Am. J. Trop. Med. Hyg., 90(6), 2014, pp. 1098–1101
doi:10.4269/ajtmh.13-0477

Short Report: A Case of Quadruple Malaria Infection Imported from Mozambique to Japan

Masayuki Oki, Satomi Asai,* Yumiko Saito-Nakano, Taira Nakayama, Yumiko Tanaka, Hiroshi Tachibana, Hiroshi Ohmae, Tomoyoshi Nozaki, and Hayato Miyachi

Department of Internal Medicine, Department of Laboratory Medicine, and Department of Infectious Diseases, Tokai University School of Medicine, Isehara, Kanagawa, Japan; Clinical Laboratory Center, Tokai University Hospital, Isehara, Kanagawa, Japan; Department of Parasitology, National Institute of Infectious Diseases, Shinjuku, Tokyo, Japan

Abstract. A 35-year-old Japanese man had an intermittent fever and mild headache for eight weeks after he returned to Japan from working in Mozambique. He had taken antimalarial prophylaxis (doxycycline) for 25 weeks, and stopped taking this drug two weeks after his return. Microscopic examination of a peripheral blood smear showed a mixed infection with *Plasmodium vivax*, *P. falciparum*, and *P. ovale*. In addition, a nested polymerase chain reaction and subsequent sequencing detected specific DNA sequences of four species of *Plasmodium*, including *P. malariae.* The patient was successfully treated with artemether-lumefantrine and primaquine phosphate. The present case is a rare instance of a mixed infection with four species of *Plasmodium*. Nonimmune persons in malaria-endemic areas may have a risk of mixed infection. All four species must be identified by using sensitive and specific tests, such as a nested polymerase chain reaction, in addition to conventional morphologic identification.

Although many cases of mixed malaria infection have been reported in malaria-endemic countries, coincidental infection with more than one species of *Plasmodium* spp. is rare in non-endemic countries.[1,2] In Japan, only approximately 50–80 cases of malaria are reported each year, and are all imported malaria.[3] In this report, we describe a Japanese man who was given a diagnosis of a mixed infection with four species of *Plasmodium* (*P. vivax*, *P. falciparum*, *P. ovale wallikeri*, and *P. malariae*) by using a nested polymerase chain reaction (PCR) after he returned from Mozambique.

CASE REPORT

A 35-year-old Japanese man had intermittent fever and mild headaches for eight weeks after returning from Mozambique. He returned to Japan at the end of June after working as an instructor in operating construction machinery during for two years (June 2010–June 2012). He had taken antimalarial prophylaxis (doxycycline) for 25 weeks from January through the second week of July in 2012. He had no symptoms suggestive of malaria while in Mozambique, but a slight fever developed on August 25, 2012, in Japan. A high fever developed on August 30 and he was admitted to Tokai University Hospital on September 3.

Physical examination at admission showed a body temperature of 37.9°C, a respiratory rate of 27 beats/minute, a heart rate of 100 beats/minute, a blood pressure of 108/68 mm Hg, anemia in the palpebral conjunctiva, hepatomegaly, and splenomegaly. The hemoglobin level was 11.3 g/dL, the mean corpuscular volume was 88.7 fL, and the platelet count was 54×10^9/L. Biochemical tests showed increased serum levels of aspartate aminotransferase (32 U/L, reference value < 30 U/L), lactate dehydrogenase (379 U/L, reference range = 110–219 U/L) and C-reactive protein (5.57 mg/dL, reference value < 0.3 mg/dL). A coagulation test result for the prothrombin time–international normalized ratio was slightly prolonged (1.18, reference range = 0.80–1.10). An ultrasonographic examination showed hepatomegaly (140 mm thickness along the mammary line), splenomegaly (160 × 75 mm), and reactive swelling of the portal hepatic lymph nodes.

A rapid diagnostic test (OptiMAL-IT; DiaMed, Cressier, Switzerland) for malaria with blood of the patient showed three positive bands for the control, *Plasmodium* spp., and *P. falciparum*. A thin blood smear stained with Giemsa showed *P. vivax*, *P. falciparum*, and *P. ovale* (Figure 1). Trophozoites of *P. vivax* were dominant in the microscopic examination, and the total parasitemia including other species was 1.7%.

The patient was treated with Coartem (artemether 20 mg/lumefantrine 120 mg) in a six-dose regimen for three days (total = 24 tablets), followed by primaquine, 15 mg/day for 14 days. The patient soon became afebrile and subsequently left the hospital on the seventh day of hospitalization. Since that time, *Plasmodium* has not been detected, and his symptoms did not recur.

To identify the exact *Plasmodium* species, *Plasmodium* DNA was extracted from the patient's blood by using a QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany)[4,5] and was subjected to a nested PCR specific for the 18S ribosomal RNA gene. *Plasmodium* genus–specific primers and four pairs of species-specific primers described in a previous study were used for the nested PCR.[6] Results showed that the patient was infected with four species of *Plasmodium*, including *P. malariae*, which was not identified in the blood smear by microscopy (Figure 2). Amplified fragments were cloned into a vector and sequenced. The fragment amplified with *P. falciparum*-specific nested primers showed 100% identity with the *P. falciparum* 18S ribosomal RNA gene (GenBank accession no. JQ627152.1, expected = 3×10^{-39}). Fragments amplified with *P. vivax*-specific, *P. ovale*-specific, and *P. malariae*-specific nested primers showed a single nucleotide substitution in the *P. vivax* 18S ribosomal RNA gene (GenBank accession no. JQ627158, expected = 1×10^{-38}), the *P. ovale* 18S ribosomal RNA gene (GenBank accession no. AB182490.1, expected = 9×10^{-44}) and the *P. malariae* 18S ribosomal RNA gene (GenBank accession no. M54897.1, expected = 9×10^{-44}), respectively.

Because two types of *P. ovale* strains (classic and variant types) are prevalent in Africa and Asia, and it has been reported that the two types are distinct species,[7] we generated specific nested-primers specific for the *P. ovale* cytochrome b

*Address correspondence to Satomi Asai, Department of Laboratory Medicine, Tokai University School of Medicine, 143 Shimokasuya, Isehara, Kanagawa 259-1193, Japan. E-mail: sa@is.icc.u-tokai.ac.jp

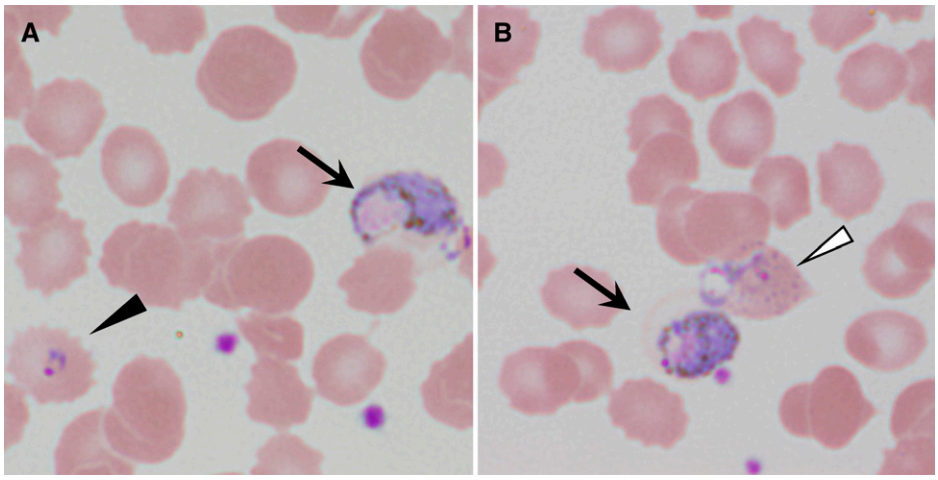

FIGURE 1. Microscopy of a Giemsa-stained peripheral blood smear of the patient. **A**, Ring form of *Plasmodium falciparum* (**arrowhead**) and a late trophozoite of *P. vivax* (**arrow**) were observed. **B**, Trophozoite of *P. vivax* (**arrow**) and trophozoite of *P. ovale* (**white arrowhead**) were observed.

gene (*cytb*) to determine the *P. ovale* species (Figure 3A). Specific bands corresponding to a 257-basepair fragment, were amplified from the sample. No specific bands were amplified from *P. vivax* genomic DNA (MRA-41, provided by the Malaria Research and Reference Reagent Resource Center) under these conditions when used as a negative control. Sequencing of the amplified fragment showed 100% identity with the *P. ovale wallikeri cytb* gene (GenBank accession no. HQ712053, expected = 1×10^{-127}), and single-nucleotide polymorphisms specific for *P. ovale wallikeri* were detected (Figure 3B). These results showed that the patient was infected with four malaria species (*P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale wallikeri*).

DISCUSSION

Some triple or quadruple mixed malaria infections have been reported among semi-immune residents in malaria-endemic areas of Asia[8,9] and Africa.[10,11] However, triple or quadruple mixed malarial infections have been rare among non-residents of such areas or among nonimmune persons. The present case is the first imported case of quadruple malaria in a nonimmune Japanese patient.

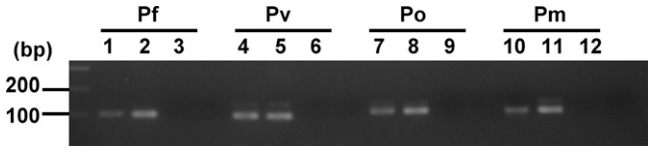

FIGURE 2. Detection of a *Plasmodium* 18S ribosomal RNA gene fragment by using nested polymerase chain reaction analysis. Amplification was performed by using a standard protocol for 35 and 20 cycles for the first and the second amplifications, respectively. Samples were first amplified with *Plasmodium* genus-specific primers and then amplified with *P. falciparum*-specific (lanes 1–3), *P. vivax*-specific (lanes 4–6), *P. ovale*-specific (lanes 7–9), and *P. malariae*-specific (lanes 10–12) oligonucleotide sets, respectively. Specific bands corresponding to *P. falciparum* (101 basepairs [bp] , lanes 1 and 2), *P. vivax* (104 bp, lanes 4 and 5), *P. ovale* (115 bp, lanes 7 and 8), and *P. malariae* (115 bp, lanes 10 and 11) were amplified. Patient samples: lanes 1, 4, 7, and 10. Positive controls: lanes 2, 5, 8, and 11. Negative controls: lanes 3, 6, 9, and 12.

In mixed malaria infections, the level of parasitemia is different for each *Plasmodium* spp. In Japan, some mixed infections have been confirmed by combined use of morphologic detection and other alternative methods, including immunoassays and the molecular analyses.[12,13] The findings in this report, as well as in other reports,[14,15] indicate that molecular detection methods, such as nested PCR and real-time PCR, have superior sensitivity and specificity, which enables identification of low levels of infection and differentiation of species.

For antimalarial chemoprophylaxis, mefloquine is recommended in Japan, but it cannot be used in areas with mefloquine-resistant malaria. Doxycycline and atovaquone/proguanil can be used in all malaria-endemic areas, and these drugs are used in Japan. These drugs are effective against erythrocytic forms and also inhibit development of the normal liver stages, but they do not affect hypnozoites. Therefore, development of clinical malaria from hypnozoites of *P. vivax* or *P. ovale* has been reported more than two months after returning among travelers who continued chemoprophylaxis based on standard regimens.[16]

Daily use of doxycycline is recommended during travel to malaria-endemic areas and for four weeks after returning to a area where malaria is not endemic. However, the patient in this study stopped taking doxycycline after two weeks. Similar prophylactic failures for doxycycline against *P. falciparum* or *P. vivax* are associated with use of inadequately low doses.[17,18] Moreover, the prevalence of *P. falciparum* isolates with reduced susceptibility to mefloquine and doxycycline has been reported in Africa.[19,20]

In nonimmune persons taking chemoprophylaxis, efficacy differs for each *Plasmodium* spp. Furthermore, different levels of drug resistance to each strain and the dose of drugs used influence the clinical manifestations in cases with chemoprophylactic failure. *P. falciparum* might dominate over *P. vivax* by inducing a primary infection, as occasionally observed in patients simultaneously infected with the two species. In the present case, however, *P. vivax* predominated over *P. falciparum*. A partial effect of doxycycline was conceivably obtained because the parasitemia with *P. falciparum* and *P. malariae* was low. Detection of *P. malariae* was difficult by

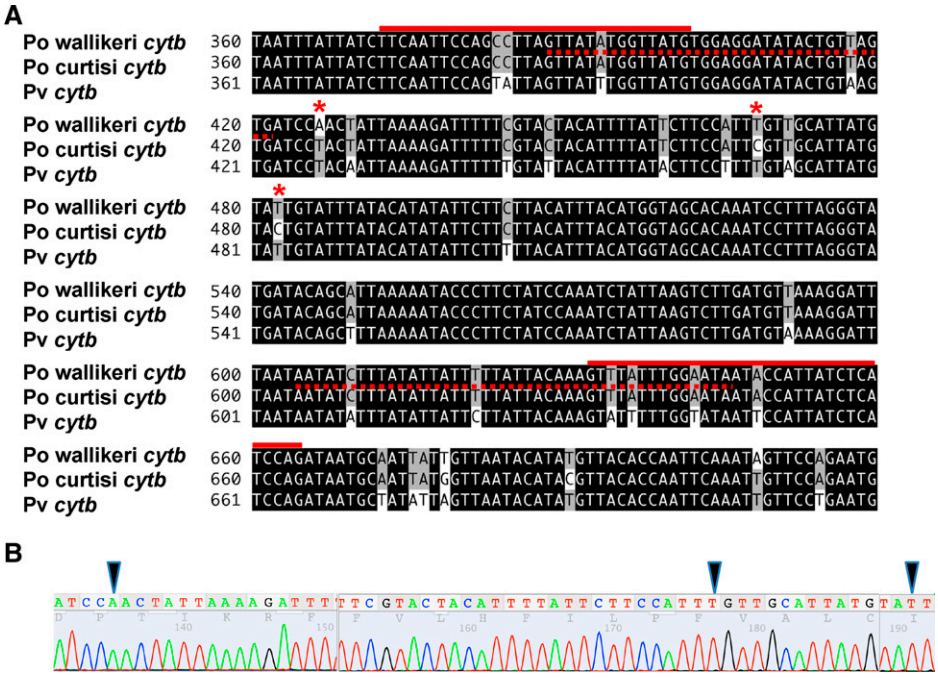

FIGURE 3. Sequence analysis of the *Plasmodium ovale* (Po) *wallikeri cytb* gene. **A**, Nucleotide alignment of the *cytb* genes from *P. ovale wallikeri* (Gen Bank accession no. GU723535), *P. ovale curtisi* (GU723514), and *P. vivax* (Pv) (AY791525). Red lines indicate regions where nested primers are annealed, respectively. Oligonucleotide sequences used to amplify polymorphic regions of *P. ovale* spp. were outer primers PoCytb-1F (5′-TTC AAT TCC AGC CTT AGT TAT ATG GTT ATG-3′) and PoCytb-2R (5′-CTG GAT GAG ATA ATG GTA TTA TTC CAA ATA AAC-3′) and inner primers PoCytb-3F (5′-GTT ATA TGG TTA TGT GGA GGA TAT ACT GTT AGT G-3′) and PoCytb-4R (5′-TAT TCC AAA TAA ACT TTG TAA TAA AAA TAA TAT AAA GAT ATT-3′). A polymerase chain reaction was performed as described above for 40 and 30 cycles for the first and second amplifications, respectively. Three single nucleotide polymorphism sites included this region specific for *P. ovale wallikeri* are indicated by red stars. **B**, Sequence analysis of a fragment amplified by using nested polymerase chain reaction identified *P. ovale wallikeri*–specific nucleotide polymorphisms, as indicated by arrowheads.

microscopy and was confirmed by using the nested PCR. A variant strain of *P. ovale* could not be detected by PCR for the classic strain, and its infection was confirmed by the more specific protocol of PCR used for the present case.

Although the Duffy-negative phenotype $FY*BE^{ES}$, which is not susceptible to infection with *P. vivax*, is dominant in sub-Saharan Africa,[21] Duffy-negative persons infected with *P. vivax* have been reported and suggested as a possible reservoir of *P. vivax* to Duffy-positive persons, such as those seen in Japanese persons.[22] As more nonimmune persons visit malaria-endemic countries in Africa, there are likely to be more cases of imported mixed infections of *Plasmodium* species, including *P. vivax*. Nonimmune persons who stay in malaria-endemic areas for long periods face an increasing risk of mixed infection, and clinical features are modified by chemoprophylaxis. When nonimmune persons show atypical clinical features for malaria or when chemoprophylaxis failure is suspected, all four species of human malaria parasites should be considered as causative agents, and the patients should be examined by using sensitive and specific tests, such as the PCR, in addition to conventional morphologic analyses.

Received August 15, 2013. Accepted for publication March 13, 2014.

Published online April 14, 2014.

Acknowledgments: The *P. vivax* genomic DNA deposited by Dr. C. Gowda was obtained from the Malaria Research and Reference Reagent Resource Center.

Financial support: This study was supported by a grant for Research on Emerging and Reemerging Infectious Diseases from the Ministry of Health, Labour and Welfare of Japan (H23-Shinko-Ippan-014 and H25-Shinko-Shitei-002).

Authors' addresses: Masayuki Oki and Taira Nakayama, Department of Internal Medicine, Tokai University School of Medicine, 143 Shimokasuya, Isehara, 259-1193, Japan, E-mails: okidoki@is.icc.u-tokai.ac.jp and taira_nakayama@hotmail.com. Satomi Asai and Hayato Miyachi, Department of Laboratory Medicine, Tokai University School of Medicine, 143 Shimokasuya, Isehara, 259-1193, Japan, E-mails: sa@is.icc.u-tokai.ac.jp and miyachi@is.icc.u-tokai.ac.jp. Yumiko Saito-Nakano, Hiroshi Ohmae, and Tomoyoshi Nozaki, Department of Parasitology, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan, E-mails: yumiko@nih.go.jp, h-ohmae@nih.go.jp, and nozaki@nih.go.jp. Yumiko Tanaka, Clinical Laboratory Center, Tokai University Hospital, 143 Shimokasuya, Isehara, 259-1193, Japan, E-mail: uitra@is.icc.u-tokai.ac.jp. Hiroshi Tachibana, Department of Infectious Diseases, Tokai University School of Medicine, 143 Shimokasuya, Isehara, 259-1193, Japan, E-mail: htachiba@is.icc.u-tokai.ac.jp.

REFERENCES

1. Marques PX, Saúte F, Pinto VV, Cardoso S, Pinto J, Alonso PL, Rosário VE, Arez AP, 2005. *Plasmodium* species mixed infections in two areas of Manhiça District, Mozambique. *Int J Biol Sci 1:* 96–102.
2. Mayxay M, Pukrittayakanee S, Newton PN, White NJ, 2004. Mixed-species malaria infections in humans. *Trends Parasitol 20:* 233–240.
3. Infectious Disease Surveillance Center, Malaria. 2006–2009. Available at: http://idsc.nih.go.jp/disease/malaria/2010week38.html. Accessed February 2013.
4. Haranaga S, Akashi M, Yara S, Tohyama M, Toyama M, Ishimine T, Miyara T, Shinzato T, Higa F, Tateyama M,

Saito A, Toma H, 2002. Two cases of mixed infection of malaria diagnosed by PCR method. *Jpn J Infect Dis 76:* 571–575.

5. Saito-Nakano Y, Tanabe K, Mita T, 2011. Identification of pyrimethamine- and chloroquine-resistant *Plasmodium falciparum* in Africa between 1984 and 1998: genotyping of archive blood samples. *Malar J 10:* 388. doi:10.1186/1475-2875-10-388.
6. Saito-Nakano Y, Tanabe K, Kamei K, Iwagami M, Komaki-Yasuda K, Kawazu S, Kano S, Ohmae H, Endo T, 2008. Genetic evidence for *Plasmodium falciparum* resistance to chloroquine and pyrimethamine in Indochina and the Western Pacific between 1984 and 1998. *Am J Trop Med Hyg 79:* 613–619.
7. Kimura M, Kaneko O, Inoue A, Ishii A, Tanabe K, 1995. Amplification by polymerase chain reaction of *Plasmodium falciparum* DNA from Giemsa-stained thin blood smears. *Mol Biochem Parasitol 70:* 193–197.
8. Sutherland CJ, Tanomsing N, Nolder D, Oguike M, Jennison C, Pukrittayakamee S, Dolecek C, Hien TT, do Rosário VE, Arez AP, Pinto J, Michon P, Escalante AA, Nosten F, Burke M, Lee R, Blaze M, Otto TD, Barnwell JW, Pain A, Williams J, White NJ, Day NP, Snounou G, Lockhart PJ, Chiodini PL, Imwong M, Polley SD, 2010. Two nonrecombining sympatric forms of the human malaria parasite *Plasmodium ovale* occur globally. *J Infect Dis 15:* 1544–1550.
9. Snounou G, Viriyakosola S, Zhua XP, Jarra W, Pinheiro L, do Rosario VE, Thaithong S, Brown KN, 1993. High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction. *Mol Biochem Parasitol 61:* 315–320.
10. Purnomo SA, Gomez-Saladin E, Bangs MJ, 1999. Rare quadruple malaria infection in Iran Jaya Indonesia. *J Parasitol 85:* 574–579.
11. Arez AP, Pinto J, Pålsson K, Snounou G, Jaenson TG, do Rosário VE, 2003. Transmission of mixed *Plasmodium* species and *Plasmodium falciparum* genotypes. *Am J Trop Med Hyg 68:* 161–168.
12. Miura T, Kimura M, Koibuchi T, Endo T, Nakamura H, Odawara T, Wataya Y, Nakamura T, Iwamoto A, 2005. Clinical characteristics of imported malaria in Japan: analysis at a referral hospital. *Am J Trop Med Hyg 73:* 599–603.
13. Higa F, Tateyama M, Tasato D, Karimata Y, Nakamura H, Miyagi K, Haranaga S, Hirata T, Hokama A, Cash HL, Toma H, Fujita J, 2013. Imported malaria cases in Okinawa prefecture, Japan. *Jpn J Infect Dis 66:* 32–35.
14. Boonma P, Christensen PR, Suwanarusk R, Price RN, Russell B, Lek-Uthai U, 2007. Comparison of three molecular methods for the detection and speciation of *Plasmodium vivax* and *Plasmodium falciparum*. *Malar J 6:* 124., doi: 10.1186/1475-2875-6-124.
15. Rosanas-Urgell A, Mueller D, Betuela I, Barnadas C, Iga J, Zimmerman PA, del Portillo HA, Siba P, Mueller I, Felger I, 2010. Comparison of diagnostic methods for the detection and quantification of the four sympatric *Plasmodium* species in field samples from Papua New Guinea. *Malar J 9:* 361.
16. Schwartz E, Parise M, Kozarsky P, Cetron M, 2003. Delayed onset of malaria: implications for chemoprophylaxis in travelers. *N Engl J Med 349:* 1510–1516.
17. Pang L, Limsomwong N, Singharaj P, 1988. Prophylactic treatment of vivax and falciparum malaria with low-dose doxycycline. *J Infect Dis 158:* 1124–1127.
18. Shmuklarsky MJ, Boudreau EF, Pang LW, Smith JI, Schneider I, Fleckenstein L, Abdelrahim MM, Canfield CJ, Schuster B, 1994. Failure of doxycycline as a causal prophylactic agent against *Plasmodium falciparum* malaria in healthynonimmune volunteers. *Ann Intern Med 120:* 294–299.
19. Fall B, Diawara S, Sow K, Baret E, Diatta B, Fall KB, Mbaye PS, Fall F, Diémé Y, Rogier C, Wade B, Bercion R, Pradines B, 2011. Ex vivo susceptibility of *Plasmodium falciparum* isolates from Dakar, Senegal, to seven standard anti-malarial drugs. *Malar J 10:* 310.
20. Fall B, Pascual A, Sarr FD, Wurtz N, Richard V, Baret E, Diémé Y, Briolant S, Bercion R, Wade B, Tall A, Pradines B, 2013. *Plasmodium falciparum* susceptibility to anti-malarial drugs in Dakar, Senegal, in 2010: an ex vivo and drug resistance molecular markers study. *Malar J 12:* 107.
21. Howes RE, Patil AP, Piel FB, Nyangiri OA, Kabaria CW, Gething PW, Zimmerman PA, Barnadas C, Beall CM, Gebremedhin A, Ménard D, Williams TN, Weatherall DJ, Hay SI, 2011. The global distribution of the Duffy blood group. *Nat Commun. 2:* 266.
22. Mendes C, Dias F, Figueiredo J, Mora VG, Cano J, de Sousa B, do Rosario VE, Benito A, Berzosa P, Arez AP, 2011. Duffy negative antigen is no longer a barrier to *Plasmodium vivax*: molecular evidences from the African west coast (Angola and Equatorial Guinea). *PLoS Negl Trop Dis 5:* e1192.