymerase and made up with sterile water [15]. For the nested PCR, we used two microliters of amplicons of PCR1 instead of DNA and 0.5μM of each primer pair (ROVA1WC: 5′-TGTAGTATTCAAACGCAGT-3′/ROVA2WC: 5′-TATGTACTTGTTAAGCCTTT-3′) [16]. Amplification was done in the thermal cycler Techne TC-512 from Bibby scientific. For PCR1, the amplification conditions comprised initial denaturation (95 °C for 5 min), 35 cycles of denaturation (95 °C for 30 s), annealing (55 °C for 30 s) and extension (72 °C for 3 min), with final extension as the last step (72 °C for 10 min). The same conditions were applied for nested PCR, with differences only during the annealing step (59 °C for 30 s) and extension step (72 °C for 1 min). In all experiments, an uninfected blood sample was systematically included to control the specificity of amplification. The PCR amplification was verified using positive and negative controls. All samples giving positive infections for *P. ovale* sp were further analyzed to a species level using the primers ROV1: 5′-GGAAAAGGACACATTAATTGTATCCTAGTG-3′/ROV2: 5′-ATCTCTTTTGCTATTTTTTAGTAT TGGAGA-3′ specific for *P. o. curtisi* and ROVA1v: 5′-ATCTCCTTTACTTTTTGTACTGGAGA-3′/ROVA2v: 5′-GGAAAAGGACACTATAATGTATCCTAAT

A-3′ for *P. o. wallikeri* [15,16]. The PCR product identity was confirmed using gel electrophoresis (2% agarose and UV fluorescence) using gel scan BioRad with Quantity One software 4.6.2.

Five hundred fifty-seven whole blood samples were collected from villagers with suspected malaria from 1996 to 2005 in Saharevo in the eastern foothill area of Madagascar. These anonymous samples were stored at −20 °C at the parasitology unit at Institut Pasteur de Madagascar until use. Microscopy examination of all samples was performed in a previous study [8]. Out of 557 blood samples collected from symptomatic patients presenting with febrile illnesses, 306 (54.9%) were from male patients and 251 (45.1%) were from female patients. The age of patients was between 5 months and 85 years with a mean age of 35 years ± 21.

2.4. Sequencing Analysis

Since it was the first time that we conducted such typing at our laboratory, to confirm our finding, nine secondary PCR products of *P. ovale*-positive samples from 1996 to 2001 were sent for sequencing at Beckman Coulter (Genewiz, UK) and sequence analysis was performed using Seaview (version 4.32.0.0) and Chromas Lite (Version 2.01) software [17,18].

2.5. Data Analysis

Data from study participants were recorded and analyzed in Excel 2010. Additional analyses, such as proportion and confidence intervals, were performed in R software version 4.0.2.

2.6. Ethical Considerations

Saharevo is part of the national sentinel network for the surveillance of malaria according to the letter no. 265/MSANP/SG/DGS/PNLP. Authorizations to carry out malaria studies in Saharevo were delivered by the Ministry of Public Health, the Regional Direction of Public Health in Mangoro, the Health District in Moramanga, and by local administrative officials. Ethical clearance was obtained from the national ethical committee. Patients who were positive for malaria parasites received standard treatment according to national treatment guidelines. The blood specimens used in this study were anonymously identified.

3. Results

3.1. *Plasmodium* Species Detection Using Nested PCR and Microscopy

Out of the 557 samples examined using PCR, 438 malaria infections [78.6%, 95%CI: 74.9–81.9%] were confirmed. *P. falciparum, P. vivax*, *P. malariae* and *P. ovale* were detected. Among these malaria cases, *P. falciparum* was the predominant species [91.1%, CI95%: 87.9–93.5%] and was present singularly and in mixed infection with other species. Twelve patients harbored *P. ovale* (Table 1). The mean age of the 12 patients infected by *P. ovale* was 12.6 years (SD 9.6; range 2–29), compared to 14.9 years (SD 15.3; range 0.4–71) in the patients infected by other species. The difference was not statistically significant (Welch's *t*-test, t = −0.80, df = 12.6, $p = 0.44$).

Among 12 *P. ovale* infections, six cases were *P. o. curtisi*, five were *P. o. wallikeri* and one was co-infected with *P. o. curtisi* and *P. o. wallikeri* (Figure 2). Cases occurred across all age groups, with the mean age not differing significantly between *P. ovale curtisi* and *P. ovale wallikeri* cases (Welch's *t*-test, t = −0.80, df = 12.6, $p = 0.4375$). Of the 12 cases, four were female and eight were male, with no significant difference in sex distribution across *P. ovale curtisi, P. ovale wallikeri* and co-infection cases (Fisher's exact test, $p = 0.697$). *P. ovale curtisi* and *P. ovale wallikeri* cases were detected

throughout the study period, with no significant variation by month (Fisher's exact test, $p = 0.9134$) or by year (Fisher's exact test, $p = 1$), as illustrated in Figure 2.

Table 1: *Plasmodium* species detection using nested PCR.

PCR Results	Frequency n (%)
Positive	438 (78.6)
P. ovale	**3 (0.7)**
P. ovale + *P. falciparum*	**6 (1.4)**
P. ovale + *P. falciparum* + *P. vivax*	**2 (0.5**
P. ovale + *P. falciparum* + *P. malariae*	**1 (0.2)**
P. falciparum	336 (76.7)
P. falciparum + *P. vivax*	43 (9.8)
P. falciparum + *P. malariae*	10 (2.3)
P. falciparum + *P. vivax* + *P. malariae*	1 (0.2)
P. vivax	23 (5.3)
P. vivax + *P. malariae*	3 (0.7)
P. malariae	10 (2.3)
Negative	119 (21.4)
Total	**557**

Note: Percentages for species distribution were calculated based on PCR-positive samples (*n = 438*). Overall positivity rate was calculated based on the total number of samples analyzed (*n = 557*).

Of these 12 *P. ovale* infections detected using PCR, three of them were singular infections and nine were mixed infections with another *Plasmodium* species. Archival data allowed for the observation of microscopy results. Microscopy examination revealed that out of the 12 samples which contained *P. ovale* as mentioned above, 11 were reported as *P. falciparum* and one sample was undetermined (Table 2).

Table 2: *Plasmodium ovale* detection using nested PCR and microscopy in Saharevo.

	Microscopy		
***P. ovale* Detection by PCR**	**Undetermined**	***P. falciparum***	**Total**
P. ovale	0	3	3
P. ovale + *P. falciparum*	0	6	6
P. ovale + *P. falciparum* + *P. malariae*	1	0	1
P. ovale + *P. falciparum* + *P. vivax*	0	2	2
Total	1	11	12

Note: "Undetermined" refers to microscopy results in which species identification was not reported at the time of examination.

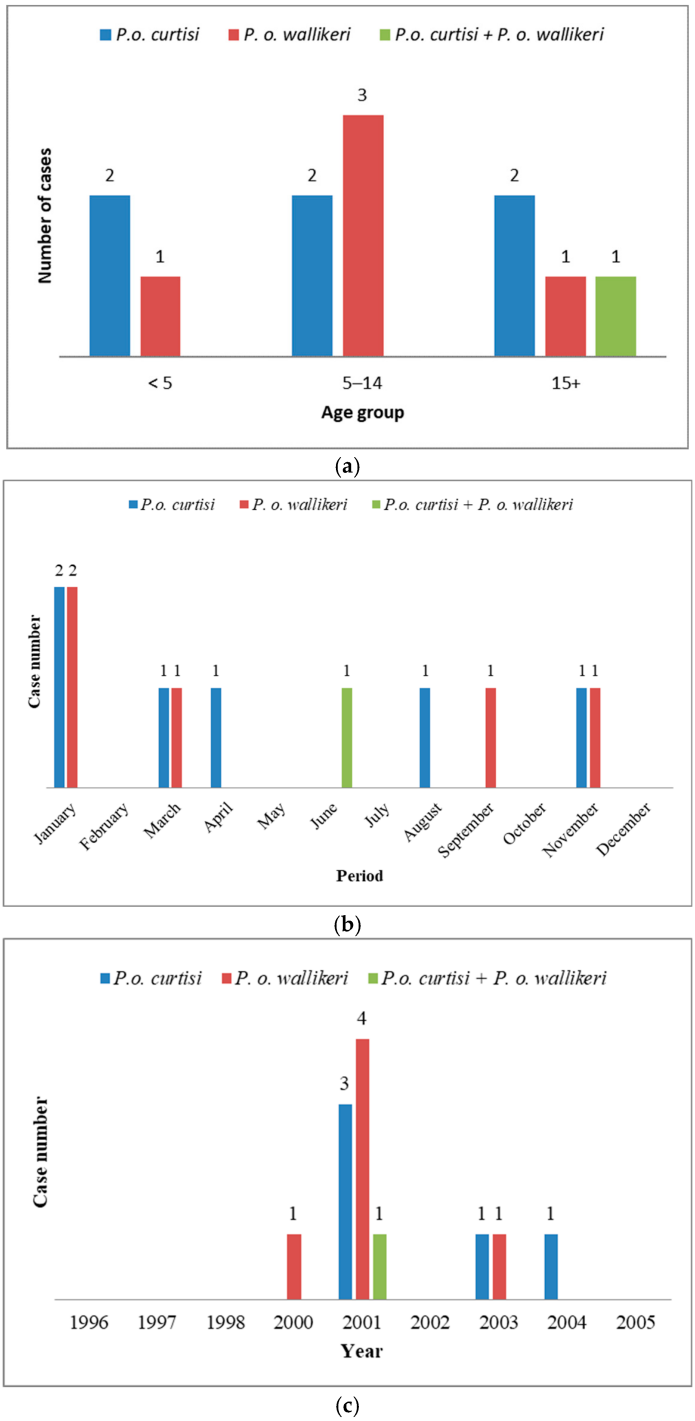

Figure 2: Reported *P. ovale* subspecies cases: (**a**): age groups of villagers infected by *P. o. curtisi* and *P. o. wallikeri*; (**b**): monthly distribution of detected *P. o. curtisi* and *P. o. wallikeri*; (**c**): annual distribution of detected *P. o. curtisi* and *P. o. wallikeri.*

3.2. Sequencing Results

The nine *P. ovale* subspecies PCR products sequenced confirmed the PCR detection results. All novel sequences were deposited and made accessible in Genbank with accession numbers PP330047-PP330048-PP330049-PP330050-PP330051-PP330052-P330053-PP330054-PP330055-PP495844 for those *P. ovale* subspecies.

4. Discussion

Our results demonstrated that *P. ovale* subspecies coexist in Saharevo. Furthermore, *P. o. curtisi and P. o. wallikeri* subspecies infected all age groups and were present throughout the year as shown in Figure 2a,b. Interestingly, one mixed infection of *P. o. curtisi* and *P. o. wallikeri* was detected. *P. o. curtisi and P. o. wallikeri* were found in 2000, 2001, 2003 and 2004, although there were likely more cases found in samples from 2001 (Figure 2c). From 1996 to 2005, only two (0.2%) *P. ovale* malaria (2/1,271 malaria cases) were identified in Saharevo according to microscopy [8]. Unfortunately, microscopic images were not available for these historical samples, and blood samples were probably missing for part of the study period. However, our results demonstrated that using nested PCR, more *P. ovale*-related infections were identified, illustrating the limitation of microscopy in detecting low-density *P. ovale*. The epidemiology of *P. ovale* remains poorly understood and there are no recent data on the distribution of this parasite in Madagascar. All old studies were based on microscopy data only. We noticed that nested PCR previously used in our laboratory to identify *P. ovale* could only detect *P. o. curtisi* and not *P. o. wallikeri*. To update the *P. ovale* subspecies mapping, we can now use the sensitive nested PCR assay with a documented limit of detection of six parasites/μL [19]. Although retrospective, these observations are consistent with the known transmission dynamics in Madagascar's eastern foothills. The long-term storage of samples (15–25 years at −20 °C) may have caused some DNA degradation; however, sequences were of high quality, and PCR products appeared clear on gels.

Also, our PCR results in this study indicate that *P. falciparum* was the predominant species in Saharevo from 1996 to 2005, followed by *P. vivax*, *P. malariae,* and *P. ovale*. At that time, chloroquine was used as a first-line treatment and long-lasting insecticidal nets (LLINs) use was not used yet in Madagascar.

Entomological investigations in Saharevo identified four malaria vector species: *Anopheles funestus*, *Anopheles mascarensis*, *Anopheles gambiae* and *Anopheles arabiensis*. Malaria transmission monitored from October 2003 to September 2004 yielded an annual entomological inoculation rate (EIR) of 2.78 infective bites per adult. *An. funestus* accounted for ~75% of transmission, occurring mainly between February and May (Randrianarivelojosia, personal communication). The sympatric circulation of *P. ovale curtisi* and *P. ovale wallikeri*, along with co-infections involving *P. falciparum*, can be interpreted in the local entomological context. Overlapping vector activity and repeated infective bites likely facilitate simultaneous exposure to multiple *Plasmodium* species and subspecies. In this entomological context, the sympatric circulation of *Plasmodium ovale curtisi* and *Plasmodium ovale wallikeri*, together with co-infections involving *Plasmodium falciparum*, is plausible. Overlapping vector activity and repeated infective bites likely increase the probability of simultaneous exposure to multiple *Plasmodium* species.

In 2023, we conducted an investigation during a malaria outbreak in Vangaindrano (southeastern Madagascar). Among 453 randomly selected villagers, 164 (36.2%) malaria infections were confirmed using PCR. *P. falciparum* was predominant, but 6.1% (10/164) of infections were due to *P. ovale* (six *P. ovale curtisi*, three *P. ovale wallikeri* and one *P. ovale curtisi* + *P. ovale wallikeri*). These findings were reported to the Ministry of Health in Madagascar. Together, these data indicate the presence of *P. ovale* in eastern Madagascar and suggest that a relative increase in its prevalence may reflect intensifying malaria transmission.

Several studies report the sympatry of *P. ovale* subspecies in Africa and Asia [20–24]. For example, *P. o. curtisi* seems predominant in India (5/7) [25], in Nigeria (33/57) and in Ghana (13/22) [26], whilst *P. o. wallikeri* is predominant in Ethiopia (7/9) and in Cameroun (7/9) [26,27]. In contrast, our studies in Saharevo and Vangaindrano showed comparable frequencies of *P. ovale curtisi* (12/22) and *P. ovale wallikeri* (8/22), with two mixed infections (*P. ovale curtisi* + *P. ovale wallikeri*), indicating no clear predominance of either subspecies.

The literature has reported severe *P. ovale* malaria cases resulting in severe respiratory symptoms [10,13] and spleen rupture [11,12]. These cases have not been investigated using molecular methods to differentiate *P. ovale curtisi* from *P. ovale wallikeri*. Given the limited knowledge of *P. ovale* subspecies in Madagascar, establishing collaborations with hospitals to genotype *P. ovale* in hospitalized patients would provide important epidemiological insights.

5. Concluding Remarks

This study provides the first molecular evidence of the sympatric occurrence of *P. ovale curtisi* and *P. ovale wallikeri* in Madagascar. On the other hand, this study also shows that *P. ovale* malaria is underreported. Therefore, globally, further studies assessing the prevalence of the non-*P. falciparum* species are needed to better understand the species distribution and to adapt malaria control strategies in Madagascar given that a hypnozoite-forming malaria parasite will require the use of amino-8-quinoline such as primaquine.

Author Contributions: D.N.A.L.R. and A.N.N.Z.R. performed laboratory work and data analysis. D.N.A.L.R. drafted the manuscript. A.A.-B. provided the positive control of the *P. ovale* species. Milijaona Randrianarivelojosia provided technical, logistical, and scientific support. M.R. and A.A.-B. conceived, planned, and supervised the study. All authors read, reviewed, contributed and approved the final manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare that they have no conflicts of interest.

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