Evidence for the Transmission of *Plasmodium vivax* in the Republic of the Congo, West Central Africa

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***Plasmodium vivax* is not thought to be transmitted in western and central Africa, because of the very high prevalence of the red blood cell Duffy-negative phenotype in local populations, a condition which is thought to confer complete resistance against blood infection with *P. vivax*. There are, however, persistent reports of travelers returning from this region with *P. vivax* infections. To investigate whether transmission occurs in this region, the presence of antibodies specific to *P. vivax* preerythrocytic-stage antigens was assessed in individuals from the Republic of the Congo. A total of 55 (13%) of 409 samples tested by enzyme-linked immunosorbent assay had antibodies to *P. vivax*–specific antigens.**

Transmission of *Plasmodium vivax* is not generally thought to occur in western or central continental Africa, where 95%–99% of the human population is refractory to *P. vivax* blood infection because of the protective effect of the red blood cell (RBC) Duffy-negative condition [1, 2]. Despite this, reports of Duffy-positive nonimmune travelers returning from these areas with infections diagnosed as being due to *P. vivax* are common and have persisted over many years of surveillance [3]. Furthermore, a recent report has implied that *P. vivax* transmission may occur in a population consisting of very high percentages of Duffy-negative individuals, with the presence of *P. vivax*–specific proteins reported in 0.65% of mosquitoes from an area of western Kenya [4]. An additional study reported evidence of *P. vivax* infections in 2 Duffy-negative individuals in Brazil [5]. Some investigators have interpreted such findings as implying that the parasite may be in the process of evolving the ability to infect Duffy-negative individuals [6]. However, we have argued elsewhere [3] that *P. vivax* transmission can be expected in populations with high levels of RBC Duffy negativity and in which malaria transmission intensities are sufficiently high, as is the case in many areas of western and central Africa. Notwithstanding this expectation, a recent polymerase chain reaction (PCR)–based parasite species–typing survey of 2588 blood samples obtained from patients in 9 western and central African countries failed to find any *P. vivax* parasites, except on the island of Sao Tome, where *P. vivax* transmission is known to occur [3].

In the present study, we used serological testing to search for evidence of *P. vivax* transmission in Pointe-Noire, a city on the west coast of the Republic of the Congo, where >95% of the population is expected to be RBC Duffy negative and, thus, refractory to *P. vivax* blood infection. In September 2007, we collected blood samples from 415 Pointe-Noire residents and searched for the presence of antibodies to the *P. vivax*–specific antigens *P. vivax* circumsporozoite protein (PvCSP) and *P. vivax* merozoite surface protein 1 (PvMSP1). Both antigens are expressed in liver-stage parasites and induce antibodies even in the absence of *P. vivax* blood infection [7]. Detection of antibodies to these *P. vivax*–specific antigens in a largely Duffy-negative human population could be evidence of its transmission there.

Materials and methods. By means of passive case detection, 415 samples were collected from the Mbota health center in Pointe-Noire, located on the west coast of the Republic of

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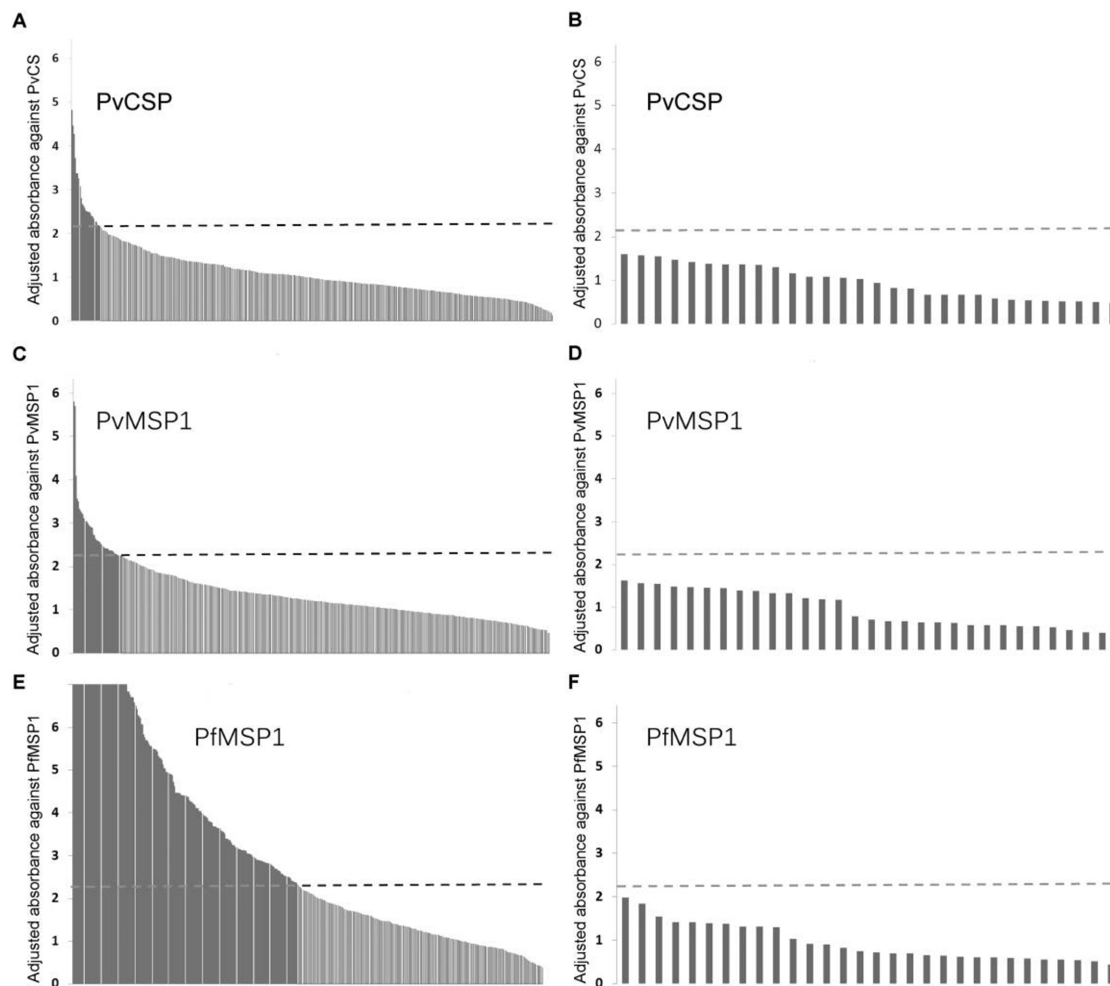

Figure 1. Adjusted absorbances against *Plasmodium vivax*–specific antigens *P. vivax* circumsporozoite protein (PvCSP) *(A), P. vivax* merozoite surface protein 1 (PvMSP1) *(C),* and the *Plasmodium falciparum* antigen *P. falciparum* merozoite surface protein 1 (PfMSP1) *(E)* for 409 individuals from Pointe-Noire, Republic of the Congo. *B, D,* and *F,* Corresponding adjusted absorbances for nonexposed individuals (from Japan and the United Kingdom). Cutoff values are denoted by horizontal dashed lines, and positive individuals are denoted by the areas shaded dark gray. The cutoff value was calculated as the mean value (+3 standard deviations) of the adjusted absorbances of 30 nonexposed individuals. All absorbances were measured at 405 nm.

the Congo, during September 2007. No age restrictions were applied to individuals from whom samples were obtained. The samples were collected on Whatman 31ETCHR filter paper. Travel histories were obtained from individuals before sample collection, and those who had traveled outside of the Republic of the Congo were excluded from the study ($n = 6$). Approval of the sample collection was obtained from the ethics committee at the Research Institute of Microbial Diseases, Osaka University (Osaka, Japan), and sampling was authorized by the administrative authority of the Ministry for Research and the Ministry for Health in the Republic of the Congo. Written informed consent was obtained from individual patients, and antimalarial treatment was provided when appropriate. An additional 10 blood samples were collected from *P. vivax*–infected patients from Siverek-Sanliurfa in the southeast of Turkey, for use as positive controls, and from 30 individuals from Japan and the United Kingdom with no previous exposure to *P. vivax* (ie, nonexposed individuals), for use as negative control samples (for collection details, see the description of supplementary methods in the Appendix, which appears only in the electronic version of the *Journal*).

All samples were screened by enzyme-linked immunosorbent assay (ELISA) for the detection of immunoglobulin G antibodies to 3 *Plasmodium*-specific proteins. The first of these proteins was PvCSP recombinant protein. This *Escherichia coli*–expressed recombinant protein encompasses the N-terminal and C-terminal regions of PvCSP flanking a chimeric repeat region [8]. The second protein, PvMSP1 recombinant protein, was expressed using a wheat germ cell-free protein translation system [9] that encompasses N-terminal blocks 1 and 2 of

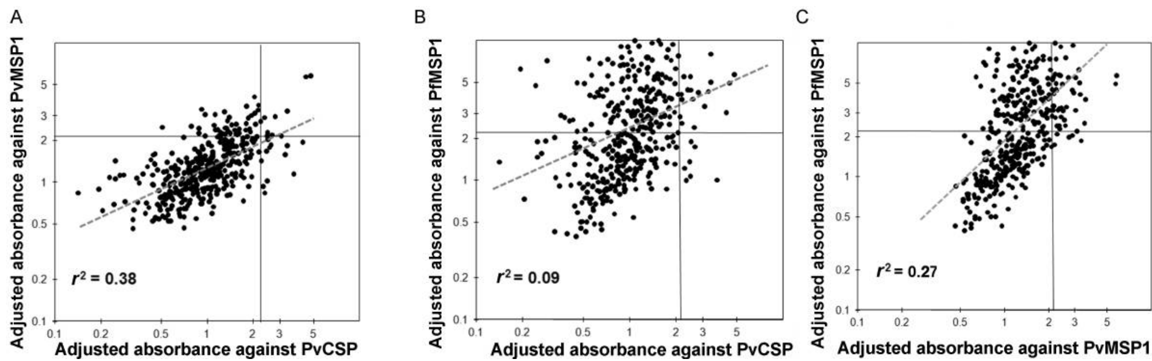

Figure 2. Linear regression analysis of the association between the adjusted absorbances against the *Plasmodium vivax* antigens *P. vivax* merozoite surface protein 1 (PvMSP1) and *P. vivax* circumsporozoite protein (PvCSP) *(A),* the *Plasmodium falciparum* antigen *P. falciparum* merozoite surface protein 1 (PfMSP1) and PvCSP *(B),* and PfMSP1 and PvMSP1 *(C)* for 409 individuals from Pointe-Noire, Republic of the Congo. Coefficient of determination values (r^2) for each antigen pair are shown on the graph, and linear regression lines are denoted by dashed gray lines. The solid horizontal and vertical lines denote the positive cutoff values for each antigen.

PvMSP1 (Ser$_{72}$ to His$_{432}$ [based on the *Sal*I sequence {GenBank accession number PVX_099980}]). The third protein, *Plasmodium falciparum* merozoite surface protein 1 (PfMSP1) recombinant protein, was an *E. coli*–expressed recombinant protein of N-terminal blocks 1–6 (M1/6) of the *P. falciparum* MAD20 sequence [10]. A detailed description of the ELISA protocol is provided in the Appendix.

To adjust for interplate variations, adjusted absorbances were obtained by dividing the mean optical density (OD) value of 2 repeats for each individual sample by the mean OD value for the same 4 nonexposed individuals assayed on the same 96-well plate (under identical conditions). Samples with adjusted OD values (+3 standard deviations) that were greater than the mean value for 30 nonexposed serum samples were considered to be positive for antibodies to the antigen tested. In the case of a positive result for PvCSP or PvMSP1, blood samples from the same patients were used for extraction of host and parasite DNA for parasite species typing and determination of host Duffy status.

Results. Figure 1 shows the results of ELISAs performed on the 409 samples collected from patients presenting to Mbota health center in Pointe-Noire, Republic of the Congo. For 25 (6%) of these samples, adjusted anti-PvCSP absorbance readings were greater than the mean value (+3 standard deviations) for 30 serum samples obtained from nonexposed individuals and were therefore considered to be positive for antibodies to this protein. For 39 (10%) of the samples, adjusted absorbance readings were greater than the cutoff value noted for PvMSP1. A total of 197 individuals (48%) were found to be positive for antibodies to PfMSP1, a *P. falciparum* antigen. All *P. vivax*–positive samples were independently tested twice more in duplicate, and the same positive results were obtained.

Of the 25 samples that were positive for PvCSP antibodies, 9 (36%) were also positive for antibodies to PvMSP1, and 16 (64%) were positive for antibodies to PfMSP1. Of the 39 samples that were positive for PvMSP1, 31 (79%) were also positive for PfMSP1. To investigate the possibility that there was cross-reactivity between antibodies to *P. falciparum* and *P. vivax* antigens, correlation and linear regression analyses were performed for the antigen pairs PvCSP/PvMSP1, PvMSP1/PfMSP1, and PvCSP/PfMSP1. Adjusted absorbance values were log transformed to meet the normality and homoscedasticity assumptions of the analysis, and coefficient of determination (r^2) values and linear regression lines were generated (Figure 2). There was a highly significant medium-strength positive correlation between antibody responses against PvCSP and PvMSP1 ($r^2 = 0.38$; 409 *df;* $P < .001$) but a much weaker, although still significant, low correlation between PvCSP and PfMSP1 ($r^2 = 0.09$; 409 *df;* $P < .001$). There was a stronger correlation between PvMSP1 and PfMSP1 ($r^2 = 0.27$; 409 *df;* $P < .001$), but this was also much weaker than the correlation between the 2 *P. vivax* antigens. Furthermore, serum antibody absorbance ELISA experiments performed with known positive serum samples incubated separately with *P. vivax* and *P. falciparum* MSP1 antigens showed no evidence of cross-reactivity between the respective antibodies (figure 3, which appears only in the electronic version of the *Journal*).

We assessed whether various factors (patient age or sex; pres-

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Figure 3. Enzyme-linked immunosorbent assay results for known *Plasmodium falciparum* antibody– and *Plasmodium vivax* antibody–positive serum samples incubated with *P. vivax* merozoite surface protein 1 (PvMSP1) and *P. falciparum* merozoite protein surface protein 1 (PfMSP1) antigens and tested for antibody responses to PvMSP1 and PfMSP1 antigens.

Table 1. Descriptive Statistics for Adjusted Absorbencies against 3 Antigens for 409 Individuals from Pointe-Noire, Republic of the Congo

This table is available in its entirety in the online edition of *The Journal of Infectious Diseases.*

ence or absence of parasites; parasite species, if infected; and district of residence) were associated with an increased probability of seropositivity against *P. vivax* antigens. There was no association between the district of residence and the presence of antibodies to *P. vivax* or *P. falciparum* (Table 1, which appears only in the electronic version of the *Journal*). Similarly, there was no strong correlation between age and the presence of antibodies to PvCSP ($r^2 = 0.09$, 409 *df; P* < .01), PvMSP1 ($r^2 = 0.13$; 409 *df; P* < .01), and PfMSP1 ($r^2 = 0.16$; 409 *df; P* < .01), as determined by Spearman rank correlation tests. The presence or absence of parasites in blood, as detected by microscopy, as well as whether those parasites were *P. falciparum, Plasmodium malariae,* or *Plasmodium ovale,* was not correlated with the presence of antibodies to either *P. vivax* or *P. falciparum;* however, the numbers of *P. malariae*– and *P. ovale*–infected individuals were low ($n = 5$ and $n = 7$, respectively), precluding statistical analysis (Table 1). Interestingly, females were significantly more likely than males to be seropositive for *P. vivax* antibodies, with 45 (17%) of 269 females positive for antibodies to PvCSP or PvMSP1, or both, compared with 10 (7%) of 145 males (6.51, by χ^2 test; 1 *df*; $P = .01$). There was, however, no difference in seropositivity for *P. falciparum* antibodies between the sexes, with 137 (51%) of 269 females and 60 (41%) of 145 males having positive responses against PfMSP1 (1.81, by χ^2 test; 1 *df;* $P = .18$).

DNA was extracted from the 55 samples for which positive antibody responses against either of the 2 *P. vivax*–specific antigens were demonstrated by ELISA. *Plasmodium* species identification was performed by polymerase chain reaction (PCR), and *P. vivax* DNA was not detected in any samples. The Duffy genotype status of the 55 individuals was determined by PCR [11], and all these individuals were found to be homozygous carriers of the $FY^{*}B^{null}$ allele and, thus, of the RBC Duffy-negative phenotype.

Discussion. We have shown that the serum samples from 55 (13%) of 409 individuals from Pointe Noire in the Republic of the Congo contained antibodies to the *P. vivax*–specific antigens PvCSP (25 samples [6%]), PvMSP1 (39 samples [9.5%]), or both (9 samples [2.2%]). These results suggest that *P. vivax* is transmitted in an area of west central Africa where the frequency of the Duffy-negative genotype is 95%–99% [1]. This finding goes against the current orthodoxy that *P. vivax* is not transmitted in western Africa and offers an explanation for the many cases of *P. vivax* contracted by Duffy-positive travelers in this region.

It has been established elsewhere [7] that Duffy-negative individuals who are refractory to the blood stages of *P. vivax* may develop antibodies to such antigens as CSP and MSP1, which are expressed in the preerythrocytic stages of this parasite in areas of endemicity. This finding is supported by evidence of the establishment of preerythrocytic immunity in individuals undergoing anti–blood-stage chemoprophylaxis for *P. falciparum* [12] and in mice with *Plasmodium yoelii* [13].

Although initial experiments indicated that there was no cross-reactivity between antibodies to the PvMSP1 and PfMSP1 antigens used in the present study, we did find a weak correlation between the antibody responses to the 2 species-specific versions of this antigen. There was also a very weak correlation between antibody responses to the PvCSP and PfMSP1 antigens. We do not consider, however, that these correlations are, in themselves, evidence for antigenic cross-reactivity between *P. vivax* and *P. falciparum* antigens. Indeed, if 2 species of malaria parasites are coendemic, this result is predicted from the fact that exposure to infection by one species of malaria parasite will be highly correlated with the risk of exposure to infection by other species.

Our data indicate that, in the region of study in western and central Africa, there is an endemic entity present that is inducing antibodies specific to the preerythrocytic stages of *P. vivax* in the RBC Duffy-negative human populations of the region. We suggest that this entity is most likely sporozoites of *P. vivax* itself, delivered by the local malaria vector mosquitoes. In conjunction with the frequent reports of travelers returning from western and central Africa with diagnosed *P. vivax* infections, these findings make a strong argument for the presence and continued transmission of *P. vivax* in this region. Given the very high malaria transmission intensity in this area, it is possible that the transmission of *P. vivax* is maintained within the local population by the ~1%–5% of Duffy-positive individuals who are presumed to be present in the local population.

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