Revista da Sociedade Brasileira de Medicina Tropical
34(6): 591-595, nov-dez, 2001.

Duffy blood group genotypes among malaria patients in Rondônia, Western Brazilian Amazon

Genótipos do sistema sanguíneo Duffy em pacientes maláricos de Rondônia, Amazônia Ocidental Brasileira

Carlos Eugênio Cavasini,[1] Fabrício José Tarelho Pereira,[1] Weber Luidi Ribeiro,[1] Gerhard Wunderlich[2] and Marcelo Urbano Ferreira[1 2]

Abstract *We have compared Duffy blood group genotype distribution, as determined by polymerase chain reaction with allele-specific primers, in 68* Plasmodium vivax*-infected patients and 59 non-vivax malaria controls from Rondônia, Brazil. Homozygosity for the allele Fy, which abolishes Duffy antigen expression on erythrocytes, was observed in 12% non-vivax controls but in no* P. vivax *patient. However, no significant association was found between Fy heterozygosity and protection against* P. vivax*. The Fy^x allele, which has recently been associated with very weak erythrocyte expression of Duffy antigen, was not found in local* P. vivax *patients.*

Key-words: Plasmodium vivax*. Malaria. Duffy antigen. Genotypes. Brazilian Amazon.*

Resumo *Compara-se neste trabalho a distribuição de genótipos do sistema sangüíneo Duffy, determinados através de reação em cadeia da polimerase com oligonucleotídeos iniciadores alelo-específicos, em 68 pacientes com infecção por* Plasmodium vivax *e em 59 controles com malária não-vivax de Rondônia, Brasil. Nenhum paciente infectado com* P. vivax*, mas 12% dos controles não-vivax, eram homozigotos para o alelo Fy, que abole a expressão do antígeno Duffy em hemácias. No entanto, não se observou evidência de proteção significativa contra* P. vivax *entre indivíduos heterozigotos para Fy. O alelo Fy^x, que tem sido recentemente associado com a expressão eritrocitária muito fraca do antígeno Duffy, não foi encontrado entre pacientes locais com infecção por* P. vivax*.*

Palavras-chaves: Plasmodium vivax*. Malária. Antígeno Duffy. Genótipos. Amazônia Brasileira.*

The incidence of malaria in Brazil has increased dramatically in recent decades, from 52,000 cases recorded in 1970 to more than 630,000 cases in 1999. A major change has also been observed in the distribution of *Plasmodium* species over the last years: in the 1980s, *P. falciparum* and *P. vivax* were similarly prevalent, while in 1999 *P. vivax* was detected in more than 80% of malaria cases diagnosed microscopically in Brazil. *P. malariae* has been officially reported in less than 0.5% of malaria patients in the 1990s (National Health Foundation, unpublished data). Malaria transmission in Brazil occurs essentially in the Amazon Basin, where more than 99% of infections are acquired. Most affected subjects are migrants living in frontier agricultural settlements and mining areas[8].

Little is known on the frequency of erythroid polymorphisms that confer either partial or complete resistance against malaria, such as sickle cell anaemia trait, α^+-thalassemia and Duffy blood group negativity[9], in Amazonian populations. Duffy blood group polymorphisms are important in areas where *P. vivax* predominates, because this molecule acts as a receptor for *P. vivax* (but not for the other human malaria parasites) on the surface of red blood cells (RBCs)[9]. The Duffy antigen (Fy) is also normally expressed in endothelial cells of kidney collecting ducts and pulmonary alveoli, and in Purkinje cells of the cerebellum; its physiologic function is unknown, but Fy is thought to serve as a scavenger for circulating chemokines[13]. The absence of Fy on the RBCs of

1. Laboratório de Parasitologia Molecular da Faculdade de Medicina de São José do Rio Preto, São José do Rio Preto, SP; 2. Departamento de Parasitologia do Instituto de Ciências Biomédicas da Universidade de São Paulo, São Paulo, SP, Brazil.
Financial support: Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), UNDP/World Bank/World Health Organization Special Programme for Research and Training in Tropical Diseases, and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). F. J. T. Pereira and W. L. Ribeiro are recipients of studentships from FAPESP, and M. U. Ferreira is a recipient of scholarships from CNPq and Fundação Faculdade Regional de Medicina (FUNFARME).
Address to: Dr. Marcelo U. Ferreira, Deptº de Parasitologia/ICB/USP. Av. Prof. Lineu Prestes 1374, 05508-900 São Paulo, SP, Brazil.
Tel: 55 11 3818-7273, Fax: 55 11 3818-7417,
e-mail: muferrei@usp.br
Recebido para publicação em 11/1/2001.

individuals from many African ethnic groups and their descendants causes no obvious ill effect, and confers natural resistance against *P. vivax* infection[13].

Duffy-negative individuals are homozygous for a single nucleotide substitution (T-33C) within the GATA-1 binding motif of the erythroid-specific promoter of the *FY* gene[14], which characterizes the allele *Fy*. The *FY* open reading frame (ORF) comprises two major alleles: Fy^a and Fy^b (or *FY1* and *FY2,* according to the new terminology proposed by the International Society of Blood Transfusion[5]). These alleles differ by a single nonsynonymous nucleotide replacement (G125A)[7]. Two other ORF nonsynonymous substitutions have been recognized: (a) the relatively rare C265T mutation in Fy^b-type sequences characterizes the Fy^x allele, associated with the weak anti-Fy^b serological reactions found in 2% of Caucasoid individuals[12,15], and (b) the G298A mutation, frequently found in either Fy^a or Fy^b-type sequences, which seems to be physiologically silent (Figure 1)[13]. The combination of these polymorphisms leads to the major Duffy phenotypes Fy (a+b+), Fy(a+b-), Fy(a-b+), Fy(a-b-) and Fy(a-b+^{WK}) (WK stands for weak)[13].

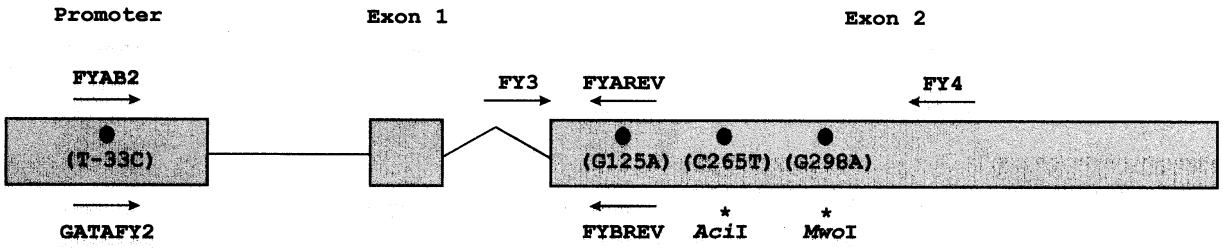

Figure 1 – Schematic representation of the Duffy blood group gene Fy, including the promoter region and the two exons. Black circles represent nucleotide substitutions (nucleotide numbers are given according to reference 13), and arrows indicate the location and orientation of the allele-specific oligonucleotide primers (FYAB2, GATAFY2, FYAREV, FYBREV) used for genotyping[11] and the FY3 and FY4 primers used for amplifying a 661-bp fragment for sequence analysis[12]. Primer sequences (from 5´ to 3´) are as follows: FYAB2, CTC ATT AGT CCT TGG CTC TTA T; GATAFY2, CTC ATT AGT CCT TGG CTC TTA C; FYAREV, AGC TGC TTC CAG GTT GGC AC; FYBREV, AGC TGC TTC CAG GTT GGC AT (note that these two pairs of forward and reverse allele-specific primers differ in a single nucleotide [T or C] at the 3´end); FY3, CCC TCT TGT GTC CCT CCC TTT, and FY4, CAG AGC TGC GAG TGC TAC CTA. The nucleotide replacements C265T and G298A in exon 2, marked with asterisks, may be detected by restriction fragment length polymorphism analysis using the restriction enzymes AciI *and* MwoI*, respectively[12].*

RBCs from individuals presenting the Duffy phenotypes Fy(a+b+), Fy(a+b-) and Fy(a-b+) are similarly susceptible to *P. vivax* invasion, while those with the Fy(a-b-) phenotype are fully refractory to this parasite. No data on *P. vivax* susceptibility are currently available for the Fy(a-b+^{WK}) phenotype. RBCs from individuals homozygous for the wild-type promoter (genotypes Fy^aFy^a, Fy^bFy^b and Fy^aFy^b) express twice the amount of Fy antigen than those heterozygous for the GATA-1 mutation (genotypes Fy^aFy and Fy^bFy)[16,17], but the biological significance of this finding is unknown[17]. Routine serology assigns the RBC phenotype Fy(a+b-) to individuals with the genotypes Fy^aFy^a and Fy^aFy, and the phenotype Fy(a-b+) to those with the genotypes Fy^bFy^b and Fy^bFy, despite the two-fold difference in Fy antigen expression between homozygotes and heterozygotes for wild-type promoter. Therefore, genotyping methods are required to assess the potential impact of these quantitative differences in erythrocyte Fy antigen expression on *P. vivax* susceptibility.

Two decades ago, Colauto and colleagues described a prevalence of 11.5% for the phenotype Fy (a-b-) in 104 non-Amerindian subjects living in Humaitá, Western Brazilian Amazon[2]. More recently, this phenotype was observed in less than 5% of 182 subjects living in a riverine community in Rondônia, about 160 km south of Humaitá. No Fy (a-b+^{WK}) individuals were found (M. M. Moura and H. Krieger, personal communication). In contrast, Duffy negativity was found in one third of blood donors in São Paulo, southeast Brazil[10]. However, phenotypes were defined by standard serology, with no information on genotype frequencies. Here we compared the distribution of Duffy blood group genotypes in patients from Rondônia infected with *P. vivax* and those infected with the other locally prevalent *Plasmodium* species which do not require Fy antigen expression for RBC invasion, namely *P. falciparum* and *P. malariae*. Patients with non-*vivax* malaria served as sympatric malaria-exposed controls. Since *P. falciparum, P. vivax* and *P. malariae* co-circulate in the same areas in Rondônia, patients infected with other malaria parasites are similarly exposed to *P. vivax*. If GATA-1 mutation heterozygosity confers some protection against *P. vivax*, due to a decreased erythrocyte Fy antigen expression, a relative excess of wild-type homozygotes among *P. vivax*-infected patients, as compared with non-*vivax* controls, would be expected[17].

Venous blood was collected, after informed consent, from 127 patients with microscopically confirmed malaria diagnosis. No attempt was made to classify donors according to ethnic groups, but Amerindians were not included in the study sample. Human DNA was isolated using standard phenol-chloroform extraction and ethanol precipitation protocols[3]. On-site *Plasmodium* species identification was made by standard thick smear microscopy[4], and further confirmed by species-specific polymerase chain reaction (PCR) (in 97 [76%] patients) or by reexamination of available blood smears by one of the authors (in 30 [24%] patients). The semi-nested PCR used for species identification[6] was performed

exactly as described elsewhere[1]. All patients had a febrile illness and were clinically examined by one of the authors at outpatient malaria clinics in Porto Velho, Rondônia, between 1995 and 1998. Antimalarial treatment was given, following the recommendations of the National Health Foundation[4], based on on-site species identification.

Duffy blood group genotyping was performed essentially as described by Olsson and colleagues[11]. Amplification was performed with the forward oligonucleotide primers GATAFY2 (that targets the mutated GATA-1 promoter sequence) and FYAB2 (that targets the wild-type promoter sequence), and the forward primers FYAREV (that targets the Fy^a ORF allele) and FYBREV2 (that targets the Fy^b ORF allele). Oligonucleotide primer locations, orientations and sequences are given in Figure 1[11]. Four separate reactions were prepared for each patient, using the following primer combinations: GATAFY2 and FYAREV, GATAFY2 and FYBREV2, FYAB2 and FYAREV, and FYAB2 and FYBREV2. The first primer pair amplifies the Fy^{anull} allele (an Fy^a-type allele with mutated GATA-1 sequence), recently found in Papua New Guinea[17] but not elsewhere, while the other combinations amplifies the alleles *Fy,* Fy^a and Fy^b, respectively. PCR mixtures included 1µl of extracted DNA, 0.2µM of each primer, 100µM of each dNTP (Amersham Pharmacia Biotech, USA), 1.5µM of $MgCl_2$ and 0.5U of AmpliTaq Gold polymerase (Perkin Elmer, USA), in the AmpliTaq Gold buffer supplied by Perkin Elmer, in a reaction volume of 25µl. Mixtures were incubated for 8 min at 95°C, followed by 10 cycles of 94°C for 1 min and 69°C for 1 min, 25 cycles of 94°C for 1 min, 64°C for 1 min and 72°C for 1 min, and a final incubation at 72°C for 10 min. A positive reaction was defined as the presence of a clearly visible 711-bp band, under UV illumination, after 1.5% agarose gel electrophoresis.

Table 1 compares results of Duffy genotyping and phenotyping, as well as *Fy* allele frequencies, in *P. vivax*-infected patients and non-vivax controls. All patients harbouring *P. vivax* infection, regardless of the presence of a second malaria species, were included in the *P. vivax* group for this analysis. As expected, no *P. vivax*-infected patient was Fy (a-b-), but the prevalence of this phenotype in non-*vivax* malaria controls (12%) was quite similar to that described in non-Amerindians in Humaitá[2]. Data presented in Table 1 do not support the hypothesis that GATA-1 mutation heterozygosity confers some protection against *P. vivax* infections[17], since similar proportions of homozygotes for the wild-type erythrocyte-specific promoter were found among *P. vivax*-infected patients (66%) and non-*vivax* controls (71%) ($P = 0.54$ by χ^2 analysis). Allele frequencies were remarkably similar in both groups.

The Duffy genotyping method designed by Olsson and colleagues[11] is unable to detect the relatively rare allele Fy^x, which would be mistyped as Fy^b. Therefore, subjects with the genotypes Fy^bFy or Fy^bFy^b may actually bear one or two copies of the allele Fy^x (instead of Fy^b), resulting in the phenotype Fy (a-b+^{WK}) instead of the

Table 1 – Duffy blood group genotypes, deduced phenotypes and allele frequencies among 127 malaria patients from Rondônia, Western Brazilian Amazon.

Malaria species[a]	Patients nº	Patients %	GATA-1 type[b]	*Fy* genotype	Deduced phenotype traditional terminology	Deduced phenotype new ISBT terminology[c]	Allele frequencies allele	Allele frequencies frequency
Plasmodium vivax								
	15	22.0	wt/wt	Fy^aFy^a	Fy (a+b-)	FY: 1,-2	Fy^a	0.404
	10	15.0	wt/m	Fy^aFy	Fy (a+b-)	FY: 1,-2	Fy^b	0.426
	15	22.0	wt/wt	Fy^bFy^b	Fy (a-b+)	FY: -1,2	*Fy*	0.169
	13	19.0	wt/m	Fy^bFy	Fy (a-b+)	FY: -1,2		
	15	22.0	wt/wt	Fy^aFy^b	Fy (a+b+)	FY: 1,2		
	0	0.0	m/m	*FyFy*	Fy (a-b-)	FY: -1,-2		
Plasmodium falciparum and/or *P. malariae*								
	16	27.0	wt/wt	Fy^aFy^a	Fy (a+b-)	FY: 1,-2	Fy^a	0.398
	3	5.0	wt/m	Fy^aFy	Fy (a+b-)	FY: 1,-2	Fy^b	0.398
	14	24.0	wt/wt	Fy^bFy^b	Fy (a-b+)	FY: -1,2	*Fy*	0.203
	7	12.0	wt/m	Fy^bFy	Fy (a-b+)	FY: -1,2		
	12	20.0	wt/wt	Fy^aFy^b	Fy (a+b+)	FY: 1,2		
	7	12.0	m/m	*FyFy*	Fy (a-b-)	FY: -1,-2		

[a]The first group included all patients harbouring *P. vivax* ($n = 68$), regardless of the presence of a second malaria species; the second group included all patients harbouring malaria species other than *P. vivax* ($n = 59$).

[b]wt = wild-type; m = mutated (i. e., presenting the T-33C substitution).

[c]ISBT = International Society of Blood Transfusion. See reference 5 for details of the new terminology.

deduced phenotype Fy (a-b+). Fy (a-b+^{WK}) individuals have not previously been assessed for susceptibility to *P. vivax* infection. We used restriction fragment length polymorphism (RFLP) analysis to look for the C265T mutation, which defines the Fy^x allele, in 29 *P. vivax*-infected patients bearing one or two copies of Fy^b-type alleles. A 661-bp fragment of the exon 2 of the *Fy* gene, that encompasses nucleotide 265 (Figure 1), was amplified with the primers Fy3 and Fy4, as described elsewhere[12]. PCR mixtures included 1µl of extracted DNA, 0.2µM of each primer, 100µM of each dNTP and 1U of *Taq* polymerase (Amersham Pharmacia Biotech, USA) in a final reaction volume of 40ml. Mixtures were incubated for 4 min at 94°C, followed by 33 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min, and a final incubation at 72°C for 10 min. The C265T mutation was searched for by digesting a 3-µl aliquot of the PCR product with 4U of the restriction enzyme Aci*I* (New England Biolabs, USA) for 4h at 37°C, since the C265T mutation eliminates an Aci*I* restriction site (CCGC -> CTGC). Restriction fragments were resolved by agarose gel electrophoresis.

No *P. vivax*-infected patient presented the genotypes Fy^xFy or Fy^xFy^x, which result in the phenotype Fy (a-b+^{WK}). Since a few equivocal RFLP results were obtained, these findings were confirmed by sequencing the 661-bp PCR fragment from six *P. vivax*-infected subjects presenting the Fy^bFy genotype, as well as one patient with the Fy^bFy^b genotype and one with the Fy^aFy^b genotype. PCR fragments were cloned into the pGEM-T Easy vector (Promega, USA) and sequenced on both strands using the ThermoSequenase Cy5.5 Dye Terminator Sequencing Kit (Amersham Pharmacia Biotech, USA). Four clones for patient were sequenced, in order to have both alleles represented. Sequencing reaction products were analyzed using a SEQ 4x4 Personal Sequencing System (Amersham Pharmacia Biotech, USA). A single patient (#32OC, genotype Fy^bFy^b) was heterozygous for the G298A mutation in exon 2; no instances of C265T (or any other) mutation were found. The G298A mutation may also be detected by RFLP analysis with the enzyme *Mwo*I[12] (Figure 1), and does not seem to affect Fy antigen expression on RBCs[13].

In conclusion, our findings argue against the hypothesis that heterozygosity for the wild-type erythrocyte-specific promoter of the *Fy* gene, which results in reduced erythrocyte expression of the Duffy antigen[16,17], may confer some protection against *P. vivax* infection[17]. No *P. vivax*-infected patient was found to bear the Fy^x allele. However, since Fy^x allele frequencies in local human populations remain undetermined, no conclusion may be drawn from our data regarding the susceptibility to *P. vivax* infection among individuals with the Fy (a-b+^{WK}) phenotype.

ACKNOWDELGEMENTS

We thank Drs. Lilian Castilho (State University of Campinas, Brazil) and Nechama S. Kosower (Tel-Aviv University, Israel) for helpful suggestions, Renata Tonhosolo (University of São Paulo, Brazil) for technical support, and Prof. Henrique Krieger (University of São Paulo, Brazil) for sharing unpublished data.

REFERENCES

1. Cavasini MTV, Ribeiro WL, Kawamoto F, Ferreira MU. How prevalent is *Plasmodium malariae* in Rondônia, Western Brazilian Amazon? Revista da Sociedade Brasileira de Medicina Tropical 33:489-492, 2000.
2. Colauto EMR, Barraviera B, Meira DA, Matsubara LS, Pellegrino Jr. J, Machado PEA, Sogayar R, Barboza AF, Silva EA, Colauto R, Pirolla JAG, Mendes RP. Malária no Município de Humaitá, Estado do Amazonas. XII – Freqüência de fatores de resistência eritrocitária na população geral e em doentes: hemoglobina S e sistema sangüíneo Duffy. Revista do Instituto de Medicina Tropical de São Paulo 23(Supl.5):72-78, 1981.
3. Ferreira MU, Liu Q, Kaneko O, Kimura M, Tanabe K, Kimura EAS, Katzin AM, Isomura S, Kawamoto F. Allelic diversity at the merozoite surface protein-1 locus of *Plasmodium falciparum* in clinical isolates from the southwestern Brazilian Amazon. American Journal of Tropical Medicine and Hygiene 59:474-480, 1998.
4. Fundação Nacional de Saúde. Diagnóstico e tratamento da malária. Manual para pessoal de saúde de nível médio. Brasília, Ministério da Saúde, 1995.
5. Garratty G, Dzik W, Issit PD, Lublin DM, Reid ME, Zelinski T. Terminology for blood group antigens and genes – historical origins and guidelines in the new millennium. Transfusion 40:477-489, 2000.
6. Kimura M, Kaneko O, Liu Q, Zhou M, Kawamoto F, Wataya Y, Otani S, Yamaguchi Y, Tanabe K. Identification of the four species of human malaria parasites by nested PCR that targets variant sequences in the small subunit rRNA gene. Parasitology International 46:91-95, 1997.
7. Mallinson G, Soo KS, Schall TJ, Pisacka M, Anstee DJ. Mutations in the erythrocyte chemokine receptor (Duffy) gene: the molecular basis of the Fy^a/Fy^b antigens and identification of a deletion in the Duffy gene of an apparently healthy individual with the Fy(a-b-) phenotype. British Journal of Haematology 90:823-829, 1995.
8. Marques AC. Human migration and the spread of malaria in Brazil. Parasitology Today 3:166-170, 1987.
9. Miller LH. Impact of malaria on genetic polymorphism and genetic diseases in Africans and African Americans. Proceedings of the National Academy of Sciences of USA 91:2415-2419, 1994.
10. Novaretti MCZ, Dorlhiac-Llacer PE, Chamone DAF. Estudo de grupos sangüíneos em doadores de sangue caucasóides e negróides na cidade de São Paulo. Revista Brasileira de Hematologia e Hemoterapia 22:23-32, 2000.
11. Olsson ML, Hansson C, Avent ND, Akesson IE, Green CA, Daniels GL. A clinically applicable method for determining the three major alleles at the Duffy (*FY*) blood group locus using polymerase chain reaction with allele-specific primers. Transfusion 38:168-173, 1998.

12. Parasol N, Reid M, Rios M, Castilho L, Harari I, Kosower NS. A novel mutation in the coding sequence of the FY*B allele of the Duffy chemokine receptor gene is associated with an altered erythrocyte phenotype. Blood 92:2237-2243, 1998.

13. Pogo AO, Chaudhuri A. The Duffy protein: a malarial and chemokine receptor. Seminars in Hematology 37:122-129, 2000.

14. Tournamille C, Colin Y, Cartron JP, Kim CLV. Disruption of a GATA motif in the *Duffy* gene promoter abolishes erythroid gene expression in Duffy-negative individuals. Nature Genetics 10:224-228, 1995.

15. Tournamille C, Kim CLV, Gane P, Le Pennec PY, Roubinet F, Babinet J, Cartron JP, Colin Y. Arg89Cys substitution results in very low membrane expression of the Duffy antigen/receptor for chemokines in Fy^x individuals. Blood 92:2147-2156, 1998.

16. Woolley IJ, Hotmire KA, Sramkoski RM, Zimmerman PA, Kazura JW. Differential expression of the Duffy antigen receptor for chemokines according to RBC age and *FY* genotype. Transfusion 40:949-953, 2000.

17. Zimmerman PA, Woolley I, Masinde GL, Miller SM, McNamara DT, Hazlett F, Mgone CS, Alpers MP, Genton B, Boatin BA, Kazura JW. Emergence of $FY*A^{null}$ in a *Plasmodium vivax*-endemic region of Papua New Guinea. Proceedings of the National Academy of Sciences of USA 96:13973-13977, 1999.