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BRIEF REPORT

# Symptomatic *Plasmodium vivax* Infection in Rwanda

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We report a *Plasmodium vivax* infection in a Rwandan child misdiagnosed with *Plasmodium falciparum* and administered artemether-lumefantrine. Antigen detection revealed an absence of *P falciparum* histidine-rich protein 2 (HRP2) and presence of *Plasmodium vivax* lactate dehydrogenase. Nested and real-time polymerase chain reactions verified that the sample only contained *P vivax* deoxyribonucleic acid.

**Keywords.** microscopy; misdiagnosis; multiplex; PCR; *Plasmodium vivax*.

A recent household survey in Rwanda estimated that *Plasmodium falciparum* causes 97% of malaria infections, whereas *Plasmodium malariae* and *Plasmodium ovale* each cause 1%–2% of remaining infections (Rwandan National Malaria Control Program, Aline Uwimana, 2018, unpublished data). The last reported case of *Plasmodium vivax* of assumed Rwandan origin occurred in 2005 in a Japanese traveler returning to Japan after traveling to Rwanda [1]. Diagnosis and speciation of malaria in Rwanda at health facilities are mainly performed using microscopy, but combination rapid diagnostic tests (RDTs) utilizing detection of pan-*Plasmodium* lactate dehydrogenase and *P falciparum* antigen histidine-rich protein 2 (HRP2) are used in community health centers and at larger health facilities when microscopy is not available. According to the World Health Organization (WHO) [2], the recommended treatment of children and adults with uncomplicated *P falciparum* infection is a 3-day course of an artemisinin-based combination therapy (ACT). In Rwanda, artemether-lumefantrine and dihydroartemisinin-piperaquine are the first- and second-line treatments for *P falciparum* malaria. Unlike *P falciparum*, *P vivax* and *P ovale* parasites can form dormant stages in the liver, known as the hypnozoites, which can reactivate weeks to years after the primary infection. To achieve a radical cure—the elimination of all parasites—in uncomplicated *P vivax* infections, the WHO recommends a 3-day course of an ACT to eliminate blood-stage parasites and a 14-day course of primaquine to clear hypnozoites [2]. In this study, we report the case of a 4-year-old boy from Masaka, Rwanda, with *P vivax* infection who was misdiagnosed with *P falciparum* monoinfection and given a 3-day course of treatment with artemether-lumefantrine.

## METHODS

### Patient Enrollment

The patient was enrolled in an ongoing antimalarial therapeutic efficacy study (TES) approved by the Rwanda National Ethics Committee (reference 195/RNEC/2017) [3]. Informed consent was obtained from the accompanying parent/guardian at the time of enrollment, and a dried blood spot (DBS) specimen was collected on filter paper.

### Treatment and Monitoring of Infection

To diagnose malaria and determine parasitemia in the patient, thin and thick blood smears were prepared and stained with 5% Giemsa. Each slide was independently read by 2 microscopists who were trained and supervised by WHO Level 1-certified laboratory technicians from the Rwandan National Reference Laboratory. Asexual parasite density per microliter of blood was calculated as the average of the 2 readings using the number of infected red blood cells per 200 white blood cells and multiplied accordingly.

### Multiplex Assays

Multiplex antigen detection of 4 antigens—*P falciparum* HRP2/3, *P vivax* lactate dehydrogenase (PvLDH), pan-*Plasmodium* antigens LDH, and aldolase—was performed on whole blood eluted from DBS as described [4]. Antigen detection using anti-PvLDH capture antibodies has previously been shown to be specific for *P vivax* and not reactive to *P malariae* or *P ovale* antigens [5]. The *P vivax* isolate included in this study as a control was isolated from an Indian patient in February 2021, and prior photo-induced electron transfer polymerase chain reaction (PET-PCR) analysis determined this sample to have a cycle threshold value of 32.69 with *P vivax* primers.

A second multiplex assay was used to capture immunoglobulin (Ig)G that recognize recombinant merozoite surface protein 1 (MSP1) 19-kD antigens from *P falciparum*, *P vivax*, *P ovale*, and *P malariae*. The anti-$MSP1_{19}$ IgG capture assay has previously been validated to be species specific and was

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performed as described previously [6]. This work was reviewed by the US Centers for Disease Control and Prevention (CDC), determined to be nonresearch (program evaluation), and conducted consistent with applicable federal law and CDC policy.

#### Deoxyribonucleic Acid Extraction and Genotyping

Total deoxyribonucleic acid (DNA) was extracted from a 6-mm DBS punch using the QIAamp Blood Mini Kit (QIAGEN Inc) according to the manufacturer's protocol. Two PCR speciation assays, PET-PCR [7] and a nested PCR [8], were carried out as described previously. Amplification of *pfmsp1*, *pfmsp2*, *pfhrp2*, *pfhrp3* exon 1-2, and exon 2 spanning regions by PCR was conducted as described previously [4]. To confirm consistent results, each PCR was repeated on a different day.

## RESULTS

#### Case Report

On May 18, 2018, a 49-month-old boy arrived at a health center in Masaka, Rwanda, with clinical signs and symptoms suggestive of malaria infection, including fever. No manifestations of severe malaria were detected, and no underlying diseases, malnutrition, allergy to antimalarial medications, or any other ongoing prophylaxis were noted. The patient was determined to be anemic after a capillary blood sample revealed a hemoglobin level of 10.2 mg/dL as measured via HemoCue (HemoCue AB). The patient's parasite density was determined to be 14200 p/µL blood. He was diagnosed with a *P falciparum* monoinfection and was enrolled in an ongoing TES and assigned a study identification number of R218. The patient was then admitted to the health center for 3 days of inpatient observation to ensure adherence to the treatment regimen, which comprised 6 doses of artemether-lumefantrine at hours 0, 8, 24, 36, 48, and 60. On day 3, upon completion of treatment, thin and thick smear were again taken and determined to be negative for malaria parasites, and the patient was discharged. As part of the TES protocol [3], the child returned for visits on days 7, 14, 21, and 28 postenrollment to provide additional blood samples for microscopy and DBS, but no infection was detected by microscopy during these visits.

#### Molecular Analysis and *Plasmodium* Species Identification

The HRP2-based RDTs are widely used for the diagnosis of *P falciparum* malaria in sub-Saharan Africa. However, *pfhrp2* and *pfhrp3* gene deletions in *P falciparum* allow parasites to evade detection by these RDTs. As part of an ongoing study to investigate the presence of *pfhrp2* and *pfhrp3* deletions in samples derived from African TESs, all DBS from the 2018 Rwanda TES were subjected to a multiplex antigen detection assay to determine the antigen carriage of *P falciparum* HRP2/3, *P vivax* lactate dehydrogenase (PvLDH), and pan-*Plasmodium* antigens LDH and aldolase.

Of the patient samples collected as part of the 2018 Rwanda TES, the blood sample from our patient of interest showed a nonpositive low assay signal for HRP2 but high signals for the pan-*Plasmodium* antigens and PvLDH (Figure 1A). This profile is consistent with the antigen profile observed for a *P vivax* isolate obtained from a patient with PCR-confirmed *P vivax* infection from India. A separate IgG capture multiplex assay utilizing the 4 *Plasmodium* spp orthologs of the MSP1 19-kD antigen revealed that the same patient's blood sample was positive for IgG antibodies recognizing the *P falciparum*, *P ovale*, and *P vivax* MSP1 antigens (Figure 1B).

Due to these antigen and antibody assay results, additional PCR assays were performed on this sample due to suspicion of *P vivax* infection. Two repeat experiments of a PET-PCR (Figure 1D) and a nested PCR (Figure 1C) speciation assays both revealed the sample to be positive for *Plasmodium* genus and *P vivax* DNA only.

In addition to speciation, PCR amplification of *pfmsp1*, *pfmsp2*, *pfhrp2*, *pfhrp3* exon 1–2, and exon 2 spanning regions [4] revealed this sample to be negative for all *P falciparum* genes tested. Genotyping for polymorphisms associated with drug resistance, including *P falciparum* kelch 13 and multidrug resistance-1 genes, was carried out as part of routine TES surveillance and also revealed a lack of *P falciparum* genes.

Upon confirmation by PCR that this patient was infected with *P vivax*, the thin smear obtained at the time of enrollment was re-examined by a WHO Level 1-certified microscopist who confirmed *P vivax* parasites by morphology as observation revealed that the cytoplasm of the parasite was fragmented and increased, with irregular ameboid appearance characteristic of *P vivax*.

## DISCUSSION

In this study, we report a PCR-confirmed symptomatic *P vivax* infection for a 49-month-old Rwandan male who was misdiagnosed with *P falciparum* infection by microscopy and treated with artemether-lumefantrine. Treatment of a *P vivax*-infected patient without a 14-day course of primaquine puts the patient at risk for a relapse infection weeks to months later. Although this patient returned for weekly monitoring until day 28 postdiagnosis, we cannot be certain whether the child experienced a relapsed infection due to *P vivax* beyond the last observation date. One limitation of our report is the lack of previous travel history for the patient.

The most recent report of *P vivax* infections linked to Rwanda is the case of a man who returned to Japan from Rwanda with a *P vivax* infection in 2005 [1]. The WHO's malaria country profile for Rwanda shows no *P vivax* cases in the country dating back to 2000 [9, 10]. A 2015 study that assessed the evidence of *P vivax* transmission in sub-Saharan Africa found 5 cases of travel-related *P vivax* infections where Rwanda was considered

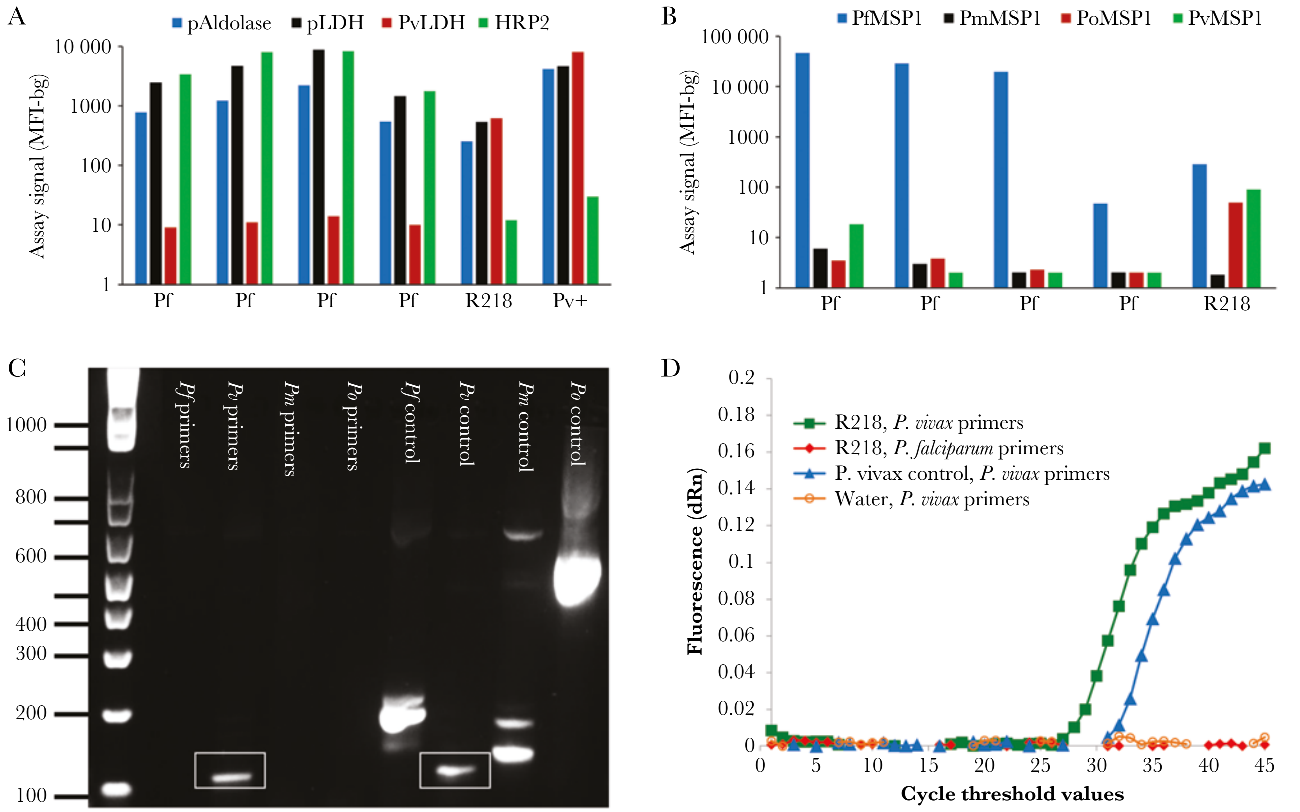


**Figure 1.** Detection of *Plasmodium vivax* antigens, antibodies, and deoxyribonucleic acid (DNA). (A) Bead-based multiplex antigen detection assay for pan-*Plasmodium* aldolase (pAldolase) and pan-*Plasmodium* lactate dehydrogenase (pLDH), *P vivax* lactate dehydrogenase (PvLDH), and *Plasmodium falciparum* histidine-rich protein 2 (HRP2) using whole blood eluted from dried blood spots. Results from 4 *P falciparum* infections from Rwanda (Pf) are compared with the Rwandan *P vivax* patient (R218) and a *P vivax* isolate obtained from an Indian patient (Pv+). Results are displayed as median fluorescence intensity minus background (MFI-bg) assay signal. (B) Capture of immunoglobulin (Ig)G from blood samples from 4 *P falciparum*-infected patients and the *P vivax*-infected patient R218 from the 2018 Rwandan therapeutic efficacy study (TES). Antigens used for IgG capture were merozoite surface protein 19 kD (MSP1) antigens from *P falciparum*, *P malariae*, *P ovale* spp, and *P vivax*. Data displayed as MFI-bg assay signal. (C) Agarose gel of nested polymerase chain reaction (PCR) products after amplification of DNA from the *P vivax* patient's blood using *P falciparum* (Pf), *P vivax* (Pv), *Plasmodium malariae* (Pm), and *Plasmodium ovale* (Po) 18S primers compared with control DNA amplified with the same primers. Gel products in the box show the amplified *P vivax* DNA target. (D) Photo-induced electron transfer PCR (PET-PCR) amplification curves from DNA from the *P vivax* patient's blood (R218) using *P vivax* 18S primers are shown in green squares. Controls presented include an Indian *P vivax* reference strain amplified using the same *P vivax* primers (blue triangles), the patient R218's blood amplified using *P falciparum* primers (red diamonds), and a water nontemplate control amplified with *P vivax* primers (orange open circles). All PET-PCR curves are shown as the average of 3 replicates.

the probable country of origin but ultimately concluded evidence of *P vivax* infection in Rwanda to be weak [11]. The CDC's *Morbidity and Mortality Weekly* reports of *Plasmodium* species responsible for malaria infection in persons returning to the United States after travel show only 2 non-PCR-confirmed cases of *P vivax* imported from Rwanda in 2017 and 2016 [12, 13].

The Duffy blood group antigens are the main invasion receptors on reticulocytes and erythrocytes for *P vivax* merozoites. In Rwanda, Duffy negativity is highly prevalent, with an estimated frequency of >95% [14]. Because consent for human DNA genotyping was not agreed upon by the patient/guardian at the time of enrollment, the Duffy status of this patient is unknown. However, *P vivax* can still cause symptomatic malaria in Duffy-negative individuals through the *P vivax* ligands and host receptor molecules that allow merozoite invasion of Duffy negative erythrocytes [15]. *Plasmodium vivax* infection of Duffy-negative individuals has been reported across sub-Saharan Africa, including in Mali, Benin, Cameroon, Equatorial Guinea, Kenya, and Angola, all countries with a predominantly Duffy-negative population [15].

## CONCLUSIONS

Although confirmatory diagnosis by microscopy or RDT is recommended before initiation of antimalarial treatment [2], malaria microscopists may have difficulty identifying low-parasite density and mixed infections—which would be especially pertinent in a country where multiple *Plasmodium* species are endemic [16]. In addition, a mixed *P falciparum*/*P vivax* clinical infection could be misdiagnosed as *P falciparum* only if an RDT based on HRP2-only was being used or if a microscopist identified *P falciparum* parasites and failed to continue monitoring for parasites from other species. Therefore, increased awareness and routine performance evaluation of microscopists in

*P falciparum* predominant areas is beneficial to ensure accurate diagnoses of infections causing clinical disease.


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