

Integrated microscopic and molecular diagnosis of imported mixed malaria caused by *Plasmodium falciparum* and *Plasmodium ovale* curtisi: an Italian case report

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Abstract

Malaria remains a major global health threat, particularly in endemic regions of sub-Saharan Africa. In 2024 alone, an estimated 282 million cases were reported worldwide, with approximately 610,000 associated deaths. Beyond the high disease burden, mixed-species *Plasmodium* infections represent an additional clinical challenge, as they are frequently underdiagnosed and may significantly complicate both diagnostic accuracy and therapeutic management even in non-endemic countries. We report the case of a 67-year-old man from Guinea who presented to the Emergency Department with high-grade fever and asthenia shortly after returning to Italy; initial microbiological investigations were negative, despite markedly elevated inflammatory markers were diagnosed. The patient reported a previous untreated malaria episode approximately six months earlier, raising suspicion of a novel *Plasmodium* infection. A comprehensive malaria diagnostic workflow was promptly initiated, integrating rapid immunoenzymatic testing (IE), microscopy, and molecular assays. The IE test suggested *Plasmodium falciparum* or a mixed infection as result, while loop-mediated isothermal amplification (LAMP-PCR) confirmed the presence of *Plasmodium spp* DNA. Microscopic examination of stained blood smears revealed typical *P. falciparum* ring forms and suspected *P. ovale* trophozoites; so, species-specific real-time PCR confirmed a mixed infection with *P. falciparum* and *P. ovale*, supporting prompt antimalarial treatment. Final molecular confirmation was achieved by Sanger sequencing, which identified the *Plasmodium ovalecurtisi* as co-aetiological agent of disease. The rapid and integrated diagnostic approach enabled timely initiation of appropriate antimalarial therapy with artemether–lumefantrine and primaquine, leading to complete clinical recovery and gene sequencing allowed a correct species confirmation and epidemiological characterization. This case highlights the importance of combining microscopic analysis with advanced molecular diagnosis for an accurate identification of mixed malaria infections even in country and contributes novel epidemiological data through molecular characterization of *P. ovalecurtisi* in an imported case.

Introduction

Despite substantial progress in prevention and treatment strategies, malaria continues to represent a major public health challenge, particularly in African settings. This disease remains responsible for a considerable burden of morbidity and mortality, with hundreds of millions of cases reported annually worldwide.

Mixed-species *Plasmodium* infections are sometimes recognized in imported malaria cases and pose significant diagnostic and therapeutic challenges, especially in non-endemic settings: these infections are frequently underdiagnosed due to low parasitemia levels and overlapping morphological features on light microscopy, potentially leading to delayed or incomplete treatment.

Although molecular diagnostic techniques have improved the detection of mixed infections, complex mixed-species *Plasmodium* infections are often challenging to diagnose and manage in non-endemic areas, where diagnostic approaches may vary according to local expertise and laboratory availability.

Here, we describe a case of imported malaria caused by a mixed infection with *Plasmodium falciparum* and *Plasmodium ovalecurtisi*, diagnosed through an integrated diagnostic workflow combining microscopy, molecular assays, and gene sequencing.

Case history

A 67-year-old man, native of Guinea who had returned to Italy on 17 August, presented to the Emergency Department (ED) of Fondazione Policlinico Universitario A. Gemelli IRCCS in Rome on 25 August with shiver fever and asthenia. His recent clinical history dated back to July, when he first developed fever accompanied by chills and asthenia, without additional symptoms. Upon arrival to the ED, his body temperature was 39°C high; laboratory tests revealed leukopenia (blood cells 3,700/mm³), with neutrophils accounting for 3,080/mm³; serum glucose was 119 mg/dL; while aspartate aminotransferase was markedly elevated at 71 U/L, procalcitonin and C-reactive protein levels were significantly increased at 7.28 ng/mL and 162.5 mg/L, respectively, indicating an ongoing systemic inflammatory response.

Due to the markedly elevated inflammatory markers, blood cultures were also obtained to investigate a possible bloodstream infection which later returned negative.

Given the patient's recent travel history and clinical presentation, a rapid multiplex molecular panel for neurotropic arboviruses, including Dengue virus, Zika virus, Chikungunya virus, West Nile virus, and yellow fever virus (FilmArray® Meningitis/Encephalitis Panel, BioFire Diagnostics, Salt Lake City, UT, USA), was performed and returned negative.

The patient reported a previous malaria episode approximately six months earlier, which had not been treated in his country of origin. Considering this history, a comprehensive malaria diagnostic workup was subsequently initiated at our centre, as is routine for any suspected malaria infection.

First, a blood sample was collected; from this sample, at least three blood smears were immediately prepared and subsequently stained with May-Grunwald-Giemsa (1). Another aliquot of the blood was used for an immunoenzymatic rapid assay (Malaria CareUS Rapydtest®, Wokingham, UK; IE) to obtain a rapid indication of malarial infection. In parallel, a loop-mediated isothermal amplification polymerase chain reaction (LAMP-PCR; Illumipro-10, Meridian Bioscience Inc., Cincinnati, USA) was performed in about 1h, providing a rapid yet more sensitive assessment of *Plasmodium* spp. infection.

The IE test indicated a result consistent with "*Plasmodium falciparum* or mixed infections," as indicated by manufacturer's instructions, while the LAMP-PCR assay detected a positive *Plasmodium* spp. infection. Thus, our rapid tests confirmed the presence of at least one *Plasmodium* species in about 1h.

Microscopic examination additionally revealed several *P. falciparum* trophozoites (Fig. 1, a), as well as forms suspected to belong to *P. ovale* (Fig. 1, b), with a total parasitaemia of < 1% under light microscopy, using an Olympus BX60 microscope (Olympus, Japan) with a 100× magnification. Although Schüffner's

stippling was not clearly appreciable, the observed oval-shaped erythrocytes and trophozoite morphology were highly suggestive of *P. ovale* infection. Specifically, *P. falciparum* parasitaemia was estimated at 0.4%, while *P. ovale* parasitaemia was approximately 0.15%. Due to these microscopic findings and the positive but ambiguous IE test result, a Real-Time PCR (RT-PCR; VIASURE Real Time PCR Detection Kits, Certest Biotec, San Mateo de Gállego, Zaragoza, Spagna) was performed for its greater sensitivity; it finally revealed a positivity for *P. falciparum* (Ct = 30.86) and *P. ovale* (Ct = 37.33), confirming the microscopic observation in May-Grunwald-Giemsa stain.

Moreover, for final molecular confirmation, we performed a Nested PCR using two specific primer pairs followed by Sanger gene sequencing (Snounou et al., 1993), with the aim to correctly identify *Plasmodium* species. Ultimately, gene sequencing confirmed the presence of *P. ovalecurtisi*.

Following the first line diagnostic results, obtained within approximately 2 hours and strongly suggestive of *Plasmodium falciparum* infection, antimalarial therapy with artemether–lumefantrine was promptly initiated. Subsequent molecular investigations, completed within two days, confirmed the mixed infection with *P. falciparum* and *P. ovalecurtisi*, supporting the addition of primaquine to the therapeutic regimen (Glucose-6-phosphate dehydrogenase deficiency was excluded before primaquine administration). The patient was eventually discharged in good general condition, afebrile, with progressive improvement in inflammatory markers.

Discussion and results

Malaria is an infectious disease caused by protozoa parasites belonging to the genus of *Plasmodium* (2); in particular, they were transmitted from female *Anopheles* mosquitoes which acquire the parasites during a blood meal from an infected host and subsequently transmit them to new hosts through additional mosquito bites (2). Five *Plasmodium* species are responsible for human malaria: *P. falciparum*, the most common, *P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi* (3). Rare cases of non-vector transmission have also been documented, including vertical transmission during pregnancy or at birth, transmission through blood transfusion, and infections resulting from needle sharing (2).

Malaria remains a critical global health threat: in 2024, an estimated 282 million cases were reported worldwide (4); in the same year, approximately 610,000 malaria-related deaths were recorded, representing an increase of 12,000 compared with 2023, particularly driven by rises in Madagascar, Yemen, and Ethiopia (4).

In this manuscript we described a case of 67-year Guinean man who was admitted to ED of our tertiary care hospital with shivering fever at 39°C and asthenia. Laboratory tests revealed elevated inflammatory markers, suggesting an ongoing inflammatory process; therefore, microbiological investigations consistent with the clinical presentation yielded negative results. The patient also reported a previous, untreated, malaria episode approximately six months earlier and for this reason were started a malaria diagnostic workup as is standard in any suspected malaria infection in our centre: a rapid IE test revealed in 15 minutes a positive result for “*P. falciparum* or mixed infection”, while a LAMP-PCR detected

the presence of *Plasmodium* spp. DNA. In parallel, a microscopic examination of a May–Grünwald–Giemsa stained blood smear revealed a ring forms, typical of *P. falciparum* trophozoites and a highly suspicious forms compatible with *P. ovale*, characterized by oval-shaped erythrocytes containing ring forms, as shown in Fig. 1a and 1b (5).

Successively, to achieve species-level confirmation a RT-PCR was performed, which showed a mixed infection with both *P. falciparum* and *P. ovale*. Antimalarial treatment based on artemether-lumefantrine was initiated promptly after first-line diagnostic confirmation in $\approx$ 2h, while molecular characterization was completed subsequently for species-level confirmation and epidemiological purposes in 2 days total.

The entire diagnostic workflow is schematised in Fig. 2.

Dealing with local epidemiology, the World Health Organization (WHO) did not include a specific country profile for Guinea in its latest report, owing to limited and inconsistent data availability related to logistical and technical constraints. However, in 2023 the WHO estimated that Guinea reported approximately 4.4 million malaria cases (4); due to substantial regional disparities within the country, detailed epidemiological analyses describing the distribution and burden of malaria species are not available, so national data remain incomplete.

Nevertheless, a local Guinean survey from 2012 reported that, as in many other African regions, *P. falciparum* remains the predominant species responsible for malaria infections (98%), whereas *P. ovale* accounts for a much smaller proportion (0.5%) (6). Moreover, *P. ovale* infections were more frequently detected as part of mixed infections rather than as mono-infections, consistent with the case-presentation described in this manuscript. Therefore, based on the available epidemiological evidence, our case aligns with the expected distribution of *Plasmodium* species within the Guinean context.

The main feature of the *P. ovale* life cycle is its hypnozoite stage in the human liver, where the parasite may remain dormant for weeks to years before reactivating and developing into hepatic merozoites, then entering into the bloodstream and initiating a blood-stage infection (2). It is likely that our patient had been infected several months earlier during his previous malaria episode; however, an inconsistent and non-specific diagnostic workup may have failed to detect the presence of *P. ovale*, which could have persisted in the liver in a dormant state, possibly facilitated by inappropriate or incomplete therapy. After approximately six months, in presence of permissive exogenous factors and host conditions, *P. ovale* may have reactivated and be able to start a new blood-stage infection. As demonstrated by Kotepui *et al.*, *P. ovale* infections carry a lower risk of progressing to severe disease compared with *P. falciparum*, which remains the most virulent *Plasmodium* species in malaria pathogenesis (7). Nevertheless, *P. ovale* may cause severe malaria, although typically with less pronounced clinical alterations than *P. falciparum*, particularly manifesting as jaundice, severe anemia, and pulmonary impairment (7). In all cases, the recommended therapy for mixed infections involving *P. ovale*, as suggested for severe *P. falciparum* infections is artesunate and lumefantrine (in combination with artemether) and primaquine to target the erythrocytic stage of infection (8). In the present case, molecular characterization did not alter the initial

antimalarial management, which was promptly initiated based on first line diagnostic findings, but provided additional species-level and epidemiological information regarding the *P. ovale* isolate.

Indeed, as reported by Snounou et al. in a recent opinion article, *P. ovale* strains are divided up into *wallikeri* and *curtisi*, which represent two genetically distinct and non-recombining species (9); accordingly, the previously used trinomial nomenclature, which has sometimes led to confusion, has been replaced by a binomial nomenclature recognizing *P. ovalecurtisi* and *P. ovalewallikeri* as separate species. For this reason, we adopted this nomenclature throughout the present manuscript.

Dealing with malaria diagnosis, microscopic observation remains the gold standard, although it requires an experienced physician and a limit of detection between 50–500 parasites/μL (7); indeed, in some cases, *P. ovale* forms may be misidentified as *P. vivax* (7). In this context, rapid diagnostic tests such as IE and molecular approaches have been developed to address the sensitivity and time limitations of microscopic detection, allowing the recognition of low amounts of circulating *Plasmodium* antigens and DNA also in not specialistic settings (10, 11). The IE test used in this case report is able to detect *P. falciparum* histidine-rich protein 2 (HRP2) antigen and pan-parasite lactate dehydrogenase (pLDH), which is shared by *P. falciparum*, *P. ovale*, *P. vivax* and *P. malariae*. Overall, IE tests are not sufficiently effective for the diagnosis of *P. ovale*, as they exhibit low overall sensitivity; however, pLDH-based tests have been reported to be more accurate in detecting *P. ovalewallikeri* than *P. ovalecurtisi* (12): this difference may be explained by polymorphisms in *P. ovale* LDH, which could affect antibody affinity in IE assays (12). In light of these data, the IE test used in this diagnostic workflow was able to correctly identify a suspected mixed infection, even though it is well known that the performance of rapid diagnostic tests may be influenced by multiple factors, including parasite density, host-related factors and the timing of laboratory diagnosis (13, 14).

Moreover, molecular tests such as PCR, enable detection of low quantities of various *Plasmodium* spp. DNA, even in doubtful cases or where microscopy suggests a non-*P. falciparum* species infection (7, 15).

Recent evidence shows that molecular approaches provide very high sensitivity and specificity compared to microscopy in malaria diagnosis (16); for this reason, molecular assays may provide useful support in selected cases of imported malaria, particularly for species confirmation, mixed infections and epidemiological characterization, as performed in this manuscript (16).

Although RT-PCR Ct values (*P. ovale* Ct = 37.33; *P. falciparum* Ct = 30.86) suggested a low parasitic burden in the patient's blood, it is not possible to derive a quantitative estimate directly correlating with microscopically determined parasitemia. So, molecular techniques remain optimal tools for confirming low-density infections, mixed infections, and for providing rapid diagnostic results; however, they do not play a primary role in clinical management, as microscopic examination which remains the gold standard for parasitemia determination (17)

Additionally, we decided also to enhance our diagnostic algorithm in this case by sequencing the *P. ovale* strain, because diagnostic workflows for imported malaria may vary according to local expertise and

laboratory availability, although evidence remains limited and sometimes conflicting, we decided to perform Sanger gene sequencing to characterize the *P. ovale* strain (18). Unfortunately, no epidemiological reports specifically addressing even *P. ovale* subspecies in Guinea are available, likely due to the lack of population-based studies and surveillance programs in some area of the region.

So, a Nested-PCR was performed, using two specific primer pairs amplifying the small subunit ribosomal RNA of *Plasmodium*, as described (19). The final PCR products indicated even in this case a co-infection of *P. falciparum* and *P. ovale* (**Supplementary figure S1, line 1 and 10**). The positive *P. ovale* product was subsequently prepared for Sanger sequencing (20), which confirmed *P. ovalecurtisi* as result; gene sequence has then been deposited in GenBank under the accession number PX920863.

Although epidemiological data from Guinea are lacking, evaluations performed on imported cases from Guinea suggest a slight predominance of *P. ovalecurtisi* in the region (21): in this context, the *P. ovalecurtisi* strain reported in this case aligns with previously reported observations in literature.

Although mixed *Plasmodium* infections have been identified in imported malaria cases in Italy using molecular methods, to our knowledge there are no previous Italian case reports of a *P. falciparum* and *P. ovalecurtisi* co-infection confirmed by microscopic identification, molecularly characterized and with sequence deposition in public databases.

In conclusion, we report a well-documented case of mixed infection with *Plasmodium falciparum* and *Plasmodium ovalecurtisi* in an imported malaria case, promptly diagnosed and successfully treated with complete clinical recovery. This case highlights the value of integrating microscopy with molecular methods for the confirmation and characterization of mixed-species malaria infections, particularly in non-endemic settings.

In our patient molecular assays and Sanger sequencing did not modify the initial antimalarial management, which was promptly initiated based on first line diagnostic results, but provided accurate species level characterization of the *P. ovale* isolate and additional epidemiological information. The deposition of the sequence in a public database may further contribute to the molecular epidemiology of imported malaria cases from Guinea and to the growing knowledge regarding the *P. ovale complex*.

Declarations

Ethics approval and consent to participate

- Consent for publication

Competing interests

- Funding

Authors' contributions

C.M.M., G.Q., and V.D.P. drafted the manuscript. G.Q. and G.F. contributed to the microbiological investigations and microscopic diagnosis. C.M.M. performed the molecular analyses. V.S., C.D.V., and G.M. managed the clinical case and antimalarial treatment under the supervision of A.C. and C.T. M.S., C.T., and L.M. supervised the overall study and critically revised the final manuscript. All authors have read and agreed to the published version of the manuscript. All authors had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Ethics approval and consent to participate

Ethics approval was not required for this case report according to institutional policy.

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Conflicts of interest

The authors declare that they have no competing interests.

Author Contribution

C.M.M., G.Q., and V.D.P. drafted the manuscript. G.Q. and G.F. contributed to the microbiological investigations and microscopic diagnosis. C.M.M. performed the molecular analyses. V.S., C.D.V., and G.M. managed the clinical case and antimalarial treatment under the supervision of A.C. and C.T. M.S., C.T., and L.M. supervised the overall study and critically revised the final manuscript. All authors have read and agreed to the published version of the manuscript. All authors had full access to all the data in the study and had final responsibility for the decision to submit for publication.

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Not applicable

Data availability

All relevant data generated or analysed during this study are included in this published article and its supplementary information files.

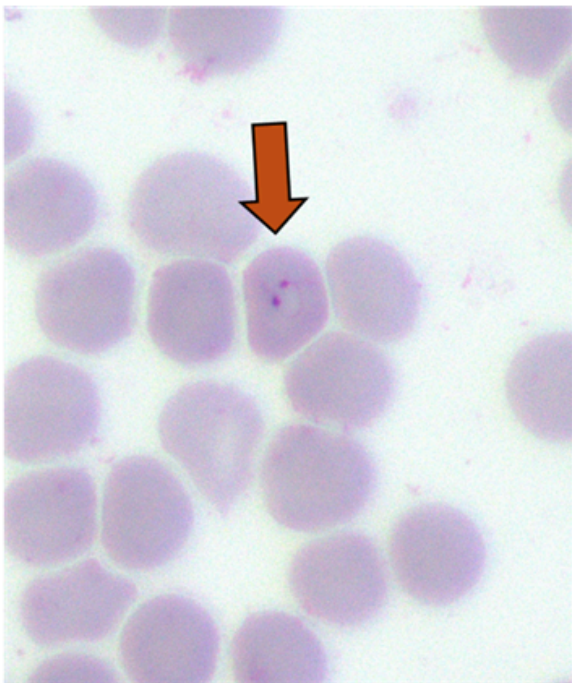
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Figures

a



b



Figure 1

(a) Microscopic examination of *P. falciparum* trophozoites (May–Grünwald–Giemsa stain, 100x objective magnification) showing the characteristic ring forms; **(b)** Microscopic examination of *P. ovale* trophozoites (May–Grünwald–Giemsa stain, 100x magnification), with the typical oval-shaped infected erythrocytes from which the species derives its name.

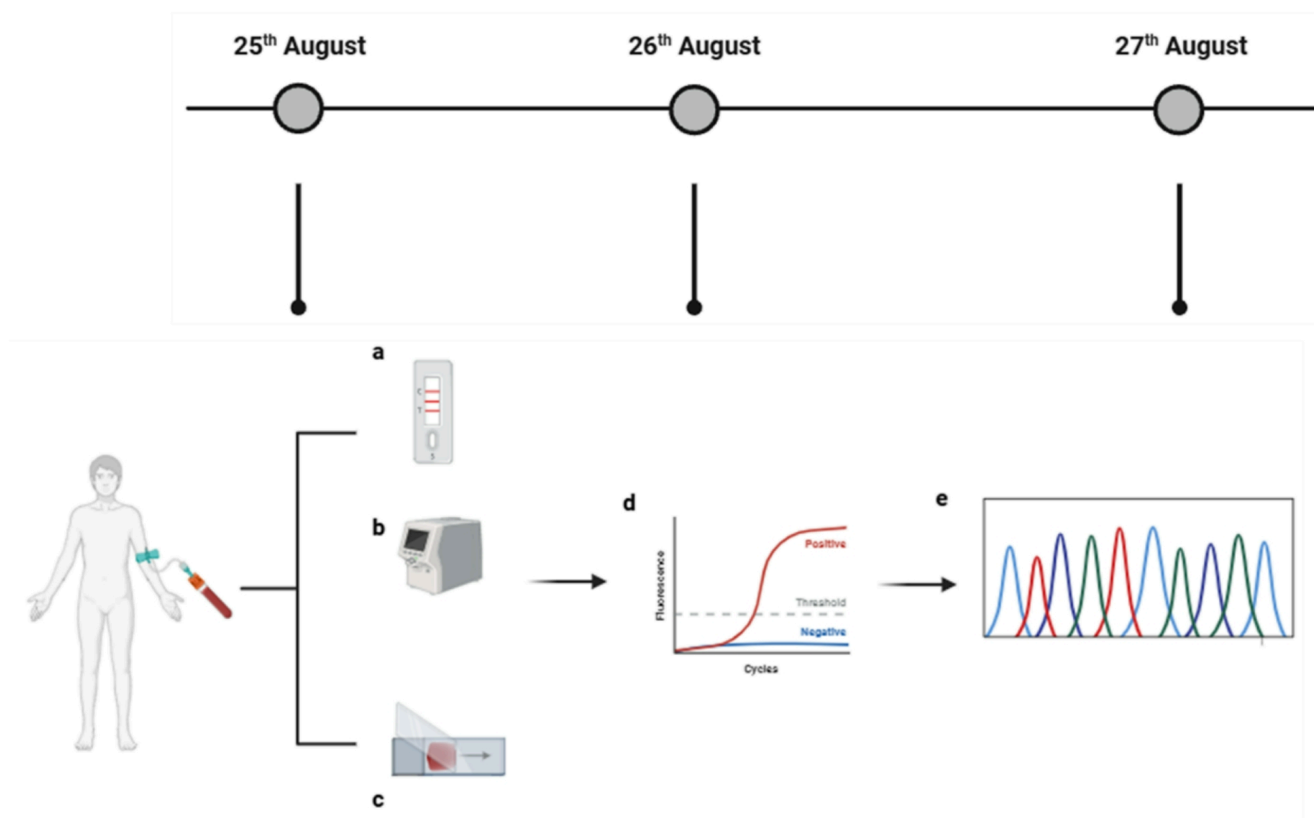


Figure 2

Schematic diagnostic workflow for malaria infection. A patient with suspected malaria was admitted to the ED, and a series of diagnostic investigations were performed on a blood sample:

- a)** an IE rapid test, which indicated *P. falciparum* or mixed infections (three coloured bands) within approximately 10 minutes;
- b)** a LAMP-PCR assay, which detected the presence of *Plasmodium* spp. DNA in about 1 hour.
- c)** In parallel with tests **a** and **b**, a May–Grünwald–Giemsa-stained thin blood smear was prepared, representing a pivotal component of the diagnostic workflow.
- d)** Following the positive results from **a** and **b**, and the microscopic detection of *P. falciparum* trophozoites together with suspected *P. ovale* forms, a second-line molecular test (RT-PCR) was performed, confirming *P. falciparum* (Ct = 30.86) and *P. ovale* (Ct = 37.33) detection.
- e)** Sanger gene sequencing which yield to *P. ovale* curtisi as result

Supplementary Files

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