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Case Report

Molecular diagnosis and therapy for *Plasmodium ovale* infection of a returned traveler from East Africa

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Malaria is an infectious disease caused by *Plasmodium* parasites that are mainly transmitted through the bites of infected female Anopheles mosquitoes. The average annual number of malaria cases was less than ten in Taiwan in the last five years. Most of the cases were caused by *Plasmodium vivax* and *Plasmodium falciparum*, and were primarily diagnosed in travelers who returned from Southeast Asia and Africa. Here, we report the first case of *Plasmodium ovale* infection within five years that was confirmed by peripheral blood smear examination and molecular identification in a 25-year-old Asian female patient who returned from Uganda.

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Introduction

Malaria is a serious infectious disease caused by *Plasmodium* parasites. Without prompt medical care, the disease can potentially be severe and fatal. In 1965, malaria eradication has been achieved in Taiwan.¹ The cases have been continuously imported from abroad without indigenous infection. In 2020, only two imported malaria cases have been documented and the cases were originated from Malaysia and Uganda, respectively. Significantly, the average annual number of malaria cases reported from the Taiwan Centers for Disease Control was less than ten in the last five years. The cases were primarily exported from the regions of Africa and then from the regions of Southeast Asia. Most of the *Plasmodium vivax* cases were imported from Southeast Asia, whereas *Plasmodium falciparum* was the predominant parasite found in Africa imported cases. Interestingly, no *Plasmodium ovale* infections were diagnosed in the past five years. Here, we report the first case of *P. ovale* infection within five years that was confirmed by peripheral blood smear examination and molecular identification in a 25-year-old Asian female patient who returned from Uganda.

Due to the rapid spread of coronavirus disease, the Taiwanese government mandates a 14-day home quarantine for inbound travelers. A traveler from Uganda was reported after presenting persistent high-grade fever during the quarantine period. Considering Uganda as a malaria-endemic country, excluding malaria as an underlying cause of fever was considered as the priority. We confirmed the fever was caused by malaria infection instead of coronavirus infection by examining the blood smear samples with light microscopy, the gold standard diagnostic test of malaria, and polymerase chain reaction (PCR) assay.² After the administration of hydroxychloroquine, falcitrim, and primaquine, malaria parasites were not detected on a blood smear or PCR assay.

Here, we report the first case with a fever that was diagnosed by the infection of *P. ovale* since the SARS-CoV-2 pandemic in 2020. Furthermore, the patient was successfully treated by administration of hydroxychloroquine, falcitrim, and primaquine. Our report suggests that there is an urgent need to aggressively tackle the coronavirus infection, but the diagnosis and therapy of malaria should not be neglected.

Case report

The patient was a 25-year-old Asian female who traveled to a refugee camp located in the Arua district of Uganda as a volunteer from April 1st, 2019 to November 5th, 2020 without taking prophylactic medications for malaria. The patient had three to four self-reported sickness presenting flu-like symptoms and were later diagnosed with malaria in

May and October 2019 at a local ambulatory care center in Uganda. With unclassified *Plasmodium* species, the patient was treated with a three-day regimen of dihydroartemisinin and piperaquine (orally; 40 and 320 mg per meal, respectively) in each episode. The patient had a follow-up appointment in one week for a second microscopic diagnostic test to confirm full recovery. On November 5th, the patient was undergoing digital surveillance complying with the home quarantine policy mandated in the Taiwanese coronavirus-prevention program after arrival. Starting on November 13th, the patient presented with symptoms of high-grade fever, chills, neck pain, and muscle weakness during the quarantine period. She was then sent to an emergency department for the management of suspected coronavirus disease at our hospital on November 15th. Based on the patient's recent travel history, a malaria diagnostic test was ordered per protocol.

The blood film result was positive for *Plasmodium* species via Wright-Giemsa stain (Fig. 1A–D), but insufficient to determine the precise species of *Plasmodium*. The chromatin appears red while the cytoplasm appears blue. The malaria pigment appears yellowish-brown in color. The blood film result was positive for *P. ovale* with an oval shape with jagged edges and few *P. falciparum* characteristics of multiple chromatin dots and rings. Base on the initial staining interpretation, the patient was admitted to our hospital for malaria management; however, we were unable to distinguish the precise *Plasmodium* species due to the complex nature of rapid staining interpretation. As the result, two tablets of hydroxychloroquine (200 mg/tablet) were given initially as a one-time empiric therapy at hospital admission.

The diagnosis of malaria was further supported by additional Giemsa stain and Liu's stain. The blood film results showed a clear presentation of Schuffner's stippling that was unique in *P. ovale* infection, which Wright-Giemsa stain failed to demonstrate.³ The staining characteristics of *P. ovale* were described accordingly: *P. ovale* targets and subsequently infects only young RBCs (reticulocytes) which are generally bigger than mature RBCs. The infected reticulocytes can be round, oval, or fimbriated with an irregular edge; *P. ovale* schizonts have 4 to 16 merozoites; Schuffner's stippling are revealable with Giemsa stain but its presence may be hidden by anticoagulants; the presentation of ring forms, trophozoites, schizonts, and gametocytes can co-exist in surrounding red blood cell (Fig. 1E–L); double chromatin dots are occasionally seen.⁴ Based on these staining characteristics, the sample was positive for *P. ovale* but fail to diagnose mixed malaria.

A patient blood sample was drawn to isolate the DNA template for PCR analysis.⁵ The nested PCR assay involves sequential PCR amplification methods, which *Plasmodium* genus-specific primers set was used for the first PCR amplification followed by species-specific primer (Table 1A).

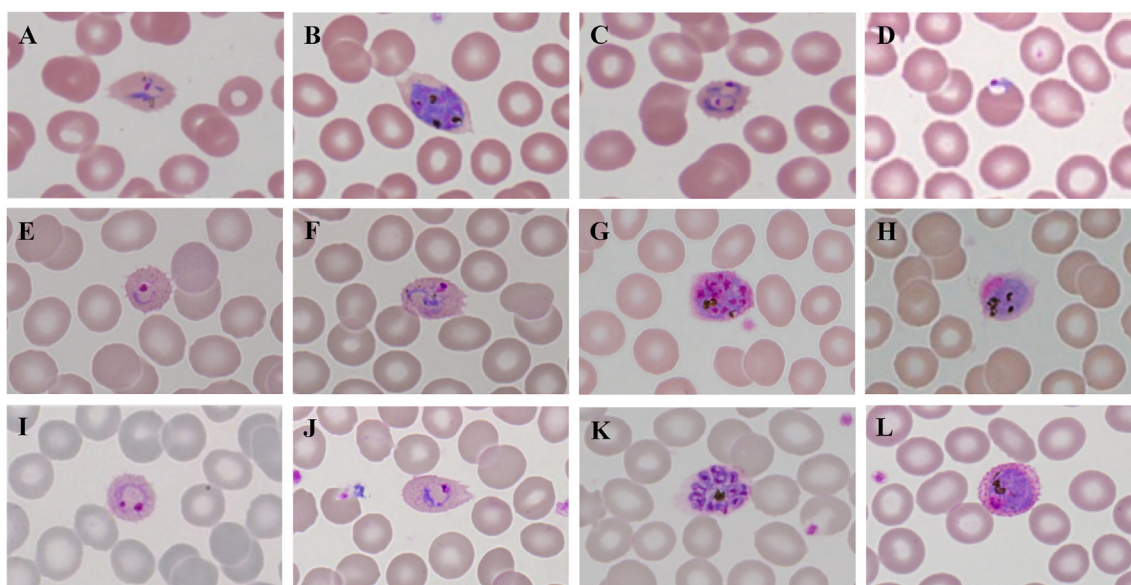


Figure 1 The stage of *Plasmodium ovale* in the peripheral blood smears revealed by stains. Peripheral blood thin smear with Wright-Giemsa stain shows infected RBC by *Plasmodium* spp. with frayed (fimbriated) edges (A, B). Double ring forms (early trophozoites) can be seen (C). Accollé forms of *Plasmodium* spp. in the infected RBC (D). Peripheral blood thin smear with Giemsa stain shows different stage of infected RBC with *Plasmodium ovale* ring form (E), trophozoite (F), schizont (G), and gametocyte (H). Peripheral blood thin smear with Liu's stain shows different stage of infected RBC with *Plasmodium ovale* ring form (I), trophozoite (J), schizont (K), and gametocyte (L). (optical microscope magnification 1000x).

The product of this first amplification served as a DNA template for four separate second PCR amplification with primers specific for each of the human malaria species: *P. falciparum*, *P. vivax*, *Plasmodium malariae*, and *P. ovale*.⁵ The presence of unique *Plasmodium* small subunit ribosomal RNA genes and corresponding molecular size helps detect and differentiates the *Plasmodium* species in the blood sample. The genus-specific primers used in the nest 1 amplification detect the presence of malaria (Table 1A), whereas the species-specific primer helps identify precise species (Table 1B). The unique molecular size of each species was listed in the following: *Plasmodium* species, positive:238 bp,⁶ *P. falciparum*, positive:205 bp,⁷ *P. vivax*, positive:120 bp,⁷ *P. malariae*, positive:144 bp,⁷ *P. ovale curtis*, positive:787 bp,⁶ *P. ovale wallikeria*, positive:782 bp.⁸ The genus-specified PCR result is positive for *Plasmodium* species with a product size of 238 base pair.⁵ After amplified the PCR assay with species-specific primer, the product size of 787 base pair is indicative for the presence of *P. ovale curtis*, where the chance of mixed malaria is excluded.^{6,8}

After the second confirmatory test using Giemsa stain (Fig. 1E–H), Liu's stain (Fig. 1I–L), and PCR assay (Fig. 2A), the presence of *P. ovale* was confirmed to start a three-day regimen of falcitrim followed by primaquine phosphate. Oral administration of falcitrim (artesunate 200 mg and mefloquine 250 mg) dose pack was prescribed, which patient take one tablet of 200 mg artesunate daily and 2 tablets of 250 mg mefloquine daily for 3 day as directed. Before each dose, thick and thin blood smear were examined to monitor drug efficacy. After the completion of falcitrim therapy, the blood test was negative for malaria

pathogens. *P. ovale* is undetectable when exhibiting the hypnozoite stage in the liver, which increases the likelihood of relapse in weeks or even years later. Oral primaquine (7.5 mg/tablet) was given three times daily for a total of 14 days to prevent *P. ovale* relapses.⁹

The patient was scheduled to follow-up monthly for one year to continuously monitors negative signs of malaria via microscopic tests and PCR tests. The results obtained from the first follow-up appointment were shown in the following (Fig. 2B).

Discussion

Although there is an urgent need to aggressively tackle the coronavirus infection, the diagnosis and therapy of malaria should not be neglected. Malaria could be fatal if left untreated and the diagnosis of malaria may be delayed because of the rare occurrence in Taiwan. We reported a case of returned travelers from Uganda having similar symptoms to SARS-CoV-2 infection during the home quarantine period in Taiwan. Moreover, this is the first case of *P. ovale* infection within five years in Taiwan. The infection occurred in May and October 2019, which was aligned with the transmission peak of two wet seasons in Uganda (from March to May, and from September to November). Interestingly, *P. ovale* is a relapsing infection that occurred as early as 17 days after the treatment of primary attack¹⁰ and it is difficult to differentiate from another malaria parasite, *P. vivax*. Therefore, for returned travelers from endemic areas, we should not ignore the possibility of malaria infection. Here, we provide evidence of molecular diagnosis and therapy for *P. ovale* infection.

Table 1 Primer sets for the detection and identification of *Plasmodium* spp.

Category	Primer pairs (forward + reverse)	PCR product size
A. Primer pairs for malaria genus.		
Primers for Genus		
1st step PCR	rPLU1 : 5'- TCA AAG ATT AAG CCA TGC AAG TGA-3' rPLU5 : 5'- CCT GTT GTT GCC TTA AAC TTC-3'	
2nd step PCR	rPLU3 : 5'- TTT TTA TAA GGA TAA CTA CGG AAA AGC TGT-3' rPLU4 : 5'- TAC CCG TCA TAG CCA TGT TAG GCC AAT ACC-3'	238 bp
B. Primer pairs for malaria Species.		
Primers for Species		
1st step PCR	rPLU6 : 5'-TTA AAA TTG TTG CAG TTA AAA CG-3' rPLU5 : 5'-CCT GTT GTT GCC TTA AAC TTC-3'	
2nd step PCR	Pf1 : 5'-TTA AAC TGG TTT GGG AAA ACC AAA TAT ATT-3' Pf2 : 5'-ACA CAA TGA ACT CAA TCA TGA CTA CCC GTC-3' Pv1 : 5'-CGC TTC TAG CTT AAT CCA CAT AAC TGA TAC-3' Pv2 : 5'-ACT TCC AAG CCG AAG CAA AGA AAG TCC TTA-3' Pm1 : 5'-ATA ACA TAG TTG TAC GTT AAG AAT AAC CGC-3' Pm2 : 5'-AAA ATT CCC ATG CAT AAA AAA TTA TAC AAA-3' Poc1 : 5'- ATC TCT TTT GCT ATT TTT TAG TAT TGG AGA-3' Poc2 : 5'-GGA AAA GGA CAC ATT AAT TGT ATC CTA GTG-3' Pow1 : 5'-ATC TCC TTT ACT TTT TGT ACT GGA GA-3' Pow2 : 5'-GGA AAA GGA CAC TAT AAT GTA TCC TAA TA-3'	205 bp 120 bp 144 bp 787 bp 782 bp

Pf.: *P. falciparum* ; Pv.: *P. vivax* ; Pm.: *P. malaria* ; Poc.: *P. ovale curtis* ; Pow.: *P. ovale wallikeria*.

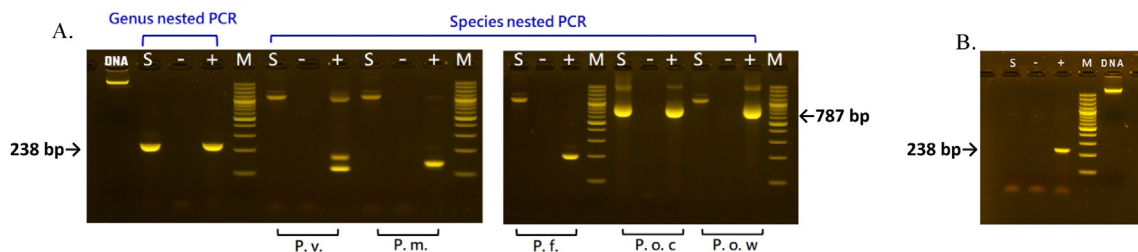


Figure 2 (A) The result of genus- and species-specific nested polymerase chain reaction in malaria detection assay. S: Patient DNA; -:Negative control (ddH₂O); +:Positive control (Plasmid); Lane M : 100 bp Marker; P.v.: *P. vivax*; P.m.: *P. malaria*; P.f.: *P. falciparum*; P.o.c.: *P. ovale curtis*; P.o.w.: *P. ovale wallikeria*. (B) The result obtained from the first follow-up appointment of genus nested polymerase chain reaction was shown in Negative. S: Patient DNA; -:Negative control (ddH₂O); +:Positive control (Plasmid); Lane M : 100 bp Marker.

Infection of malaria leaves a national burden with illness and death in Uganda. While the majority of cases in Uganda were *P. falciparum*, both *P. vivax* and *P. ovale* were diagnosed, especially in several districts of Uganda.¹¹ *P. ovale* is commonly found as mono-infection but may exhibit multiple infections; therefore, the presentation of ring-form trophozoites with double-chromatin dots can be a challenge to differentiate this species from *P. falciparum*. The schizonts also have a similar presentation to *P. vivax* making it more difficult to interpret. Importantly, the schizonts in hepatocytes can cause relapse in many months after primary infection by releasing the invasive mature merozoites to blood.^{12,13} Additional testing may require to further inspect the parasite morphology to properly identify true species. Although microscopy test is the gold standard for the diagnosis of malaria due to its inexpensive and rapid detection characteristics, it often fails to accurately identify *Plasmodium* species. This limitation can be overcome

with PCR assays based on its high sensitivity to examine blood smears based on species-specific sequences in the small subunit ribosomal RNA genes.¹⁴ PCR is indeed relatively expensive which requires highly trained medical professionals to operate testing, it is still considered as a valuable method for early identification of mixed malaria, new parasite species, and malaria relapses.

Although malaria cases are rare after it has been eradicated in Taiwan, the imported infection cannot be neglected as of world travel and international trade advances, including migration of workers from malaria-endemic regions. The initial clinical presentation of malaria is nonspecific and often manifests as a flu-like illness, misdiagnosed can delay treatment plan. About 80% of malaria patients presented with thrombocytopenia; therefore, healthcare professionals should be cautious when encountering patients with recent travel history to endemic countries along with thrombocytopenia and fever. The rapid

microscopic test is recommended for this type of malaria diagnosis.^{15–17}

In the diagnosis of malaria, the most common initial laboratory abnormalities were thrombocytopenia, mild hyperbilirubinemia, and leukopenia.¹⁸ In our case report, the patient had a low platelet level of 120,000, which is lower than the normal range (130,000–400,000 platelets/ μ l). Through the laboratory analysis, Liu's stain and Giemsa stain are capable of staining the Schüffner's stippling that Wright-Giemsa stain failed to present. For the treatment of malaria, immediate diagnosis and optimized medication are critical. Although the drug treatment for *P. ovale* had barely been discussed in Taiwan due to the rare occurrence, the patient was successfully treated by administration of hydroxychloroquine (400 mg for one dose), falcitrim (artesunate 200 mg and mefloquine 500 mg for 3 days), and primaquine (7.5 mg three times daily for 14 days). A longer follow-up tracing is also necessary to prevent the possible *P. ovale* relapses.¹⁹

Author contributions

Y.T, N.T, and C.H, designed the study. Y.T, S.H, P.C, M.C and C.H, performed the experiments. Y.T, C.L, S.H and C.H analyzed the data. Y.T, C.L, and P.B.L wrote the manuscript. All authors discussed the results and commented on the manuscript.

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Declaration of competing interest

The authors declare that they have no competing interests.

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