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Plasmodium vivax Malaria in Duffy-Positive Patients in Rwanda

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Abstract. *Plasmodium vivax* is the second-most common malaria pathogen globally, but is considered very rare in the predominantly Duffy-negative sub-Saharan African population. In 259 malaria patients from highland southern Rwanda, we assessed *Plasmodium* species and Duffy blood group status by polymerase chain reaction (PCR). *Plasmodium falciparum*, *P. vivax*, *Plasmodium malariae*, and *Plasmodium ovale* were seen in 90.7%, 8.1%, 11.6%, and 5.0%, respectively. *Plasmodium vivax* occurred more frequently as a monoinfection than in combination with *P. falciparum*. All *P. vivax*–infected individuals showed heterozygous Duffy positivity, whereas this was the case for only 3.1% of patients with *P. falciparum* monoinfection and malaria-negative control subjects ($P < 0.01$). Based on PCR diagnosis, *P. vivax* is not rare in southern Rwanda. All episodes of *P. vivax* were observed in heterozygous Duffy-positive patients, whereas elsewhere in Africa, *P. vivax* is also reported in Duffy-negative individuals. Refined mapping of *Plasmodium* species is required to establish control and elimination strategies including all malaria species.

Plasmodium vivax causes only a fraction of global malaria episodes, but is the second-most common malaria pathogen after the dominant *Plasmodium falciparum*. The proportion of vivax malaria among all reported malaria cases varies widely, from 71.5% in the Americas and 40% in Southeast Asia to only 0.3% in sub-Saharan Africa (SSA).[1] Long considered rather benign, increasing evidence shows *P. vivax* to contribute substantially to the disease burden, including severe malaria and adverse pregnancy outcomes.[2–4] The low frequency of *P. vivax* in SSA is attributed to the virtual absence in African populations of the parasite's erythrocyte invasion receptor—that is, the Duffy blood group antigen (encoded by the Duffy-associated receptor chemokine [DARC]).[5] However, recent data show *P. vivax* infection among Duffy-negative individuals across Africa, suggesting alternative invasion pathways.[6] This points to a significant underestimation of vivax malaria on the African continent,[7] which is a result, in part, of the comparatively low sensitivity of microscopy in *P. vivax* detection, particularly in mixed infections.[8]

As for Rwanda, East Africa, cases resulting exclusively from *P. falciparum* have been reported to the WHO during the past decade.[1] Although *P. vivax* has been reported occasionally,[9,10] the actual epidemiology of that parasite in Rwanda is largely obscure. Rwanda has achieved a remarkable decline in the malaria burden during the past few years,[1] which can be attributed largely to the scale-up of vector control measures and widely available artemisinin combination therapy. Eliminating the *P. vivax* reservoir—that is, hepatic hypnozoites—requires the administration of primaquine, which, however, is not included in the national drug policy for *P. falciparum* malaria. Moreover, primaquine can cause dose-dependent hemolysis in individuals with glucose-6-phosphate dehydrogenase (G6PD) deficiency, which is common in Africa, and respective pretreatment testing would be needed.

The main objective of this study was to assess *P. vivax* prevalence among patients with uncomplicated malaria in 2018 and 2019 in Huye, southern Rwanda. In addition, we explored Duffy antigen genotypes among *P. vivax* patients, *P. falciparum* patients, and healthy control subjects.

In March through June 2018 and September through December 2019, we recruited consenting malaria patients (> 1 year of age) at Sovu Health Center and Kabutare District Hospital, Huye District, southern Rwanda (population, ~390,000; average altitude, 1,700 m; yearly rainfall, 1,200 mm; mean temperature, 19°C). Detailed study procedures are reported elsewhere.[10,11] In short, patients were febrile (≥ 37.5°C, axillary) or reported fever in the preceding 48 hours, and tested positive for *Plasmodium* infection by a rapid diagnostic test (RDT) (SD Bioline Malaria Ag Pf/Pan; Abbott Global Point of Care, Chicago, IL). Malaria was confirmed by thick blood film microscopy and treated with artemether–lumefantrine. Aliquots of blood were preserved as dried blood spots (Whatman 3MM chromatography paper, Cytiva, Marlborough, MA). Healthy control subjects (negative RDT, no fever) were recruited in 2019—preselected by age, gender, and village—to match respective parameters in malaria patients. DNA was extracted from whole blood (patients) and dried blood spots (patients and healthy control subjects) using the QIAamp blood mini kit (Qiagen, Hilden, Germany). *Plasmodium* infection and species were confirmed by nested PCR in 2018)[12] and real-time PCR assays in 2019 (commercial primers and probes; TIB MOLBIOL, Berlin, Germany).[13] The nested PCR targets the *18S ribosomal RNA (rRNA)* gene in the *Plasmodium* spp. genome. The real-time assay targets the *CytB* gene for *P. falciparum*, the *18S rRNA* gene for *P. vivax* and *Plasmodium ovale*, and the *MSP1* gene for *P. malariae*. To exclude potential *P. vivax* DNA contamination of blood samples, we confirmed *P. vivax* positivity in dried blood spot extracts. We assessed the *Duffy genotype (DARC)* in all *P. vivax* cases, in 100 *P. falciparum* cases (in 2018, $n = 36$; in 2019, $n = 64$), and in 98 control subjects (in 2019) by high-resolution melting curve assays on a Roche LightCycler 480 (Figure 1).[14,15] In addition, we assessed

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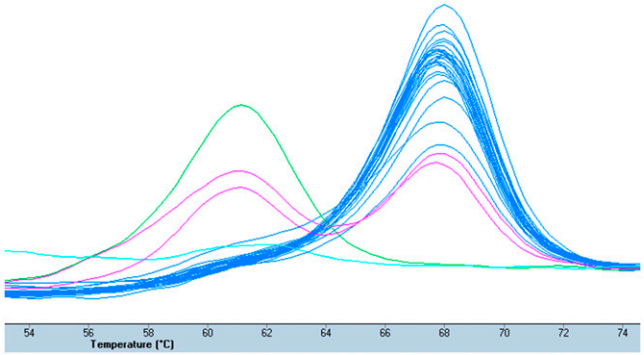

FIGURE 1. High-resolution melting curve of Duffy genotyping assay. Melting peaks of homozygous Duffy-positive (one peak at 61°C), homozygous Duffy-negative (-33T > C; one peak at 68°C), and heterozygous (two peaks) samples, and a negative control.

20 samples randomly for the Duffy genotype by Sanger sequencing (forward primer, 5′-CAGGAAGACCCAAGGCCA G-3′; reverse primer, 5′-CCATGGCACCGTTTGGTTCAGG-3′) (Figure 2). Primers and probes as well as sequencing were provided by Eurofins Genomics, Ebersberg, Germany.

In 2018 and 2019, we recruited 259 treatment-seeking malaria patients with PCR-confirmed *Plasmodium* infection. *Plasmodium falciparum*, *P. vivax*, *P. malariae*, and *P. ovale* were observed in 90.7%, 8.1%, 11.6%, and 5.0%, respectively (Table 1). One in seven falciparum malaria patients showed a mixed-species infection. *Plasmodium vivax* occurred more frequently as a monoinfection than in combination with *P. falciparum*.

All 21 *P. vivax*–infected individuals were heterozygous for the Duffy antigen (*DARC* -33T > C)—that is, they were Duffy positive. Among the *P. falciparum* cases (monoinfection), 98 were genotyped successfully for Duffy. Heterozygosity was 3.1% (3 of 98; allele frequency, 1.5%), and no Duffy-positive homozygosity was seen. Identically, among 98 successfully

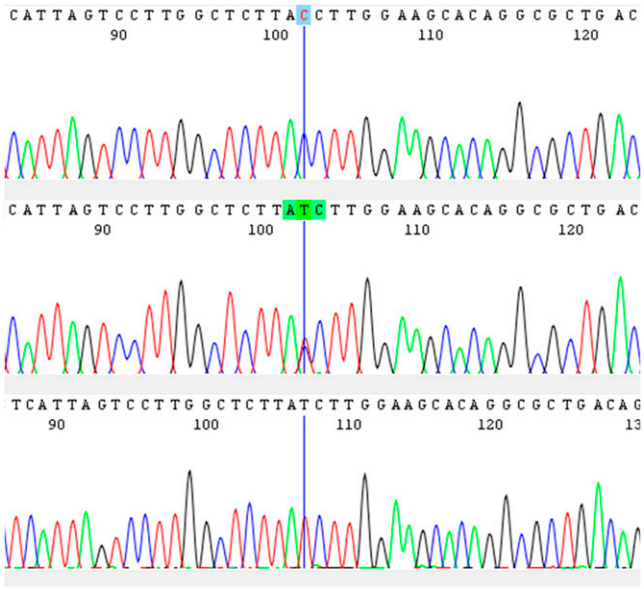

FIGURE 2. Alignment of a homozygous Duffy-negative (upper), a heterozygous (middle), and a homozygous Duffy-positive (lower) sample.

TABLE 1
Plasmodium species detected by PCR in patients in Huye, Rwanda, 2018 and 2019

Plasmodium species	2018 (*n* = 183), *n* (%)	2019 (*n* = 76), *n* (%)	Total (*N* = 259), *n* (%)
Plasmodium falciparum	168 (91.8)	67 (88.2)	235 (90.7)
Plasmodium vivax	15 (8.2)	6 (7.9)	21 (8.1)
Plasmodium malariae	24 (13.1)	6 (7.9)	30 (11.6)
Plasmodium ovale	12 (6.6)	1 (1.3)	13 (5.0)
Monoinfection			
P. falciparum	135 (73.8)	64 (84.2)	199 (76.8)
P. vivax	7 (3.8)	5 (6.6)	12 (4.6)
P. malariae	2 (1.1)	4 (5.3)	6 (2.3)
P. ovale	2 (1.1)	0 (0)	2 (0.8)
Mixed-species infection with *P. falciparum*			
P. vivax	6 (3.3)	1 (1.3)	7 (2.7)
P. malariae	20 (10.9)	2 (2.6)	22 (8.5)
P. ovale	8 (4.4)	1 (1.3)	9 (3.5)
Mixed-species infection without *P. falciparum*			
P. malariae + *P. vivax*	1 (0.5)	0 (0)	1 (0.4)
P. malariae + *P. ovale*	1 (0.5)	0 (0)	1 (0.4)

PCR = polymerase chain reaction.

genotyped healthy control subjects, Duffy heterozygosity was 3.1% (3 of 98; allele frequency, 1.5%), with no Duffy-positive homozygous individuals. Duffy positivity was associated significantly with *P. vivax* infection compared with control subjects without *Plasmodium* infection, and with malaria patients with *P. falciparum* infection (for both, $P < 0.01$).

Among 259 PCR-confirmed malaria cases from southern Rwanda, 8.1% had a *P. vivax* infection, including 4.6% as a monoinfection. This contrasts with the absence of reported vivax cases in Rwanda and has implications for malaria control. All vivax cases were Duffy positive (heterozygous carriage of *DARC* -33T > C), whereas 97% of healthy control subjects and patients with *P. falciparum* monoinfection were Duffy negative. To the best of our knowledge, this is the first report on Duffy genotypes in a Rwandan population.

Plasmodium vivax is considered to have more potential for geographic spreading in the Rwandan highlands compared with *P. falciparum* because of its broader temperature tolerance.[16] Also, *P. vivax* has a transmission advantage over *P. falciparum* because of its earlier onset of gametocyte development.[17] For the treatment of vivax malaria, chloroquine as well as 2 weeks of primaquine for relapse prevention are recommended in Rwanda.[1] Such treatment would eventually eliminate the *P. vivax* reservoir if cases were diagnosed. However, primaquine can cause dose-dependent acute hemolysis in individuals with G6PD deficiency. This trait is common but generally mild in SSA; in the study population, it is present in ~10% of the general population that overlaps with our study population.[18] Radical primaquine treatment thus requires G6PD deficiency testing in place, for example, by available rapid tests, and cautious primaquine administration in G6PD deficiency. The WHO recommends giving primaquine at 0.75 mg base/kg body weight once a week for 8 weeks in people with G6PD deficiency, with close supervision for hemolysis (instead of daily 0.25–0.5 mg base/kg body weight for 2 weeks).[19]

The occurrence of *P. vivax* infection exclusively in Duffy-positive hosts in our study should be interpreted with care. In nearby *P. vivax*–endemic Ethiopia, Duffy negativity among vivax carriers reached > 10%.[20] Our sample size of 21 is too small to rule out with confidence the presence of 10% Duffy-negative

P. vivax cases (e.g., at 2 of 21, 95% CI, 0.01–30.3). Elsewhere, *P. vivax* parasitemia has been found to be lower in Duffy-negative than in Duffy-positive hosts.[20,21] We may have missed low-density *P. vivax* infections at recruitment as a result of species-dependent sensitivity limitations of RDTs and microscopy.[8] Notwithstanding these limitations, our data do reveal a reservoir of *P. vivax* in a largely Duffy-negative population. When both Duffy alleles are present in a population, the Duffy-positive reservoir might facilitate *P. vivax* strains to develop mechanisms to invade Duffy-negative erythrocytes.[21]

The paradigm of *P. vivax* being benign has been challenged repeatedly,[2–4] and the use of molecular tools in diagnosis reveals its underestimation. Particularly in regions of Africa where malaria elimination is in reach, control and elimination strategies will require the inclusion of all malaria species. Refined mapping of *Plasmodium* species distribution is required.

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