Tropical Medicine and International Health doi:10.1111/j.1365-3156.2012.03016.x

VOLUME 17 NO 8 PP 989–1000 AUGUST 2012

Plasmodium vivax Duffy binding protein: baseline antibody responses and parasite polymorphisms in a well-consolidated settlement of the Amazon Region

Flora S. Kano[1], Bruno A. M. Sanchez[2], Tais N. Sousa[1], Michaelis L. Tang[1], Jéssica Saliba[1], Fernando M. Oliveira[1], Paulo A. Nogueira[3], Alessandra Q. Gonçalves[3], Cor J. F. Fontes[2], Irene S. Soares[4], Cristiana F. A. Brito[1], Roberto S. Rocha[3] and Luzia H. Carvalho[1]

1 *Laboratório de Malária, Centro de Pesquisas René Rachou/Fiocruz, Belo Horizonte-MG, Brazil*
2 *Universidade Federal do Mato Grosso, Cuiabá-MT, Brazil*
3 *Instituto Leônidas e Maria Deane/Fiocruz, Manaus-AM, Brazil*
4 *Universidade de São Paulo, São Paulo-SP, Brazil*

Abstract

OBJECTIVE To investigate risk factors associated with the acquisition of antibodies against *Plasmodium vivax* Duffy binding protein (PvDBP) – a leading malaria vaccine candidate – in a well-consolidated agricultural settlement of the Brazilian Amazon Region and to determine the sequence diversity of the PvDBP ligand domain (DBP_{II}) within the local malaria parasite population.

METHODS Demographic, epidemiological and clinical data were collected from 541 volunteers using a structured questionnaire. Malaria parasites were detected by conventional microscopy and PCR, and blood collection was used for antibody assays and molecular characterisation of DBP_{II}.

RESULTS The frequency of malaria infection was 7% (6% for *P. vivax* and 1% for *P. falciparum*), with malaria cases clustered near mosquito breeding sites. Nearly 50% of settlers had anti-PvDBP IgG antibodies, as detected by enzyme-linked immunosorbent assay (ELISA) with subject's age being the only strong predictor of seropositivity to PvDBP. Unexpectedly, low levels of DBP_{II} diversity were found within the local malaria parasites, suggesting the existence of low gene flow between *P. vivax* populations, probably due to the relative isolation of the studied settlement.

CONCLUSION The recognition of PvDBP by a significant proportion of the community, associated with low levels of DBP_{II} diversity among local *P. vivax*, reinforces the variety of malaria transmission patterns in communities from frontier settlements. Such studies should provide baseline information for anti-malarial vaccines now in development.

keywords *Plasmodium vivax*, Duffy binding protein, antibodies, polymorphisms, frontier settlement

Introduction

After more than a century of control, *Plasmodium vivax* remains more widely distributed than *P. falciparum* and is a potential cause of morbidity and mortality amongst the 2.85 billion people living at risk of infection (Guerra *et al.* 2010). In Brazil, the Amazon Basin remains the largest malaria-endemic area in the Americas with 334 000 cases registered in 2010, more than 80% of which were caused by *P. vivax* (SVS/MS 2010).

In the Brazilian Amazon Region, the incidence of malaria is significantly influenced by political, economic, social and ecological factors (Taiul 2011). For five centuries, episodic waves of explorers, conquerors and colonists have penetrated various sections of the forest, exploiting its natural resources and native peoples, in a series of cultural successions called 'frontiers' (Browder *et al.* 2008). This process of frontier expansion has induced dramatic ecological transformations in the Amazon, which have facilitated human malaria transmission in the area (Marques 1987; De Castro *et al.* 2007). Although the dynamics of malaria transmission in these frontier settlements remains poorly understood (De Barros *et al.* 2011), it has been associated with land clearing and farming. Symptomatic infections decrease after several years of residence in those areas, indicating the development of some degree of clinical immunity (Silva-Nunes *et al.* 2008; Ladeia-Andrade *et al.* 2009).

In communities originating from frontier settlements, the available data on immune response to malaria vaccine candidates are still limited, and those few available studies suggest that antigenic polymorphisms and poor immunogenicity of

vaccine candidates might compromise the development of subunit vaccines (Bastos *et al.* 2007; Souza-Silva *et al.* 2010). As a contribution to current efforts towards vaccine development against *P. vivax*, we analysed the immunological response to the *P. vivax* Duffy binding protein (PvDBP), a leading malaria vaccine candidate that plays a critical role in *P. vivax* erythrocyte invasion (Grimberg *et al.* 2007; Chitnis & Sharma 2008). For this purpose, we compared the profiles of antibody responses to PvDBP, as well as to another vaccine candidate, the 19-kDa C-terminal fragment of *P. vivax* merozoite surface protein 1 ($PvMSP1_{19}$), among individuals living in a well-consolidated frontier settlement of the Brazilian Amazon Region. Owing to the polymorphic nature of PvDBP (Ampudia *et al.* 1996; Xainli *et al.* 2000; Sousa *et al.* 2006, 2010), we also investigated polymorphisms in the region II ligand domain of PvDBP (DBP_{II}).

Methods

Study area and population

The study took place in the agricultural settlement of Rio Pardo, Presidente Figueiredo municipality, in the north-east of Amazonas state, Brazil (Figure 1). The rural locality of Rio Pardo is located roughly 160 km from Manaus, the capital of the state, with main access via a paved road (BR-174) that connects the states of Amazonas and Roraima. The agricultural settlement was officially created in 1996, by the National Institute of Colonization and Agrarian Reform (INCRA), as part of the large-scale colonisation projects focused on agriculture and wide-ranging human settlement in the Amazon (De Castro *et al.* 2006). In this area, the human population lives on subsistence farming and fishing along the Rio Pardo stream. Housing quality is poor, thereby rendering ineffective indoor residual spraying of insecticides against the mosquito vectors of malaria. Health services are extremely limited, and a single government-run malaria diagnosis outpost provides free malaria diagnosis and treatment to the inhabitants of the study site. In the Rio Pardo area, the mean annual temperature is 31 °C, with a humid climate and average annual rainfall of 2000 mm per year. There are two recognised seasons: the rainy season (November–May) and the dry season (June–October). The settlement is composed of households located along both sides of unpaved roads, known locally as *Ramal* (Appendix 1). These unpaved roads are organised according to the typical deforestation pattern of Amazon settlements – the 'fishbone' pattern (Browder *et al.* 2008) – which consists of a main road (*Principal*) connected perpendicularly to side roads that are surrounded by tropical rain forest. The *Ramal* area of Rio Pardo consists of six side roads (Samuel, Novo Paraíso, Gusmão, Terra Preta, Taxista and Novo Progresso). The settlement also includes a riverine population, known as *Igarape*, living along the Rio Pardo stream, with dwellings located within 1.5 km of the stream margins (Appendix 1). In the Rio Pardo settlement, a population census (conducted September–October, 2008) identified 701 inhabitants, with 360 (51.4%) living in the Ramal area and 341 (48.6%) in the Igarape area.

In the study area, malaria transmission occurs year-round, with an Annual Parasitological Index (API) of 127 cases per 1000 inhabitants (SVS/MS 2008). Although *P. vivax* and *P. falciparum* are transmitted year-round, *P. vivax* causes about 80% of malaria cases (SVS/MS 2009).

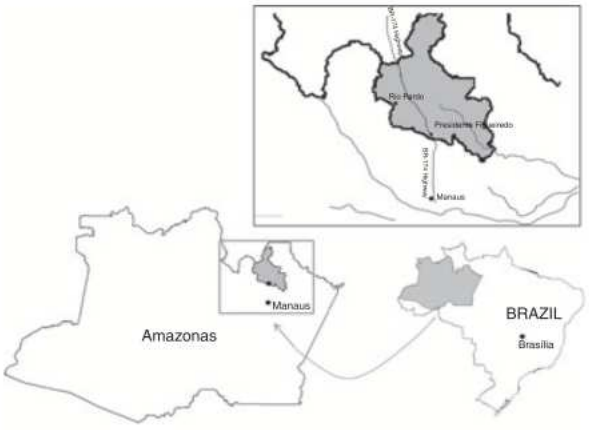

Figure 1 Map of the state of Amazonas, north-western Brazil, showing the Rio Pardo settlement, Presidente Figueiredo municipality (grey area in the inset), located roughly 160 km from the capital Manaus.

Study design

During the cross-sectional survey – November 18th to 26th 2008 – the field team visited all households in the study area, and the project objectives were explained to each family. Of 701 residents invited to participate in the study, 541 (77.2%), living in 176 dwellings, accepted, giving their written informed consent, as specified by the Brazilian National Council of Health (Resolution 196/96; Approved protocols No.007/2006 and No. 07/2009). The following procedures were performed during this initial field survey: (i) application of a structured questionnaire (including demographic, epidemiological and clinical information); (ii) physical examination, including body temperature and spleen sizes recorded according to standard clinical measurements; (iii) a search for malaria parasites by light microscopy; and (iv) venous blood collection (10 ml using

EDTA) from individuals aged 5 years or older, or blood spotted onto filter papers (finger-prick) in those aged < 5 years. The geographical location of each dwelling was also recorded during the survey, using a hand-held global positioning system (GPS, Garmin 12XL), with a positional accuracy within 15 m.

Cumulative exposure to malaria was estimated by age, the length of residence in malaria-endemic areas (either in the Rio Pardo settlement or elsewhere in the Amazon Region) and the self-reported number of lifetime malaria episodes. Clinical assessment was recorded based on symptoms suggestive of malaria, adapted from the original criteria described by Karunaweera *et al.* (1998). Most subjects (527, 97.4%) were native to the Amazon Region, but for 14 individuals information on ethnic origin was not available. Of 541 studied subjects, 432 (79.9%, median age 22 years) had serum samples collected for antibody assays. Data obtained during this cross-sectional survey provided the baseline for the prospective cohort, which was initiated in November of 2008 by parasitological and epidemiological surveillance.

Laboratory diagnosis of malaria

Two methods were used to detect malaria infection: microscopic examination of Giemsa-stained thick blood smears and real-time PCR amplification of a species-specific segment of the multicopy *18S rRNA* gene of human malaria parasites. The Giemsa-stained thick blood smear technique was performed by experienced local microscopists, according to the malaria diagnosis guidelines of the Brazilian Ministry of Health (SVS/MS 2005). The real-time PCR was performed according Mangold *et al.* (2005), in which a consensus pair of primers (PL1473F18 [5′ TAACGAACGAGATCTTAA 3′] and PL1679R18 [5′ GTTCCTCTAAGAAGC TTT 3′]) was used.

Amplification, sequencing and 3-D structural model of DBPII

DNA samples extracted from whole blood with *P. vivax* infection were used as templates to amplify sequences encoding DBP_{II}, corresponding to nucleotides 870–1545 (aa 290–515, reference sequence: Sal-I) (Fang *et al.* 1991). For PCR, platinum high fidelity Taq DNA polymerase (Invitrogen Corporation, Carlsbad, CA) was used and the following primers, forward: 5′ ATGTATGAAGGAAC TTACGAAT 3′, and reverse: 5′ACCTGCCGTCTGAACC TTTT 3′. The PCR products were purified using a GFX-96 PCR kit (GE Healthcare, Little Chalfont, UK), and directly sequenced using DYEnamic™ ET dye terminator kit (GE Healthcare) and MegaBace automated DNA sequencer (GE Healthcare). The sequences were analysed using Bioedit sequence alignment editor (http://www.mbio.ncsu.edu/bioedit/bioedit.html) to identify DBP_{II} polymorphisms relative to the SAL-1 sequence. As no heterozygous sites in DBP sequences were observed, we could infer that those sequences came from patients with either single infections or from predominant variants in patients with multiple clone infections. All single segregating sites were confirmed by sequencing from independent PCR amplifications (sixfold sequence coverage for each of the analysed isolates). Estimates of the nucleotide diversity (π), haplotype (H), haplotype diversity (Hd), and their corresponding standard deviations (SDs) were obtained using DNASP 4.10 software (Librado & Rozas 2009). The DBP_{II} sequences were deposited in GenBank with accession numbers JQ405271 to JQ405293.

Based on recent findings showing that dimerisation is conserved in DBL-domain receptor engagement and drives receptor affinity and specificity (Batchelor *et al.* 2011), the 3-D DBP_{II} dimer structure (PDB ID: 3RRC) was used to map polymorphic residues using PYMOL v1.0 software (Wijeyesakere *et al.* 2007).

Recombinant proteins and serological assays

Two recombinant *P. vivax* proteins were used to detect total IgG antibodies. The recombinant PvDBP, which includes amino acids 132–771 (regions II–IV, $DBP_{II\ IV}$), was expressed as a 140 kDa soluble glutathione S-transferase (GST) fusion protein. A detailed description of this GST fusion protein has been reported elsewhere (Fraser *et al.* 1997; Ceravolo *et al.* 2005). The recombinant protein representing the 19-kDa C-terminal region of the *P. vivax* merozoite surface protein-1 ($PvMSP1_{19}$), which represents amino acids 1616–1704 of the full-length MSP1 protein, was expressed as a 6xHisTag fusion protein, as described elsewhere (Cunha *et al.* 2002; Rodrigues *et al.* 2003; Barbedo *et al.* 2007). To assess IgG antibodies against PvDBP and $PvMSP1_{19}$, ELISAs were carried out as described previously (Ceravolo *et al.* 2005), using serum samples at a 1:100 dilution and recombinant proteins at a concentration of 5 μg/ml (PvDBP) and 1 μg/ml ($PvMSP1_{19}$). For PvDBP, the final optical density (OD) at 492 nm was calculated by subtracting the OD obtained with GST (antigen control). The results were expressed as reactivity index (RI = OD_{492nm} values of test sample divided by the value of the cut-off). Cut-off points were set at three standard deviations above the mean OD_{492nm} of sera from 30 individuals who had never been exposed to malaria. Values of RI > 1.0 were considered positive.

Statistical analysis

A database was created using Epidata software (http://www.epidata.dk). Proportions were compared using 2 × 2 contingency tables with either chi-squared tests, adjusted by Yates' continuity correction, or Fisher's exact tests, as appropriate. Pairwise correlations were evaluated with Spearman's correlation coefficient ρ. Differences in medians were tested by Kruskal–Wallis with Dunn's post hoc test to identify the significant differences between groups. Multiple logistic regression models with stepwise backward deletion were built to describe independent associations between covariates and the presence of malaria infection, antibodies to PvDBP and $PvMSP1_{19}$. Covariates were selected for inclusion in logistic models if they were associated with the outcome, at the 20% level of significance, in exploratory unadjusted analysis. Logistic regression models included the following covariates: age, gender, time of residence in Amazon Region, previous malaria episodes, recent malaria infection and household location within the study area. Multivariate logistic regression was performed using STATA v.10 software. Only variables associated with statistical significance at the 5% level were maintained in the final models.

Results

Malaria prevalence and risk factors associated with malaria infection

We have studied baseline malarial infection in 541 subjects, 9–44 years of age (median 22 years), with a male/female ratio of 1.3:1 (Table 1). The subjects had 4–10 years of residence in the settlement of Rio Pardo (median 7 years) and 9–38 years of residence in the Brazilian Amazon Region (median 19 years), where they have been continuously exposed to malaria infection. Based on the classical way to estimate malaria endemicity – the spleen rate (SR) in children aged 2–9 years (WHO 1951) – the study area was classified as mesoendemic (SR = 25%, 28 of 111).

The overall frequency of malaria infection, as detected by microscopy or PCR, was 7.0% (38 of 541), with 34 (6%) infections caused by *P. vivax* and only 4 (1%) by *P. falciparum*. Twenty of 38 (52.6%) infections were diagnosed by thick blood smear, and PCR allowed the detection of 18 (47.4%) additional malaria infections; most of the PCR-positive but microscopy-negative samples (10 *P. vivax* and one *P. falciparum*) were from individuals who had subclinical malaria infection. No mixed *Plasmodium* spp. infections and no episodes of severe or complicated malaria were detected in the study area. Of the 20 patients who reported signs and/or symptoms of uncomplicated malaria infection, headache (40%), fever (25%), chills (20%) and myalgia (20%) were the most prevalent symptoms.

Table 1 Demographical, epidemiological and immunological data of the 541 inhabitants of the agricultural settlement of Rio Pardo, Amazon, Brazil

Characteristic	
Median age, years (range)	22 (9–44 years)
Gender, male:female	1.3:1
Acute malaria infection, *n* (%)	38 (7.0%)*
Years of malaria exposure, median (range)	19 (9–38 years)
Years of residence in Rio Pardo, median (range)	7 (4–10 years)
Previous malaria episodes, median (range)	5 (1–11)
Antibody responses, *n* = 432; positive (%)†	
Anti-PvDBP	214 (49.5)
Anti-PvMSP119	261 (60.0)
Anti-PvMSP119 and/or anti- PvDBP	328 (75.9)

*Thirty-four infections by *P. vivax* and four by *P. falciparum*, as detected by conventional light microscopy and/or real-time PCR.
†Evaluated by ELISA using recombinant proteins against the Duffy binding protein (PvDBP) and 19-kDa fragment of C-terminal region of the merozoite surface protein 1 (PvMSP119).

In the study area, the risk of malaria infection was not associated with gender, age, time of residence in the endemic area, previous malaria experience or antibody responses (Table 2). However, the place of residence was significantly associated with susceptibility to malaria infection. People living in the Igarape area, along and around of the local stream, had a higher risk of malaria infection than people living in the *Ramal* area (unpaved roads) (OR = 5.1, 95% CI = 2.20–11.57, $P < 0.0001$). The association between dwelling location and malaria infection was further indicated by adjusted analysis in a stepwise logistic regression model: specifically, the probability of being infected with malaria was six times higher in individuals living along the Rio Pardo stream than in those living along the unpaved *Ramal* roads (OR = 6.17, 95% CI = 2.10–18.14, $P = 0.010$). Accordingly, a significantly lower frequency of malaria symptoms was detected among residents living along the Rio Pardo stream (25.8% *vs.* 34.4% in the Igarape and *Ramal*, respectively; $P = 0.029$).

Naturally acquired IgG antibody responses to PvDBP and $PvMSP1_{19}$

Of 541 subjects, 432 (79.8% of the eligible) had their serum samples tested for IgG antibodies to PvDBP and $PvMSP1_{19}$. The frequency of PvDBP antibodies was 49.5% (214/432); that of $PvMSP1_{19}$ antibodies 60% (261/432)

Table 2 Risk factors associated with malaria infection in 541 inhabitants of the Rio Pardo settlement, Amazon, Brazil

Variables	*N*	Acute malarial infection	
		OR (95% CI)*	*P*-value
Gender			
Male	306	1	0.87
Female	235	1.06 (0.54–2.05)	
Age (years)			
0–9	126	1	
9–22	140	1.59 (0.60–4.10)	0.46
22–44	135	1.36 (0.50–3.70)	0.72
>44	139	1.18 (4.25–3.30)	0.96
Residence in Amazon Region (years)			
0–9	120	1	
9–19	124	1.26 (0.45–3.50)	0.797
19–38	130	1.35 (0.49–3.65)	0.621
>38	126	1.24 (0.45–3.44)	0.797
Residence in Rio Pardo (years)			
0–9	367	1	
9–19	153	1.02 (0.49–2.11)	0.97
>19	14	1.01 (0.13–8.02)	1.00
Previous malaria episodes			
Zero	78	1	
1–4	119	1.10 (0.25–4.73)	1.00
5–10	121	2.75 (0.75–10.09)	0.17
>10	130	3.26 (0.91–11.65)	0.07
Dwelling localisation			
Ramal area†	276	1	**<0.0001**
Igarapé area‡	265	5.1 (2.2–11.78)	
Antibodies anti-PvDBP and/or anti-PvMSP1$_{19}$§			
No	104	1	
Yes	328	2.24 (0.77–6.57)	0.19

In bold, there was statistical significance level of 5% (*P*-value).
*The odds ratio (OR), respective 95% confidence intervals (95% CI).
†Unpaved roads.
‡Located within 1.5 km of stream margins.
§Presence of anti-PvDBP and/or anti-PvMSP1$_{19}$.

(Table 1). The overall antibody response was 75.9% (328/432), and, as expected, the reactivity indices to PvDBP and PvMSP1$_{19}$ were weakly correlated ($\rho = 0.24$, $P < 0.0001$; Spearman's correlation test).

We further analysed whether the antibody responses against PvDBP and PvMSP1$_{19}$ were related to clinical, demographic and epidemiological variables. According to the analysis, there was no association between antibodies and malaria symptoms ($\chi^2 = 0.003$, $P = 0.96$). However, the likelihood of having PvDBP antibodies increased with age, time of residence in the Amazon Region (but not in the Rio Pardo settlement), and in those subjects who had reported more than 10 previous malaria episodes or who had been exposed to malaria parasites in the previous 6 months (Table 3). Nevertheless, using multiple logistic regression models, subject age was the only predictor significantly associated with the presence of anti-PvDBP antibodies (adjust OR = 1.05, 95% CI = 1.02–1.08, $P = 0.005$). Each additional year of age increased the probability of having anti-PvDBP antibodies by 5%. Figure 2 illustrates the increase in the magnitude of anti-PvDBP antibody response according to subject's age.

With regard to PvMSP1$_{19}$, age was not associated the magnitude of the anti-PvMSP1$_{19}$ antibody response, but the odds of being seropositive increased with recent exposure to malaria, the number of previous malaria episodes, and dwelling location (Table 3). However, adjusted logistic regression analysis identified recent exposure to malaria and dwelling location as independent predictors of seropositivity to PvMSP1$_{19}$ (respectively, adjusted OR = 2.74, 95% CI = 1.41–5.33; and adjusted OR = 1.71, 95% CI = 1.01–2.86).

DBP$_{II}$ polymorphism among local parasites

By sequencing PvDBP ligand domain (region II, DBP$_{II}$), we identified 15 polymorphic sites (two synonymous and 13 non-synonymous substitutions). For further analysis, polymorphic residues identified in the studied samples were included in a 3-D structure of DBP$_{II}$ (Figure 3a) and showed in details in Appendix 2. Although polymorphic residues were widely distributed throughout the DBP$_{II}$ sequence, three residues (404, 417 and 424) were clearly located surround the DARC-binding site (Figure 3a, coloured in yellow) or at the DBP$_{II}$ dimer interface (coloured in purple). The polymorphisms identified in the study area were arranged in eight haplotypes (Figure 3b), corresponding to a haplotype diversity of 0.715 (SD 0.093). Remarkably, the reference Sal-1 DBP$_{II}$ variant (haplotype 1) was highly prevalent (52.17%) in the Rio Pardo community.

Discussion

The selection of an antigen for vaccination requires a detailed understanding of natural immune responses elicited by the protein in different epidemiological and ecological settings. A baseline study of PvDBP – the major *P. vivax* vaccine candidate – in a well-organised Amazon community showed prevalence rates of 6% for *P. vivax* and 1% for *P. falciparum*, with a spleen rate of 25% among children 2–9 years of age. Despite the limitations of classical indices for measuring malaria endemicity (Hay *et al.* 2008), the spleen and parasite rates allowed classification of the study area as hypo- to mesoendemic, consistent with the general profile of infection for

Table 3 Risk factors for the presence of antibodies to PvDBP and PvMSP1$_{19}$ in the 432 inhabitants of the Rio Pardo settlement, Amazon, Brazil

		Anti-PvDBP		Anti-PvMSP1$_{19}$	
Variables	*N*	OR (95% CI)*	*P*-value	OR (95% CI)*	*P*-value
Gender					
Male	249	1	0.68	1	0.66
Female	183	0.90 (0.62–1.32)		0.90 (0.61–1.32)	
Age (years)					
0–9	30	1		1	
9–22	132	1.38 (0.58–3.25)	0.60	0.96 (0.43–2.14)	0.92
22–44	133	2.84 (1.21–6.66)	**0.02**	1.45 (0.65–3.24)	0.49
>44	137	3.59 (1.53–8.42)	**0.004**	1.15 (0.52–2.57)	0.88
Residence in the Amazon Region (years)					
0–9	30	1		1	
9–19	116	1.53 (0.64–3.64)	0.45	0.96 (0.43–2.14)	0.92
19–38	128	2.64 (1.12–6.22)	**0.038**	1.16 (0.53–2.50)	0.87
>38	124	3.45 (1.46–8.16)	**0.006**	1.27 (0.57–2.85)	0.71
Residence in Rio Pardo (years)					
0–9	264	1		1	
9–19	150	1.06 (0.71–1.58)	0.86	0.96 (0.64–1.45)	0.93
>19	13	1.20 (0.39–3.68)	0.97	0.75 (0.24–2.28)	0.77
Previous malaria episodes					
0	38	1		1	
1–4	96	1.74 (0.81–3.37)	0.15	11.13 (3.66–33.82)	**<0.0001**
5–10	96	1.19 (0.55–2.56)	0.65	29.95 (9.54–94.03)	**<0.0001**
>10	123	2.09 (1.00–4.40)	**0.049**	15.26 (5.08–45.84)	**<0.0001**
Recent malaria†					
No	280	1	**0.047**	1	**<0.0001**
Yes	100	1.59 (1.00–2.53)		3.35 (1.89–5.95)	
Dwelling localisation					
Ramal‡	223	1	0.44	1	**<0.0001**
Igarapé§	210	1.80 (0.81–1.74)		2.93 (1.96–4.38)	

In bold, there was statistical significance level of 5% (p-value).
*The odds ratio (OR) and respective 95% confidence intervals (95% CI).
†Malaria diagnosed 6 month before the enrollment, November of 2008.
‡Unpaved roads.
§Located within 1.5 km of stream margins.

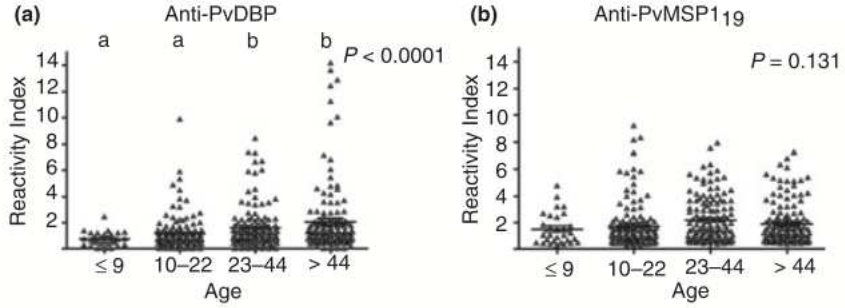

Figure 2 The levels of antibody against the Duffy binding protein (PvDBP) but not against the merozoite surface protein 1 (PvMSP1$_{19}$) increase with subject's age. IgG antibodies to PvDBP (a) or PvMSP1$_{19}$ (b) were evaluated by enzyme-linked immunosorbent assay (ELISA), as described in Material and Methods. Sera reactivity was expressed as Reactivity Index (RI) at 492 nm, with RI > 1.0 being considered positive. Different letters on the top of the figure indicate significant difference ($P < 0.05$), as determined by Kruskal–Wallis test with Dunn's post-test.

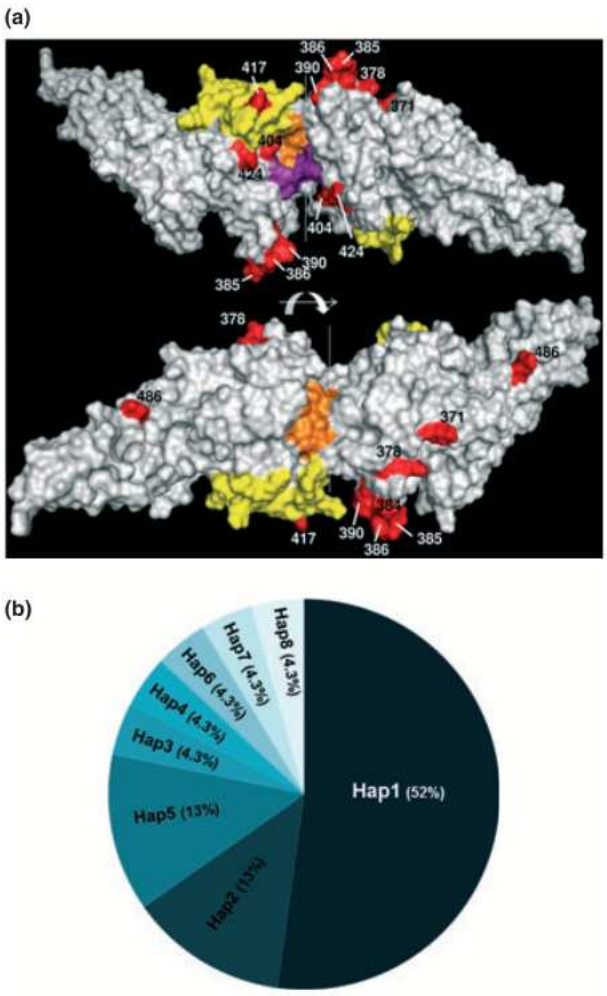

Figure 3 DBP_{II} 3-D structure and haplotypes. (a) DBP_{II} dimeric structure showing: (i) residues that form the putative sulfotyrosine-binding pocket at the dimer interface (Lys273, Arg274 and Gln356 – purple); (ii) residues that surround the DARC-binding groove and are required for DARC binding (Asn291, Asn292, Tyr293, Arg294, Tyr295, Asn296, Lys297, Asp298, Phe299 and Val365, Lys366, Lys367, Arg368, Leu369, Lys370, Gly371, Asn372, Phe373, Ile374, Trp375, Ile376, Cys377 – yellow); (iii) residues that make contact creating the dimeric architecture (Phe267, Leu270, Il277, Tyr278, Val282, Tyr363 and Arg274, Glu249 – orange) (Batchelor *et al.* 2011); (iv) and polymorphic residues identified in the Rio Pardo isolates (in red). A dashed line indicates the dimer interface. (b) DBP_{II} haplotype frequencies (%). Eight haplotypes were identified in the study area (named from hap1 to hap8).

well-established frontier settlements (da Silva-Nunes *et al.* 2008; De Barros *et al.* 2011). However, in the Rio Pardo settlement, we observed a significant positive correlation between the subject's age and cumulative exposure to malaria. This contrasts with the typical description of frontier malaria across the Amazon Basin, in which most subjects are migrants from malaria-free areas, and their ages do not necessarily correlate with exposure to malaria (Camargo *et al.* 1994; De Castro *et al.* 2006; da Silva-Nunes *et al.* 2008). In Rio Pardo, most incoming migrants are native to the Amazon Region and have probably had a lifetime of exposure to malaria infection. Our observations reinforce the diversity of malaria transmission patterns in communities from frontier settlements.

In the study area, residents along and around the Rio Pardo stream were more likely to be infected with malaria that those living along the unpaved roads, suggesting that they are much more exposed to malaria transmission, presumably because of proximity to anopheline breeding sites. Previous studies have showed that only 20% of *Anopheles darling*, the main vector in these areas, would fly over 500 m from its natural larval habitats (De Barros & Honório 2007). This explains our results, where cases are clustered near breeding sites, while the majority of the population remains unaffected. Consequently, epidemiological models derived from urban or riverine malaria are probably inadequate for describing disease transmission in agricultural settlements (De Barros *et al.* 2011). Overall, these data corroborate earlier studies, concluding that malaria transmission is a local problem, which varies within a village according to the microepidemiological factors (Carvalho *et al.* 1999; da Silva-Nunes *et al.* 2008; Moss *et al.* 2011).

Interestingly, our results showed that the occurrence of malaria symptoms (such as fever, headache chills or myalgia) was less common among residents along the stream than along the unpaved roads. Although the occurrence of asymptomatic infection suggest some degree of clinical protection, the presence or absence of symptoms in the Amazon Region may not correspond to stable phenotypes and may not necessarily indicate clinical immunity (da Silva-Nunes & Ferreira 2007).

Almost 50% of the studied individuals had antibody responses to PvDBP, and the prevalence of responders did not differ significantly among residents of stream and unpaved roads, suggesting that dwelling location was not a good predictor of the presence of anti-PvDBP antibodies in the study area. On the other hand, the subject's age was a strong predictor of seropositivity to PvDBP. This profile of antibody response was quite different to that obtained to another vaccine candidate, the 19-kDa C-terminal region of the merozoite surface protein 1 ($PvMSP1_{19}$), in which subject's age was not related with positivity or intensity of antibody response. Consistent with these observations, previous studies were unable to demonstrate an association between anti-$PvMSP1_{19}$

antibodies and cumulative exposure to malaria (Fraser *et al.* 1997; Ceravolo *et al.* 2005), possibly because antibodies to $PvMSP1_{19}$ are directed mainly to conserved epitopes (Soares *et al.* 1999), whereas anti-PvDBP antibodies are biased towards polymorphic epitopes (VanBuskirk *et al.* 2004; Ceravolo *et al.* 2009). Consequently, it was not surprising that recent malaria infection was a good predictor of anti-$PvMSP1_{19}$ antibodies in Rio Pardo community.

Recently, poor antibody recognition of PvDBP (< 20% of responders) was detected in a frontier Amazonian settlement, Acre State, where most settlers were migrants from a malaria-free area (Souza-Silva *et al.* 2010). Given the fact that DBP was recognised by approximately 50% of the Rio Pardo community, it is reasonable to assume that a significant number of settlers from the Rio Pardo area are able to mount an adequate anti-PvDBP antibody response. The reasons for the difference of anti-PvDBP antibody responses between these two settlements are not clear but may relate to several factors including: (i) differences in exposure to malaria (migrant *vs.* native populations), (ii) different genetic backgrounds of the human host populations, and (iii) diversity of the malaria parasite populations. While we cannot rule out significant genetic differences between the two human populations, it seems unlikely because most of the Brazilian population contains a significant amount of racial admixture (Trachtenberg *et al.* 1988). On the other hand, the levels of genetic diversity of DBP_{II} were quite different between *P. vivax* isolates circulating in these two settlements, with the main DBP_{II} haplotype of the Rio Pardo settlement (Sal-1 background) being detected in low frequency in the Acre community (Sousa *et al.* 2010; Souza-Silva *et al.* 2010). As antibodies to PvDBP are allele-specific (VanBuskirk *et al.* 2004; Ceravolo *et al.* 2009), it is reasonable to conclude that the lower parasite diversity associated with a major exposure to malaria seems to be responsible for the relatively higher anti-PvDBP antibody response among the Rio Pardo community.

Intriguingly, the DBP_{II} Sal-1 haplotype found in more than half of the Rio Pardo malaria parasites sampled has only been found at a low frequency in other Amazon regions (approximately 10%) (Sousa *et al.* 2010), and, of note, it seems to be largely restricted to some geographical areas of the world (Nóbrega de Sousa *et al.* 2011). This finding is striking because DBP_{II} Sal-1 variant is currently being used to develop a PvDBP-based vaccine (Arevalo-Herrera *et al.* 2005; Yazdani *et al.* 2006; Moreno *et al.* 2008). Although our results in Rio Pardo are limited to a small number of *P. vivax* infections, the data are consistent with the existence of a possible barrier to malaria parasite gene flow caused by the relative isolation of the Rio Pardo settlement and the rarity of motorised transport in the area, typical of frontier zones, which severely limits the mobility of the settlers (De Barros *et al.* 2011).

Overall, we have demonstrated significant antibody recognition of PvDBP in a community from a frontier settlement in the Amazon Region, where host age seems to be a good predictor of the presence of anti-PvDBP IgG response. The unexpected low levels of DBP_{II} diversity among the local malaria parasites emphasise the importance of understanding the molecular epidemiology of *P. vivax* vaccine candidates in different malaria transmission settings. Such studies should provide baseline information that will be crucial for understanding the potential effects of the vaccines now in development.

Acknowledgements

We thank the inhabitants of Rio Pardo for enthusiastic participation in the study; the local malaria control team in Presidente Fiqueiredo for their logistic support; the scientific units of Oswaldo Cruz Foundation in Manaus, AM (Fiocruz-AM) and Belo Horizonte, MG (Fiocruz-Minas) for overall support. Luke A. Baton reviewed the manuscript. This work was supported by the Research Foundation of Minas Gerais State (FAPEMIG), Research Foundation of Amazonas State (FAPEAM), the Brazilian National Research Council (CNPq), and Pronex Malaria/CNPq/DECIT/MS. PAN, CJFF, IS, CFAB and LHC are research fellows of the CNPq.

References

Ampudia E, Patarroyo MA, Patarroyo ME & Murillo LA (1996) Genetic polymorphism of the Duffy receptor binding domain of *Plasmodium vivax* in Colombian wild isolates. *Molecular and Biochemical Parasitology* **78**, 269–272.

Arevalo-Herrera M, Castellanos A, Yazdani SS *et al.* (2005) Immunogenicity and protective efficacy of recombinant vaccine based on the receptor-binding domain of the *Plasmodium vivax* Duffy binding protein in Aotus monkeys. *American Journal of Tropical Medicine and Hygiene* **73**, 25–31.

Barbedo MB, Ricci R, Jimenez MCS *et al.* (2007) Comparative recognition by human IgG antibodies of recombinant proteins representing three asexual erythrocytic stage vaccine candidates of *Plasmodium vivax*. *Memorias do Instituto Oswaldo Cruz* **102**, 335–339.

Bastos MS, da Silva-Nunes M, Malafronte RS *et al.* (2007) Antigenic polymorphism and naturally acquired antibodies to *Plasmodium vivax* merozoite surface protein 1 in rural Amazonians. *Clinical and Vaccine Immunology* **14**, 1249–1259.

Batchelor JD, Zahm JA & Tolia NH (2011) Dimerization of *Plasmodium vivax* DBP is induced upon receptor binding and drives recognition of DARC. *Nature Structural & Molecular Biology* **18**, 908–914.

Brasil. Ministério da Saúde. Secretaria de Vigilância em Saúde. DATASUS. Sistema de Informação de Vigilância Epidemiológica – SIVEP – Malária 2008. Available from: http://dw.saude.gov.br/portal/page/portal/sivep_malaria/TAB 99449:tab_resumo_n?Ano_n=2008.

Brasil. Ministério da Saúde. Secretaria de Vigilância em Saúde. DATASUS. Sistema de Informação de Vigilância Epidemiológica - SIVEP - Malária. 2009. Available from: http://dw.saude.gov.br/portal/page/portal/sivep_malaria/TAB 99449:tab_resumo_n?Ano_n=2009.

Brasil. Ministério da Saúde. Secretaria de Vigilância em Saúde. DATASUS. Sistema de Informação de Vigilância Epidemiológica - SIVEP - Malária. 2010. Available from: http://dw.saude.gov.br/portal/page/portal/sivep_malaria/TAB 99449:tab_resumo_n?Ano_n=2010.

Brasil. Ministério da Saúde. Secretaria de Vigilância em Saúde (2005) *Manual de diagnóstico laboratorial da malária / Ministério da Saúde, Secretaria de Vigilância em Saúde.* Ministério da Saúde, Brasília, 112.

Browder JO, Pedlowski MA & Walker R (2008) Revisiting Theories of Frontier Expansion in the Brazilian Amazon: A Survey of the Colonist Farming Population in Rondônia's Post-frontier, 1992–2002. *World Development* **36**, 1469–1492.

Camargo L, Ferreira M, Krieger H, De Camargo E & Da Silva L (1994) Unstable hypoendemic malaria in Rondonia (western Amazon region, Brazil): epidemic outbreaks and work-associated incidence in an agro-industrial rural settlement. *The American Journal of Tropical Medicine and Hygiene* **51**, 16–25.

Carvalho LH, Fontes CJ & Krettli AU (1999) Cellular responses to *Plasmodium falciparum* major surface antigens and their relationship to human activities associated with malaria transmission. *The American Journal of Tropical Medicine and Hygiene* **60**, 674–679.

Ceravolo IP, Bruna-Romero O, Braga EM *et al.* (2005) Anti-*Plasmodium vivax* Duffy binding protein antibodies measure exposure to malaria in the Brazilian Amazon. *The American Journal of Tropical Medicine and Hygiene* **72**, 675–681.

Ceravolo IP, Sanchez BA, Sousa TN *et al.* (2009) Naturally acquired inhibitory antibodies to *Plasmodium vivax* Duffy binding protein are short-lived and allele-specific following a single malaria infection. *Clinical and Experimental Immunology* **156**, 502–510.

Chitnis CE & Sharma A (2008) Targeting the *Plasmodium vivax* Duffy-binding protein. *Trends in Parasitology* **24**, 29–34.

Cunha MG, Rodrigues MM & Soares IS (2002) Comparison of the immunogenic properties of recombinant proteins representing the *Plasmodium vivax* vaccine candidate MSP1(19) expressed in distinct bacterial vectors. *Vaccine* **20**, 385–396.

De Barros FS & Honório NA (2007) Man biting rate seasonal variation of malaria vectors in Roraima, Brazil. *Memorias do Instituto Oswaldo Cruz* **102**, 299–302.

De Barros FS, Arruda ME, Gurgel HC & Honório NA (2011) Spatial clustering and longitudinal variation of *Anopheles darlingi* (Diptera: Culicidae) larvae in a river of the Amazon: the importance of the forest fringe and of obstructions to flow in frontier malaria. *Bulletin of Entomological Research* **101**, 1–16.

De Castro MC, Monte-Mór RL, Sawyer DO & Singer BH (2006) Malaria risk on the Amazon frontier. *Proceedings of the National Academy of Sciences of the United State of America* **103**, 2452–2457.

De Castro MC, Sawyer DO & Singer BH (2007) Spatial patterns of malaria in the Amazon: implications for surveillance and targeted interventions. *Health and Place* **13**, 368–380.

Fang XD, Kaslow DC, Adams JH & Miller LH (1991) Cloning of the *Plasmodium vivax* Duffy receptor. *Molecular and Biochemical Parasitology* **44**, 125–132.

Fraser T, Michon P, Barnwell JW *et al.* (1997) Expression and serologic activity of a soluble recombinant *Plasmodium vivax* Duffy binding protein. *Infection and Immunity* **65**, 2772–2777.

Grimberg BT, Udomsangpetch R, Xainli J *et al.* (2007) *Plasmodium vivax* invasion of human erythrocytes inhibited by antibodies directed against the Duffy binding protein. *Plos Medicine* **4**, 1940–1948.

Guerra CA, Howes RE, Patil AP *et al.* (2010) The international limits and population at risk of *Plasmodium vivax* transmission in 2009. *PLoS Neglected Tropical Disease* **4**, e774.

Hay SI, Smith DL & Snow RW (2008) Measuring malaria endemicity from intense to interrupted transmission. *The Lancet Infectious Disease* **8**, 369–378.

Karunaweera ND, Carter R, Grau GE & Mendis KN (1998) Demonstratrion of anti-disease immunity to Plasmodium vivax malaria in Sri Lanka using a quantitative method to assess clinical disease. *The Amercian Journal of Tropical Medicine and Hygiene* **58**, 204–210.

Ladeia-Andrade S, Ferreira MU, De Carvalho ME, Curado I & Coura JR (2009) Age-dependent acquisition of protective immunity to malaria in riverine populations of the Amazon Basin of Brazil. *The American Journal of Tropical Medicine and Hygiene* **80**, 452–459.

Librado P & Rozas J (2009) DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* **25**, 1451–1452.

Mangold KA, Manson RU, Koay ES *et al.* (2005) Real-time PCR for detection and identification of *Plasmodium* spp. *Journal of Clinical Microbiology* **43**, 2435–2440.

Marques AC (1987) Human migration and the spread of malaria in Brazil. *Parasitology Today* **3**, 166–170.

Moreno A, Caro-Aguilar I, Yazdani SS *et al.* (2008) Preclinical assessment of the receptor-binding domain of *Plasmodium vivax* Duffy-binding protein as a vaccine candidate in rhesus macaques. *Vaccine* **26**, 4338–4344.

Moss WJ, Hamapumbu H, Kobayashi T *et al.* (2011) Use of remote sensing to identify spatial risk factors for malaria in a region of declining transmission: a cross-sectional and longitudinal community survey. *Malaria Journal* **10**, 163.

Nóbrega de Sousa T, Carvalho LH & Alves de Brito CF (2011) Worldwide Genetic Variability of the Duffy Binding Protein: insights into *Plasmodium vivax* Vaccine Development. *PLoS ONE* **6**, e22944.

Rodrigues MHC, Cunha MG, Machado RLD, Ferreira OC, Rodrigues MM & Soares IS (2003) Serological detection of Plasmodium vivax malaria using recombinant proteins

corresponding to the 19-kDa C-terminal region of the merozoite surface protein-1. *Malaria Journal* **2**, 1–7.

da Silva-Nunes M & Ferreira MU (2007) Clinical spectrum of uncomplicated malaria in semi-immune Amazonians: beyond the 'symptomatic 'vs 'asymptomatic 'dichotomy. *Memorias do Instituto Oswaldo Cruz* **102**, 341–347.

Silva-Nunes M, Codeco CT, Malafronte RS *et al.* (2008) Malaria on the Amazonian frontier: transmission dynamics, risk factors, spatial distribution, and prospects for control. *The American Journal of Tropical Medicine and Hygiene* **79**, 624–635.

da Silva-Nunes M, Codeço C, Malafronte R *et al.* (2008) Malaria on the Amazonian frontier: transmission dynamics, risk factors, spatial distribution, and prospects for control. *The American Journal of Tropical Medicine and Hygiene* **79**, 624–635.

Soares IS, Barnwell JW, Ferreira MU *et al.* (1999) A *Plasmodium vivax* vaccine candidate displays limited allele polymorphism, which does not restrict recognition by antibodies. *Molecular Medicine* **5**, 459–470.

Sousa T, Cerávolo I, Fontes CF, Couto A, Carvalho L & Brito C (2006) The pattern of major polymorphisms in the Duffy binding protein ligand domain among *Plasmodium vivax* isolates from the Brazilian Amazon area. *Molecular and Biochemical Parasitology* **146**, 251–254.

Sousa TN, Tarazona-Santos EM, Wilson DJ *et al.* (2010) Genetic variability and natural selection at the ligand domain of the Duffy binding protein in Brazilian *Plasmodium vivax* populations. *Malaria Journal* **9**, 334.

Souza-Silva FA, da Silva-Nunes M, Sanchez BA *et al.* (2010) Naturally acquired antibodies to *Plasmodium vivax* Duffy binding protein (DBP) in Brazilian Amazon. *The Amercian Journal of Tropical Medicine and Hygiene* **82**, 185–193.

Taiul PL (2011) The Prospect of eliminating malaria transmission in some regions of Brazil. *Memórias do Instituto Oswaldo Cruz* **106**, 105–106.

Trachtenberg A, Jobim LF, Kraemer E *et al.* (1988) The HLA polymorphism in five Brazilian populations. *Annuals of Human Biology* **15**, 213–221.

VanBuskirk KM, Cole-Tobian JL, Baisor M *et al.* (2004) Antigenic drift in the ligand domain of *Plasmodium vivax* Duffy binding protein confers resistance to inhibitory antibodies. *Journal of Infectious Diseases* **190**, 1556–1562.

Wijeyesakere SJ, Richardson RJ & Stuckey JA (2007) Modeling the tertiary structure of the patatin domain of neuropathy target esterase. *Protein Journal* **26**, 165–172.

World Health Organization (1951) *Expert Committee on Malaria, Report on the Fourth Session.* Technical report series no. 39, World Health Organization, Uganda.

Xainli JL, Adams JH & King CL (2000) The erythrocyte binding motif of *Plasmodium vivax* Duffy binding protein is highly polymorphic and functionally conserved in isolates from Papua New Guinea. *Molecular and Biochemical Parasitology* **111**, 253–260.

Yazdani SS, Shakri AR, Pattnaik P *et al.* (2006) Improvement in yield and purity of a recombinant malaria vaccine candidate based on the receptor-binding domain of *Plasmodium vivax* Duffy binding protein by codon optimization. *Biotechnology Letters* **28**, 1109–1114.

Corresponding Author Luzia H. Carvalho, Laboratório de Malária, Centro de Pesquisa René Rachou/Fundação Oswaldo Cruz, Avenida Augusto de Lima 1715, Belo Horizonte 30190-002, Brazil. Tel.: +55 31 33497793; Fax: +55 31 32953115; E-mail: lhcarvalho@cpqrr.fiocruz.br

Appendix I The Rio Pardo settlement is composed of two areas: (a) unpaved roads, known locally as 'Ramal', which includes households located on both sides; and (b) dwellings along of the Rio Pardo stream, known as Igarape

.

Appendix 2 Description of DBP_{II} polymorphisms identified in *P. vivax* isolates of Rio Pardo

Polymorphisms	915	924	1111	1134	1151	1153	1156	1158	1169	1192	1211	1251	1270	1309	1456
Nucleotide*	AAT† AAC	AGG AG**T**	AAA GAA	CGC CG**T**	GAT GGT	GAA AAA	AAG CAG	AAG AA**T**	CGT CA**T**	**T**CT A**C**T	ACA AGA	AA**T** AAA	**T**TA A**T**A	TGG CGG	CAA GAA
AA Haplotype (N)‡	N305N	R308S	K371E	R378R	D384G	E385K	K386Q	K386N	R390H	S398T	T404R	N417K	L424I	W437R	Q486E
1 (12)															
2 (3)				..T§	.G.	A..		..T	.A.	A..	.G.		A..		G..
3 (1)			G..		.G.	A..		..T				..A	A..	C..	
4 (1)			G..				C..						A..		
5 (3)		..T		..T	.G.	A..		..T	.A.	A..	.G.		A..		G..
6 (1)	..C														
7 (1)					.G.				.A.						
8 (1)					.G.				.A.						G..

*Nucleotide and amino acid (AA) numbers according to SAL-1 sequence (Fang *et al.* 1991).
†First codon corresponds to SAL-1 sequence and the others to the polymorphic ones observed in Brazilian isolates, substitutions are shown in bold.
‡Number of isolates with the specified haplotype.
§Dots indicate the invariant nucleotide and the letter indicates the polymorphic nucleotide.