Polymorphism of the Duffy blood group system influences the susceptibility to *Plasmodium vivax* infection in the specific area from Brazilian Amazon

Polimorfismo do Sistema de Grupo Sanguíneo Duffy influencia a susceptibilidade à infecção pelo *Plasmodium vivax* em área específica da Amazônia Brasileira

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RESUMO

*Plasmodium vivax* invasion requires interaction between the human Duffy antigen on the surface of erythrocytes and the *P. vivax* Duffy binding protein (PvDBP), a receptor, expressed by the parasite. Given that Duffy-negative individuals are resistant and that Duffy-negative heterozygotes show reduced susceptibility to blood-stage infection. This study show significant associations between Duffy Blood Group variants and susceptibility and resistance to malaria. In the present study, Duffy Blood group genotyping and phenotyping in 244 individuals living within a Brazilian endemic area and the *P. vivax* identification was determined by thick blood smears. In total, we found about eighty patients with vivax malaria (n=80) and one hundred sixty-four negative patients to malaria (n=164). Our results showed a high frequency of genotypes FYAFYB (0.475), followed by FYBFY (0.156); FYAFYA (0.143); FYBFYB (0.115); FYAFY (0.086) and 2.5% with FYFY. The frequency of FYA, FYB and FY alleles were respectively, 55%, 38.8% e 6.3% in infected patients. In the group tested negative the results showed 36.3%, 45.1% e 18.6%. These results demonstrated that FYA allele is more significantly frequent among infected subjects. The null genotype has not been found among the infected subjects; however it occurs in 3.7% in the group tested negative. This study has also found 11.5% discordance between genotype and phenotype. The estimate of the genetic frequency proved that the population participating in the study does not show a genetic balance according to Hardy-Weinberg.

Keywords: Malaria, Duffy, DARC, *Plasmodium vivax*

ABSTRACT

A invasão eritrocítica pelo *Plasmodium vivax* requer interação entre o receptor molecular de superfície do eritrócito, antígeno Duffy, com a proteína de ligação (PvDBP), uma receptora expressa pelo parasita. Sabemos que, indivíduos que não possuem o antígeno Duffy na superfície dos eritrócitos são resistentes e que os heterozigotos mostram uma susceptibilidade reduzida à infecção durante a fase sanguínea. Este estudo mostrou associações significativas entre as variantes Duffy e a susceptibilidade e resistência à malária vivax. No presente estudo, foram fenotipados e genotipados para o Sistema de Grupo Sanguíneo Duffy um total de 244 indivíduos residentes em área endêmica para a malária no Brasil e realizado exame da gota espessa para pesquisa de plasmódio. Do total de indivíduos pesquisados, 164 eram negativos e 80 positivos para *P. vivax*. Os achados mostram uma alta frequência do genótipo FYAFYB (47,5%); seguida de FYBFY (15,6%); FYAFYA (14,3%); FYBFYB (11,5%); FYAFT (8,6%) e com 2,5% o genótipo FYFY. A frequência dos alelos FYA, FYB e FY foi 55%, 38,8% e 6,3% em infectados e 36,3%, 45,1% e 18,6% em negativos, respectivamente. Esses resultados mostram que o alelo FYA é mais frequente no grupo de infectados. O genótipo nulo não foi encontrado em infectados, porém aparece com 3,7% no grupo negativo. Neste estudo foram encontrados 11,5% de não concordância entre fenótipo e genótipo. Os cálculos de frequência genética demonstraram que a população estudada não se encontra em equilíbrio gênico de acordo com Hardy-Weinberg.


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INTRODUCTION

While Plasmodium falciparum uses a complex array of receptors to invade human erythrocytes (Rayner et al., 2001), erythrocyte invasion by Plasmodium vivax, and the closely related simian parasite Plasmodium knowlesi, are understood to depend upon interaction with the Duffy blood group antigen (Miller et al., 1976). In the homologous P. knowlesi system, merozoites interact with Duffy-negative human red blood cells, but are unable to invade (Miller et al., 1976).

In Africa, where Duffy-negativity has reached fixation in many different ethnicities, transmission of P. vivax malaria is uncommon (Mendis & Sina, 2001; Welch et al., 1977). Of further interest, in Papua New Guinea, heterozygous carriers of a Duffy-negative allele are shown to express half the amount of the Duffy antigen on erythrocytes compared to wild-type homozygotes (Zimmerman et al., 1999), and exhibit reduced susceptibility to P. vivax blood-stage infection (Kasehagen et al., 2007). These observations suggest that completely or partially disrupting access to the Duffy antigen reduces the ability of the parasite to invade new erythrocytes and may constrain P. vivax parasitemia. This parasite requires interaction with the Duffy antigen receptor for chemokines (DARC) to enable its invasion of human erythrocytes. Interaction with DARC is mediated by the P. vivax Duffy-binding protein (PvDBP) and is essential for junction formation, which is a key step in the invasion process (Chitnis & Sharma, 2008).

The recent study provides evidence that antibodies against PvDBP inhibit binding to the Duffy receptor and interfere with P. vivax invasion of human red blood cells (Grimber et al., 2007). These results suggest an important role of the Duffy blood group antigen in the transmission of malaria. The Duffy Blood group system is controlled by five alleles or variants named FYA, FYB, FY and FYnull and FYX (Tournamille et al., 1998).

In the Amazon, infections are caused by P. vivax is the most prevalent responding for 80% of all known cases of people infected with parasites of malaria disease. This high occurrence of the P. vivax has been observed by Tadei et al. (1998) while they studied the Amazon anofelines mosquitoes. They reported that the mosquitoes were infected by the P. vivax two and a half times more than by P. falciparum and almost thirty times more than Plasmodium malariae. Whether this polymorphism affects susceptibility to clinical vivax malaria is unknown, but in this study we suggested a significant association between Duffy antigens and susceptibility or resistance to vivax malaria. Here, we have compared the frequency of occurrence of Duffy alleles in inhabitants of State of Amazon, relating it to the individual’s vulnerability and resistance in acquiring the infection resulting in the disease.

MATERIALS AND METHODS

Human blood samples

All human blood samples used in this study were collected after obtaining consent from study participants under protocols approved by the Ethical Committee of Instituto Nacional de Pesquisas da Amazônia (INPA) under process number 046/2006. All the collection of materials and samples in this study where taken in the town of Presidente Figueiredo in the State of Amazonas. It was collected 5 mL of blood with the EDTA (ethylenediaminetetraacetic acid) anticoagulant from the patients who came to the hospital with the symptoms of malaria. It was also collected a sample of blood (1 µL) using digital puncture to diagnose the malaria through the thick blood smears test. This study has collected samples from a total of 244 individuals.

Duffy serology

It was used the Duffy phenotype method in order to study the Duffy protein contained in the surface membrane of the erythrocytes. This data was used to contrast the phenotype and the genotype as well as evaluate the percentage of similarity between them. The blood samples were subjected to the phenotype through the hemagglutination test performed using gel cards, according to the specifications given by the manufacturer. The methodology “Micro Typing System” is described by DiaMed who is the seller of the reagents.

DNA extraction

The obtaining of genomic DNA samples was made as soon as the blood samples were collected and then it was kept on a -20°C freezer to be used later. The extraction of the DNA samples was carried out using the Easy-DNA Kit (Invitrogen).

Duffy genotyping

The molecular methodology described by Olsson et al. (1998) was applied in this study to genotype the system of the Duffy blood group since it is a very simple and quick way to identify the four possible alleles of the FY locus through the reaction of the polymerase chain reaction (PCR). For the amplification of the alleles FYAnull, FY, FY e FYB, were used four combinations (A to D) of following primers: GATAFY2, FYAB2, FYAREV e FYBREV, according showed in the Table 1.

The primer sequences are: GATAFY2 (5’-CTCATTAGTCTTGGCTTATAC-3’); FYAB2 (5’-CTCATTAGTCTTGGCTTATAT-3’); FYAREV (5’-AGCTCCTCCAGGTGCACT-3’) and FYBREV (5’-AGCTCCTCCAGGTGCACT-3’) described by Olsson et al. (1998). PCRs reactions were performed with DNA samples as follow: 30 cycles of 94°C for 30 s, 65°C for 30 s and 72°C for 1 min. A 700-bp PCR product was detected by 1% (w/v) agarose gel electrophoresis stained by ethidium bromide.

Statistical analysis

The results arrived at in this study were analyzed by the EPINFO program. The comparisons between the genotypic and phenotypic frequency were reached at on the basis of the test chi-square ($\chi^2$) by Pearson which makes use of a significance level of 5%. It was considered a significant difference concerning the p-value ($P$) which was smaller than the significance level ($P < \alpha$). It was also made use of the method brought forward by Bernstein, to estimate the populous frequencies of the alleles researched.
starting from the knowledge of the phenotypic distribution Duffy. After the analysis, we then verify if the distribution of the phenotypes in the sample studied is equal to the genetic hypothesis, that is, if the sample really represents a balanced population, according to Hardy-Weinberg, using Pearson’s chi-square test to the level of 5% of significance.

### Table 1. Primers Combination to amplification of Duffy alleles

<table>
<thead>
<tr>
<th>Combination</th>
<th>Primers</th>
<th>Allele detected</th>
<th>Amplicon (size)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>GATAFT2 FYABEV FYA&lt;sup&gt;α&lt;/sup&gt;</td>
<td>711 bp</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>GATAFT2 FYBBEV FY</td>
<td>711 bp</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>FYAB2 FYABEV FYA</td>
<td>711 bp</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>FYAB2 FYBBEV FYB</td>
<td>711 bp</td>
<td></td>
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</tbody>
</table>

**RESULTS AND DISCUSSION**

The samples were collected from November 2007 to June 2008. Among those individuals who were researched 80 were diagnosed as being positive to *P. vivax* (32.79%) and 164 had negative results on the thick blood smears (67.21%). The Duffy phenotypes, found by serology, in decreasing order of prevalence are: Fyab (36 %), Fya (34.5 %), Fyb (27 %) and Fy (2.5 %) as showed in the Figure 1.

The Table 2 compares the results of the genotypes and the phenotypes deduced from the Duffy blood group as well as the allelic frequency of infected negative individuals. This study has found 11.5% discrepancy between the phenotype and genotype in relation to the Fyb antigen. It is important to emphasize the importance of the Duffy phenotypic in phenotyped patients like Fyb(-), due to weak expression of the Fyb antigens in the surface erythrocytes and that aren’t detect by antibodies, just by analyze of FYX gene presence.

The frequencies of the alleles change differently within the populations studied, the researches carried out in regions that are endemic to malaria showed that defense mechanisms to the in infection to *P. vivax* were present, determining the appearance of phenotypes different from whose that were expected (Castilho et al., 2004; Cavasini et al., 2007; King et al., 2011).

None of the patients infected but 3.7% of those tested negative to malaria were homozygote to the allele FY (FYFY), as it was already expected, since this phenotype presents resistance to infection caused by *P. vivax* which has been described earlier (Barnewell et al., 1989; Cavasini et al., 2001), although some authors have reported the occurrence of *P. vivax* in individuals with FY allele in homozygote form (Cavasini et al., 2007; Ryan et al., 2006; Ménard et al., 2010). The allele FY had its occurrence significantly increased in the negative patients. It was also observed a decrease in the occurrence of the FY allele in heterozygosis among the individuals infected. This data suggests an increase of protection to infection by *P. vivax* in heterozygosis (FYAFY e FYBFY) in this specific population, which is consists exclusively of native individuals, underscoring the strong dependence this parasite displays on Duffy-dependent invasion.

We hypothesize that, once Duffy Blood Group System is considered to be one of the most interesting chromosomal locus to evaluate the impact of the natural selection in different geographic, can be that there are numerous mutations affecting this locus depending on geographic region and human admixture. These mutations produce different Duffy phenotypes and alleles, which leads us to suppose the existence of alternative mechanisms for erythrocyte invasion by *P. vivax*. This alternative emphasizes the importance of the different vaccines that can reduce the strategy malariae infection in different parts of the world.

The allele FYA was prevalent in the infected group of patients, while the FYB allele appeared in greater quantity, but no significance, in the negative group. The allele FYB in homozygosis didn’t indicate significant changes (P>0.05) when comparing with infected, however, it has difference when the FYB is associated with the allele FY in heterozygosis. The allele FYB did not present protection to the infection when in homozygosis form (FYBFY). However, the protecting effect appears when it is associated to the FY (P < 0.05) allele in heterozygosis individuals FYBFY.

The allele FYA in homozygosis suggests susceptibility to infection by the *P. vivax*, while in heterozygosis with FY. This susceptibility is lessened since the FY confers protection as mentioned earlier. Therefore, for the genotype FYAFY there wasn’t difference between the two groups (P > 0.05).

The results showed a high frequency of the phenotype Fyb (47.5%), while the genotype (FYAB), differently from what was expected, didn’t show significant differences between the groups studied (P > 0.05).

On the basis of the concepts of gene frequency and evolution, we analyzed the genetic balance of the population that was studied using Hardy-Weinberg’s law. The values observed were significantly different from those expected. Therefore, the results observed in this

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**Figure 1.** Duffy phenotypic frequencies in all individuals surveyed by serology. The Fyb phenotype was the most prevalent, while the Fy (null) showed a lower prevalence. Student’s t test was used for statistical comparison, which showed that the Fya, Fyb and Fy phenotypes were significantly different compared to Fyab (P < 0.05).

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**Table 2**
study show that the population studied is not in genetic balance and in accordance to Hardy-Weinberg.

Table 2. Duffy genotypes and phenotypes and frequency allelic between infected and negative individuals from Brazilian Amazon specific area. 2007-2008

<table>
<thead>
<tr>
<th>Duffy Blood Group</th>
<th>Genotypes</th>
<th>Deduced Phenotypes</th>
<th>Frequency Allelic</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>FYA/FYA</td>
<td>FY(a+b-)</td>
<td>36.3%</td>
</tr>
<tr>
<td></td>
<td>FYB/FYA</td>
<td>FY(a+b-)</td>
<td>45.1%</td>
</tr>
<tr>
<td></td>
<td>FYB/FYB</td>
<td>FY(a+b-)</td>
<td>18.6%</td>
</tr>
</tbody>
</table>

* The first group includes individuals tested negative to malaria and in the second are those who were infected by the P. vivax in a total of 244 individuals studied.

# CONCLUSIONS

The frequencies of the alleles change differently among the populations studied, because Duffy Blood Group system is considered to be one of the most interesting locus chromosomal to evaluate the impact of the natural selection in different geographic.

These findings established a significant association between Duffy Blood Group variants and susceptibility and resistance to malaria. Our results showed that FYA allele was prevalent among the infected subjects, while the FYB appeared in greater quantity in the negative group, but no significant. Therefore, the allele FYA in homozygosis suggests susceptibility and FYB doesn’t award protection to infection. In the other hand, FY allele in homozygosis was not found among the infected patients, and showed a decrease in the susceptibility or increase the protection when associated with FYA and FYB alleles, respective. These found suggesting that this mutation could still be an advantageous selection in the population from areas that are endemic to P. vivax.

There is a natural selection pressure in this population studied and that the natural adaptation in malaria endemic area may lead to the partial defense mechanisms against P. vivax.

REFERENCES


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