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Restricted genetic and antigenic diversity of *Plasmodium falciparum* under mesoendemic transmission in the Venezuelan Amazon

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SUMMARY

The study of genetic diversity in malaria populations is expected to provide new insights for the deployment of control measures. *Plasmodium falciparum* diversity in Africa and Asia is thought to reflect endemicity. In comprehensive epidemiological surveys reported here the genetic and antigenic structure of *P. falciparum* in the Venezuelan Amazon were studied over a 2-year period. DNA polymorphisms in glutamate-rich protein (*GLURP*), merozoite-surface protein 1 (*MSP1*) and *MSP2* genes, in a multicopy element (*PfRRM*), all showed low diversity, 1 predominant genotype, and virtually no multi-clonal infections. Moreover, linkage disequilibrium was seen between *GLURP*, *MSP1* and *MSP2*. Specific antibody responses against *MSP1* and *MSP2* recombinant antigens reflected the low genetic diversity observed in the parasite population. This is unexpected in a mesoendemic area, and suggests that the low diversity here may not only relate to endemicity but to other influences such as a bottleneck effect. Linkage disequilibrium and a predominant genotype may imply that *P. falciparum* frequently propagates with an epidemic or clonal population structure in the Venezuelan Amazon.

Key words: *Plasmodium falciparum*, genetic diversity, antigenic diversity, linkage disequilibrium, epidemic clonality, Venezuela.

INTRODUCTION

There is considerable interest in understanding how natural populations of *Plasmodium falciparum*, the most important human malaria parasite, are genetically structured. An assessment of parasite population structure is relevant to aspects of malaria control such as vaccine design, spread of multidrug resistant strains (Curtis & Otoo, 1986; Dye & Williams, 1997), strain typing for epidemiological tracking (Konaté *et al.* 1999), and acquisition of immunity against malaria (Greenwood, Marsh & Snow, 1991; Gupta & Day, 1994*a, b*). Numerous

field studies have been undertaken to determine the extent of *P. falciparum* diversity and its mating structure (Creasey *et al.* 1990; Ferreira *et al.* 1998; Conway & McBride, 1991; Felger *et al.* 1994; Babiker, Satti & Walliker, 1995; Konaté *et al.* 1999; Paul *et al.* 1998). Different degrees of genetic diversity (reviewed by Babiker & Walliker, 1997) between and within defined geographical areas have been positively correlated to the intensity of malaria transmission (Babiker *et al.* 1994, 1997; Paul *et al.* 1995, 1998; Arnot, 1998). Interestingly though, even in areas with hypoendemic seasonal transmission, higher than expected levels of *P. falciparum* diversity and mean number of genotypes per human host were encountered (Babiker *et al.* 1991; Paul *et al.* 1998).

Most studies of mating patterns and population structure of *P. falciparum* have been carried out in Africa and South East Asia. Malaria in South America has particular epidemiological characteristics (Gilles, 1993), and these may be reflected in the

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parasite's population structure and dynamics compared to the African/Asian settings. These differences could be exploited in the design of effective malaria control measures in S. America. Recent studies in the latter subcontinent suggest that diversity in *P. falciparum* is generally less than in other regions, and have mainly related this to a low intensity of transmission (Haddad *et al.* 1999; Ariei *et al.* 1999; Da Silveira *et al.* 1999; Laserson *et al.* 1999). We report results of genetic analysis based on the comprehensive cross-sectional sampling over 2 successive seasons of a population of *P. falciparum* in a defined district of the Venezuelan Amazon, where malaria is the major public health problem (Gabaldón, 1983). We find evidence of strong departures from panmictic expectations in the parasite population, as shown by significant linkage disequilibrium among loci, with a remarkably low level of genetic diversity, despite entomological inoculation rates and antibody prevalences indicating mesoendemic (intermediate) *P. falciparum* transmission in the area. We argue that diversity here may not only relate to endemicity but to other influences, and that *P. falciparum* frequently propagates with an epidemic or clonal population structure in the Venezuelan Amazon.

MATERIALS AND METHODS

Study area

The Padamo River area (2°54'–3°30'N, 65°10'–65°20'W) located in the Amazon region of southern Venezuela (Upper Orinoco River basin) forms part of the southern focus of malaria, which currently accounts for 70% of the malaria cases in Venezuela (DER, 1996). The area is characterized by seasonal malaria transmission with a rainy season from May to October, followed by a relatively dry season.

In 1995/6, the overall crude malaria prevalence was 12.7% where 90/708 individuals had positive slides for malaria parasites. *Plasmodium vivax* accounted for 43% of malaria infections, 37% were due to *P. falciparum*, 18% to *P. malariae* and 2% were mixed infections. *P. falciparum* transmission has been estimated to be mesoendemic from the splenomegaly prevalence in 2 to 9-year-old children (26% (56/215)) (WHO, 1963; Gilles, 1993) and using 2 parameters, the force of infection, and the entomological inoculation rate (EIR). The force of infection was estimated using a constant risk catalytic conversion model (Snow *et al.* 1996, 1997) and age-specific anti-*P. falciparum* immunoglobulin G (IgG) seroprevalence (Cavanagh & McBride, 1997; Taylor *et al.* 1995) in 3 to 11-month-old infants indicating the occurrence of 0.037 (95% CI = 0.007–0.11) new infections/person/month (comparable to other mesoendemic areas (Snow *et al.* 1997)). The main

vector species is *Anopheles darlingi*. EIR was measured between February 1994 and February 1995 in the same area by Magris *et al.* (unpublished data) using CDC light traps, yielding an estimated 69 infective bites/person/year for *P. falciparum* and 122 infective bites/person/year for all *Plasmodium* species.

Sample collection

Cross-sectional malaria surveys that included all residents of 6 Yanomami and 3 Yekwana Amerindian communities (ranging from 36 to 260 inhabitants) were carried out between November 1995 and February 1996 ($n = 708$) and July–October 1997 ($n = 925$) along the Padamo river, Amazonas State, Venezuela. In 1997, an additional Yanomami community was included in the study. The communities were located relatively close to each other at 5–10 km intervals. After individual or parent consent, census data, structured interviews, clinical examination and collection of blood samples from finger-pricks were performed from individuals of all ages (15 days–69 years) present at the time of the study.

Blood samples were collected directly on glass-fibre membrane (GFM, Titertek®) discs (Warhurst, Awad-El-Kariem & Miles, 1991) for DNA analysis, on 3MM chromatography papers (BDH) for serology, and to prepare thick and thin films for microscopical diagnosis. Microscopy in the field was confirmed by a subsequent microscopical examination at the Malaria Reference Laboratory (London School of Hygiene and Tropical Medicine, London, UK) of a random sample of blood slides and by means of a *Plasmodium* species-specific nested polymerase chain reaction (PCR) (Snounou *et al.* 1993).

Multilocus genotyping of *P. falciparum*

An isolate is defined as a blood sample taken from a *P. falciparum*-infected person on a single occasion. A genotype refers to the combination of specific alleles at the genetic loci (genetic markers) used here. The term strain is used here for a population of parasites (within an isolate) that are indistinguishable with the genetic markers used in this study.

P. falciparum-infected subjects were identified by a positive thick film. Parasite DNA templates were prepared for PCR as described elsewhere (Warhurst *et al.* 1991). The extent of *P. falciparum* genetic diversity was determined by nested PCR reactions that amplified regions of 3 unlinked, highly polymorphic single copy genes, glutamate-rich protein (*GLURP*), merozoite-surface protein 1 (*MSP1*) and *MSP2* (Snounou *et al.* 1999). Genotyping was performed directly on the field samples without previous *in vitro* culture to avoid selection of specific

genotypes (Day *et al.* 1992). Typing of parasites was also based on amplification of a polymorphic micro-satellite marker (*PfRRM*) contained within a multi-copy *PfRR*, or *rif*, repetitive element of *P. falciparum* (Su, Carucci & Wellem, 1998). Diversity among isolates was determined by amplification product size differences in the case of *GLURP* and size and sequence differences that defined allelic types in the Block2 region of *MSP1* (K1, MAD20 and RO33) or 2 main allelic families of *MSP2* (IC and FC27) (Snounou *et al.* 1999). Multilocus genotypes (MLG) were described as the combination of alleles at all loci for each isolate investigated. Parasite isolates were classified further using DNA banding patterns from PCR amplification with *PfRRM* and their genetic similarity calculated using the Dice coefficient (Dice, 1945). Positive and negative controls for PCR were *P. falciparum* standard laboratory lines and blood from uninfected European individuals. PCR products were resolved by electrophoresis on 2% agarose gels and automated laser fluorescent analysis (ALFA) (Grundmann *et al.* 1995). DNA sequencing of PCR-amplified Block 2 of the *MSP1* gene was performed on 9 field isolates from survey 1995/6. Amplified DNA fragments were electroeluted, ligated into the pGEM[®]-T Easy vector (Promega), and cloned in *Escherichia coli* XL1-Blue Competent Cells (Stratagene). Plasmids were purified (Wizard mini-prep kit, Promega) and inserts from two or more independent clones were sequenced in both directions (Big Dye sequencing kit, Perkin-Elmer).

Antigenic diversity: specificity of human antibody responses to P. falciparum MSP1 and MSP2

Antigenic diversity of *MSP1* and *MSP2* in the parasite population was estimated indirectly by measuring specific antibody (Ab) responses in the human subjects, using an enzyme-linked immunosorbent assay (ELISA) recombinant antigens representing the same polymorphic regions (sequence only) of *MSP1* and *MSP2* that were genotyped, as previously described (Cavanagh & McBride, 1997; Cavanagh *et al.* 1998; Taylor *et al.* 1995).

Serum was recovered by elution of the chromatography paper blood-spot (containing approximately 43.5 µl of whole blood) in 0.5 ml of phosphate-buffered saline solution (0.7 M KH₂PO₄, Na₂HPO₄, pH 7.4), 0.05% Tween 20, and 0.5% bovine serum albumin for 24 h at 4 °C. Sera from most *P. falciparum* slide-positive individuals detected in both surveys were included. Sera of *P. falciparum* slide-negative individuals were randomly selected from the study population ($n = 259$ in 1995/6 and $n = 192$ in 1997). It has not been excluded that some of these individuals had subpatent *P. falciparum* infections. Pooled and/or individual sera known to contain specific Ab to *MSP1* or *MSP2* were used as positive controls. Negative control sera came from European

blood donors. Specific Ab responses were evaluated using an ELISA at 1:500 test dilution and read at 492 nm on a Titertek Multiscan Plus ELISA Reader (Cavanagh & McBride, 1997). Cut-off levels for antibody-positivity were defined as mean optical density values plus 2 standard deviations of European negative control sera. Concordant Ab responses were defined as in Cavanagh *et al.* (1998). The overall frequency of Ab responses was calculated as the presence of Ab binding to at least 1 of the antigens tested. Specific Ab responses were measured as individual prevalences and as frequencies of association between Ab responses to each of the *MSP1* and *MSP2* allelic families in the same individual.

Statistical analyses

Unilocus analysis. Allele frequencies were calculated as n/N where n was the number of isolates containing indistinguishable alleles of one locus and N was the total number of all different alleles of the same locus found in the isolates. The *gene diversity* (h) was calculated as described elsewhere (Selander *et al.* 1986; Li & Graur, 1991) to estimate the probability that 2 alleles, at any 1 locus, differ when chosen at random from the population. The extent of genetic variability within a population, or mean genetic diversity, was estimated as the average (H) of the h values of the loci studied over the total number of loci (Selander *et al.* 1986; Li & Graur, 1991).

Multilocus analysis. Linkage analyses were performed to test for non-random association among allelic variants identified at different loci. Pairwise comparisons between the single-locus markers with Fisher's exact test for $r \times c$ tables were applied using the statistical package Stata 5.0 (Mehta & Patel, 1983) and the P -values obtained were multiplied by 3 to allow for the 3 pairwise comparisons. The coefficient of linkage disequilibrium, D and D' was quantified for each pairwise comparison (Hedrick, 1985; Hartl, 1988; Maynard-Smith *et al.* 1993) and recombination tests proposed previously, namely $d1$, $d2$, e and f (Tibayrenc, Kjellberg & Ayala, 1990; Tibayrenc & Ayala, 1991) were applied. These recombination tests are based on the null hypothesis of random genetic exchange, and appraise different consequences of linkage disequilibrium between loci. The $d1$ test relies on a combinatorial analysis, while $d2$, e , and f are based on Monte Carlo simulations (with 10^4 iterations). Test f is applied using the strains as unit of analysis and then treating each genotype as a single unit rather than the strains (test f^*).

Testing for association with other variables. Association of type of MLG with age, sex, ethnic group, time and place of residence was examined to detect

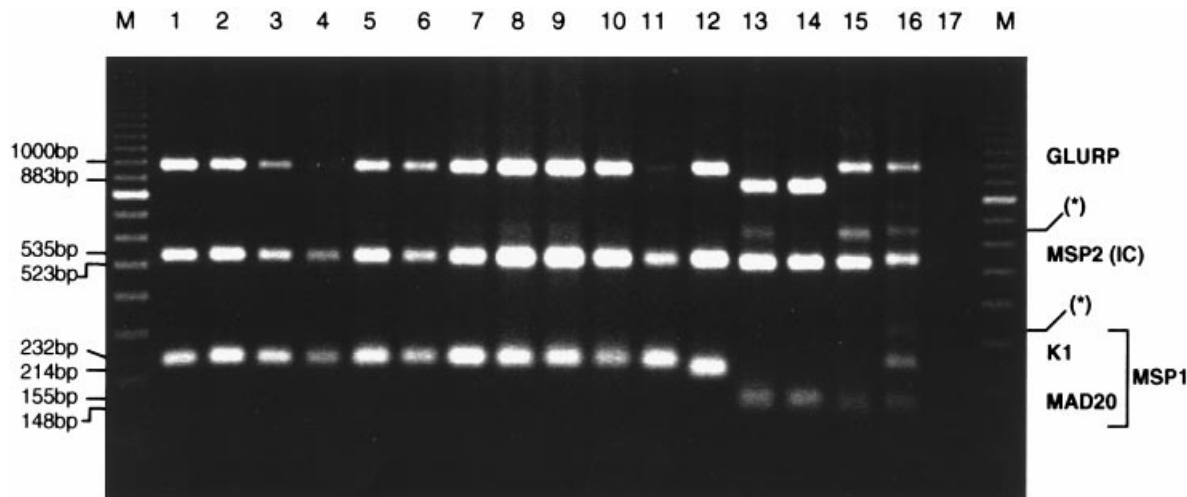


Fig. 1. Agarose-gel electrophoresis of *GLURP*, *MSP1* and *MSP2* nested-PCR amplification products from *Plasmodium falciparum* isolates, survey 1995/6, Padamo basin, Venezuela. Separate Nest-2 reactions that yielded products for each genetic marker were loaded and electrophoresed together, 1 lane per isolate. Fragment sizes in base pairs (bp), determined with ALFA, are shown on the left side of the picture. The corresponding genetic marker and allelic family of the amplification products are presented on the right side. M = 100 bp ladder marker. (*) Denotes an extra band product of primer carry-over from the Nest-1 reaction. Lanes 1–11 show the highly homogeneous pattern of PCR fragments in all genetic markers displayed by these isolates. Isolates in lanes 12–15 show different alleles for some of the markers. The only multiply-infected isolate is shown in lane 16. Multiplicity in this isolate was based on the products of *MSP1* amplification showing 2 fragments, a 232 bp and a 155 bp belonging to the K1 and MAD20 families.

confounding. The statistical significance was determined using Fisher's exact tests with *P*-values < 0.05 considered to be significant.

RESULTS

Genetic diversity

Survey 1995/6. Of 810 individuals resident in the 9 selected communities along the Padamo River, 729 were present at the time of the study, and of these, 708 agreed to participate in the investigation. Out of 708 people surveyed in 1995/6, 33 *P. falciparum*-infected individuals were detected by microscopy. Ten additional *P. falciparum* infections were detected in people that had become infected 1–2 months after being originally investigated, giving a total of 43 infected individuals, of which 18 (42%) were asymptomatic. Results of species-specific PCR applied to the 43 *P. falciparum* isolates agreed with microscopy, except for 1 sample diagnosed as *P. falciparum* and *P. malariae* by microscopy with a PCR result positive for *P. falciparum* only.

Amplification of the polymorphic regions of the 3 single copy genes, *GLURP*, *MSP1* and *MSP2*, produced 2 main results. Firstly, only a single DNA product was generated for each of the loci in all but 1 of the *P. falciparum* isolates (Fig. 1), indicating that all other individuals were infected by a *single* strain of the parasites. The 1 multiply infected isolate produced 2 different *MSP1* amplification products, while single fragments were obtained from *GLURP*

and *MSP2* reactions, suggesting the presence of at least 2 different parasite strains (Fig. 1, lane 16; Table 1). Secondly, most isolates shared the same (i.e. indistinguishable, both in size and allelic family) PCR fragment for each marker, suggesting that 1 allele predominated in each of the 3 genes examined (Fig. 1, Table 1). The *gene diversity* (*h*) was 0.095 for *GLURP*, 0.227 for *MSP2* and 0.298 for *MSP1*. The predominant 'alleles' corresponded to a 1000 bp fragment for *GLURP*, a 535 bp fragment of the IC family of *MSP2*, and a 232 bp fragment of the K1 type of Block2 of *MSP1*. The *MSP1* Block2 sequences were determined for the 232 bp K1/*MSP1* amplification products from 9 separate isolates, and all were found to be identical.

Combination of alleles from the 3 single-copy loci defined MLG in 40 isolates. Three isolates were excluded, as typing was incomplete. Forty-one MLG were distinguished in the 40 isolates (39 isolates each contained a single MLG while 1 isolate contained 2 MLG). As shown in Table 2(a), MLG type 'A' predominated, with 34 out of 41 (83%) parasites having this distinct combination of alleles. The remaining parasites were assigned to 5 other MLG, all with individual frequencies below 5%. The mean genetic diversity *H* was equal to 0.203 indicating low genetic variability. *PfRRM* typing was carried out (Fig. 2) to measure the genetic homogeneity of the parasite population with a multicopy genetic marker. Four different *PfRRM* banding patterns (types I–IV) were observed in the 40 isolates examined (Table 2(b)). *PfRRM* type I

Table 1. Allele frequencies of *GLURP*, *MSP1* and *MSP2* in *Plasmodium falciparum* field isolates ($n = 43$) (Survey 1995/6. Padamo River basin, Amazon, Venezuela.)

Gene	No. of isolates PCR (+)ve	No. of isolates with multiple infection	Allele		Frequency	
			Size (bp)	Sequence	n^*	(%)
<i>GLURP</i> ($N = 41$)	41	None	1000	–	39	95.2
			883	–	2	4.8
<i>MSP1</i> ($N = 42$)	41	1†	232	K1	35	83.3
			155	MAD20	3	7.1
			156	RO33	2	4.8
			214	K1	1	2.4
			148	MAD20	1	2.4
<i>MSP2</i> ($N = 42$)	42	None	535	IC	37	88.1
			523	IC	3	7.1
			350	FC27	2	4.8

* n = Number of isolates containing indistinguishable alleles. N = total number of all different alleles of the same locus found in the *P. falciparum* isolates.

† Two different amplification products, and therefore 2 alleles, were obtained from this isolate, a 232 pb and a 155 pb belonging to the K1 and MAD20 families, respectively. Occurrences of each allele in independent isolates were computed to calculate the allele frequencies.

Table 2. Frequency of *Plasmodium falciparum* multilocus genotypes (MLG) in field isolates

(Survey 1995/6. Padamo River basin, Amazon, Venezuela, $n = 41$ isolates.)

(a) *GLURP*, *MSP1* and *MSP2* multilocus genotypes

MLG type	<i>GLURP</i> Size*	<i>MSP1</i>		<i>MSP2</i>		Frequency	
		Size*	Sequence	Size*	Sequence	n	%
A	1000	232	K1	535	IC	34	83.0
A'	1000	214	K1	535	IC	1	2.4
B	883	155	MAD20	523	IC	2	4.9
C	1000	156	RO33	350	FC27	2	4.9
D†	1000	155	MAD20	535	IC	1	2.4
E	1000	148	MAD20	523	IC	1	2.4

* Base-pairs.

† MLG obtained from 1 doubly infected isolate; the second MLG of this isolate was characterized as type A.

n , Number of genotypes.

(b) Correlation of *GLURP*, *MSP1*, *MSP2* multilocus genotypes with typing of *PfRRM*

<i>GLURP/MSPs</i> type	<i>PfRRM</i> type	Frequency	
		n	%
A + A'	Ia	26	63.4
A	Ib	5	12.2
A	Ic	4	9.8
B	II	2	4.9
C	III	2	4.9
D†	Ib	1	2.4
E	IV	1	2.4

n , Number of genotypes.

† Doubly infected isolate.

was subdivided as types Ia, Ib and Ic as these 3 patterns were 90% similar when compared using the Dice coefficient (data not shown; Dice, 1945). Although a higher number of types was observed using this method, there was an excellent concordance with MLG based on the other genetic

markers. The multiply infected isolate showed a type Ib banding pattern though 2 MLG were found in this isolate using *GLURP/MSP1/MSP2*. It seems probable that the type Ib pattern corresponded to the strain carrying MLG type A obscuring a *PfRRM* pattern that could represent MLG type D.

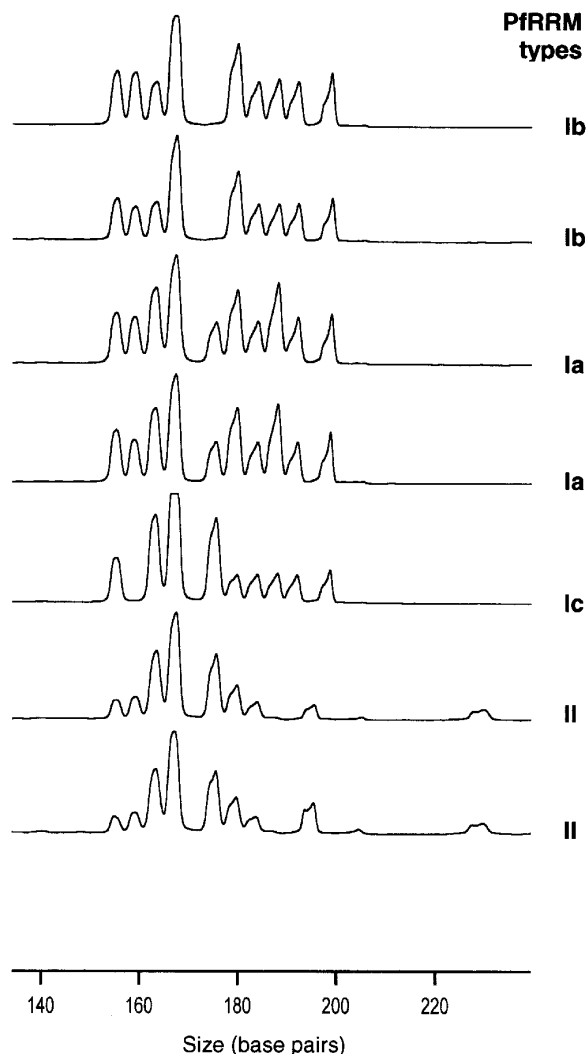


Fig. 2. Fluorescence densitogram of *PfRRM* amplification products from *Plasmodium falciparum* isolates separated by electrophoresis through an ALF™ (automated laser fluorescence) sequencer. Survey 1995/6. Padamo basin, Amazon, Venezuela. Peaks represent PCR products of the multicopy genetic marker *PfRRM*. Lane numbers indicate *PfRRM* types (only 4 types are shown).

We employed linkage disequilibrium analysis to examine any non-random associations between alleles from different loci which might provide insight into the mating structure of the parasite population (Maynard-Smith *et al.* 1993; Paul *et al.* 1998; Tibayrenc *et al.* 1991). Pairwise comparisons between *GLURP*, *MSP1*, *MSP2* using Fisher's exact test displayed significant non-random associations between the genes (adjusted *P*-value was < 0.001 for *MSP1 vs MSP2*; $P = 0.006$ for *GLURP vs MSP2*; and $P = 0.027$ for *GLURP vs MSP1*). *D'* calculated for each comparison indicated that all 3 allelic associations are at maximum disequilibrium (for *MSP1 × MSP2* and *MSP1 × GLURP*: $D = 0.04$ and $D' = 1$; *MSP2 × GLURP*: $D = -0.003$ and $D' = -1$). The test *f* (which estimates the probability of observing a linkage disequilibrium as high or higher

than actually found, based on Monte Carlo simulations) was applied to the pairwise comparisons. Statistically significant associations were found between all markers ($P < 0.05$). Finally, all recombination tests applied to the MLG for the single copy loci studied showed significant departures from random association and therefore, linkage disequilibrium, among all loci (*P*-values: $d1 = 0.037$; $d2 < 0.0001$; $e < 0.0001$; $f < 0.0001$). However, when single MLG rather than the strains were taken as the unit of analysis (test *f**), the association failed to be significant (*P*-value *f** = 0.604). Confounding due to association of MLG with place of residence, time of collection, age, sex or ethnic group was excluded (Fisher's exact $P > 0.09$). Selection bias was minimized since fewer than 3% of the individuals present in the communities at the time of the investigation refused participation and fewer than 10% of the individuals that reside in these communities were absent when the survey took place. However, statistical type II error due to a small sample size of 43 *P. falciparum* isolates cannot be excluded.

Survey 1997. To determine if the limited *P. falciparum* diversity and the predominance of one strain was a transient feature only found during the 1995/6 survey, a second cross-sectional survey was carried out 1 year later. Due to unexpected low *P. falciparum* transmission in 1997, only 12 *P. falciparum*-infected individuals were detected out of 925 people surveyed. Ten out of the 12 *P. falciparum* isolates yielded PCR products. Similar to the 1995/6 survey, no multiple *P. falciparum* infections were found by PCR. The alleles predominant in the 1995/6 survey, 1000 bp (*GLURP*), 232 bp/K1 (*MSP1*) and 535 bp/IC (*MSP2*), were also the most prevalent ones in 1997. MLG by *GLURP*, *MSP1* and *MSP2* fell into only 5 discrete types. Half of the infected individuals carried an identical MLG, the same type 'A' that was predominant amongst the 1995/6 samples studied. The rest of the individuals were infected by strains with allele combinations different from the ones found during the 1995/6 survey. *PfRRM* typing was concordant with the MLG based on *GLURP*, *MSP1* and *MSP2* (data not shown). Though the small sample size reduced the power of the pairwise comparisons a significant association between *MSP1* and *MSP2* was still observed (Fisher's exact $P = 0.051$).

Antigenic diversity

Correlation between specific antibody responses of hosts and the infecting parasites' genotypes. Specificity of Ab responses to *MSP1* and *MSP2* of 49 *P. falciparum*-infected subjects (37 from 1995/6 and 12 from 1997) was investigated. Samples from individuals who had no detectable antibodies against any tested antigen and/or for whose parasites no PCR

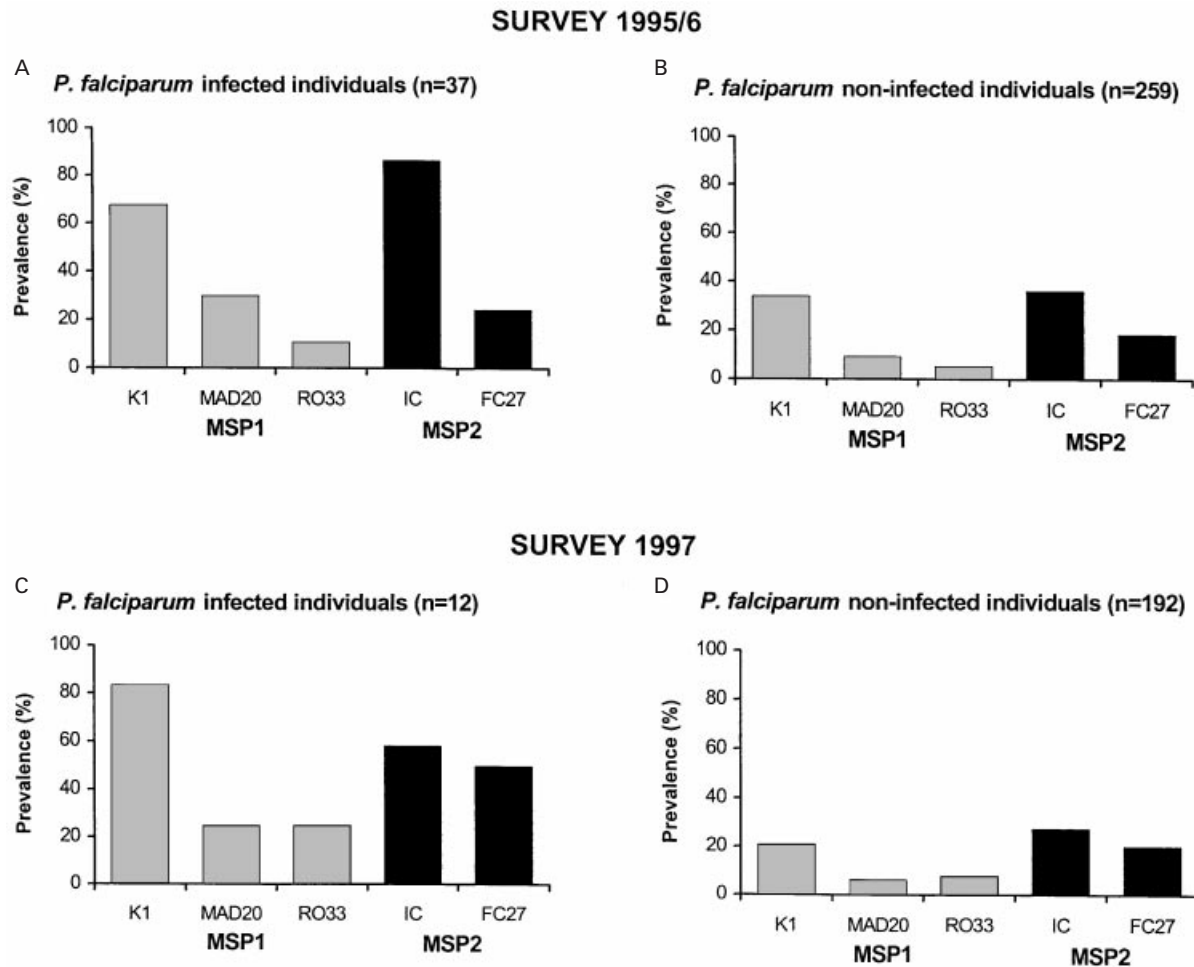


Fig. 3. Prevalence of antibody responses against MSP1-Block2 and MSP2 allelic families in *Plasmodium falciparum*-infected (slide-positive) and non-infected (slide-negative) individuals during the 1995/6 and 1997 surveys.

results were available were excluded from statistical analyses. Specificity of anti-MSP1 Block2 Ab responses matched the parasites' *MSP1*-Block2 genotype in 33/36 (91.6%) of the cases (24/26 in 1995/6 and 9/10 in 1997), and in 38/38 (100%) cases responses to MSP2 were concordant. Of 36 individuals infected with parasites displaying the *MSP1*/K1-*MSP2*/IC genotype (the most prevalent allelic association), 24 (67%) had antibodies that recognized specifically both of these antigens. Ten of these 24 individuals also had antibodies that recognized antigenic product(s) of other alleles. The correlation between specific antibodies and genotypes of *P. falciparum* in the infected subjects confirms that these responses were induced by the infecting parasite.

Frequency and associations of antibody responses to MSP1 and MSP2 in P. falciparum slide-positive and slide-negative individuals. In the first survey, 34/37 (92%) infected and 138/259 (53%) non-infected individuals presented antibodies to at least 1 of the MSP1 or MSP2 antigens, while in 1997 all 12 infected subjects and 87/192 (45%) uninfected individuals had antibodies to these antigens. In both

P. falciparum-infected and non-infected individuals the prevalence of antibodies specific for the different allelic forms of MSP1 and MSP2 mirrored the genetic allele frequencies detected in the parasites (Fig. 3). In both surveys, the most frequent association (in the same individual) was between Ab responses to the K1-like type of MSP1 and IC-like of MSP2 (Fig. 3). In 1995/6, this combination was identified in 24/33 (73%) and 60/138 (43.5%) slide-positive and -negative individuals, respectively, who were seropositive for both MSP1 and MSP2 (Fig. 4A, B). In 1997, 7/12 (58%) infected and 26/87 (30%) uninfected individuals had antibodies against the same combination of the two antigens (Fig. 4C, D). Of the 60 slide-negative individuals that raised Ab responses to the K1-IC combination in 1995/96, 37 (44%) were sampled before a seasonal peak in malaria transmission initiated in January 1996. These results strongly agree with and extend the evidence for allelic associations found by DNA typing of the parasites during both years. Overall, the data are consistent with an aggregated distribution of genotypes and the presence of a dominant *P. falciparum* strain that circulated within the Padamo for a minimum of 2 years.

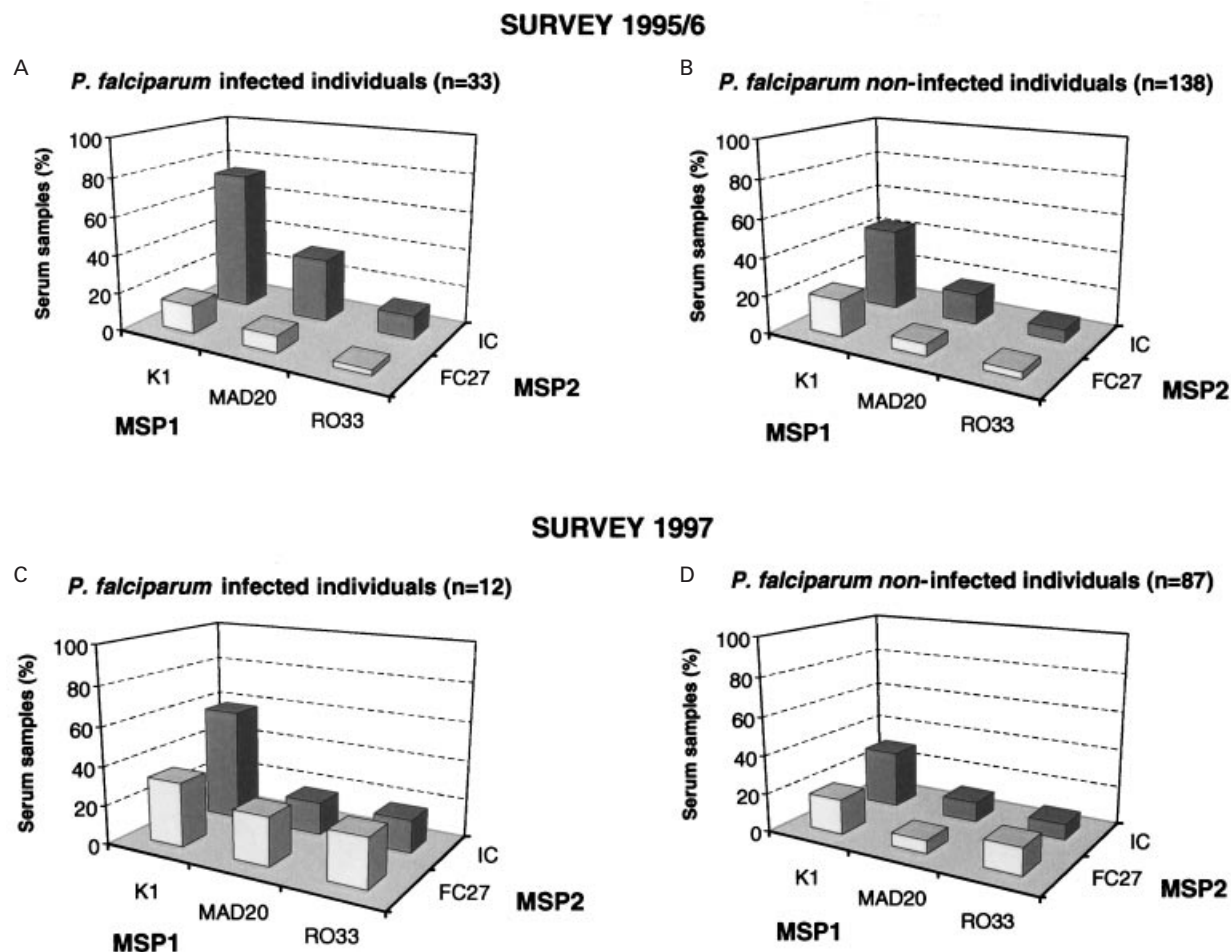


Fig. 4. Combined antibody responses against MSP1-Block2 and MSP2 allelic families in *Plasmodium falciparum* infected (slide-positive) and non-infected (slide-negative) individuals during the 1995/6 and 1997 surveys. The associations of antibodies against the 3 serotypes of Block2 of MSP1 with antibodies directed to the 2 serogroups (IC and FC27) of MSP2 are shown. Note that the denominator of infected subjects ($n = 33$) in 1995/6 and of uninfected individuals ($n = 138$ in 1995/6 and $n = 87$ in 1997) corresponded to the number of serum samples that reacted positively with any MSP antigen.

DISCUSSION

Our study focused on the analysis of the genetic and antigenic structure of the natural *P. falciparum* population in the Padamo basin, an area of mesoendemic malaria transmission of the Venezuelan Amazon, over a 2-year period. Our findings indicate that this parasite population exhibits unusually low genetic diversity with virtually no multiplicity of infections, and the predominance of one multilocus genotype together with strong linkage disequilibrium among genetic markers used. These findings contrast with results of studies in endemic areas of Africa, Asia and other parts of South America which have suggested a positive correlation between diversity, mean number of genotypes per infected person and transmission intensity (Babiker *et al.* 1994, 1997; Paul *et al.* 1995; Arnot, 1998; Ariei *et al.* 1999; Haddad *et al.* 1999). The degrees of genetic diversity (parameters h and H) among regions differing in transmission intensity have been calculated from the literature and are presented in com-

parison to our study. The studies, presented in Table 3, used the same genetic markers (except for *PfRRM*) as our investigation. Although malaria is mesoendemic in the Padamo, the extent of genetic variability of *P. falciparum* (H) was much lower than in all other regions, including areas of Asia and South America where transmission is very low (hypoendemic) (Table 3). In these other study populations, between 10 and 90% of infected individuals carried multiple *P. falciparum* genotypes in contrast to the Padamo where only 2.5% (1 infected individual) had a co-infection with a second strain.

Genetic diversity has been found to be generally lower in South America than in Africa or Asia (Ferreira *et al.* 1998; Anderson *et al.* 2000). This fact has been commonly related to the lower transmission intensity in the Americas, such as in recent studies in Honduras and French Guiana (Ariei *et al.* 1999; Haddad *et al.* 1999). However, in the Padamo area *P. falciparum* diversity and the number of multiple infections are even lower than in Honduras and

Table 3. Genetic diversity of *Plasmodium falciparum* in geographically and endemically different regions in comparison with the present study

(Padamo River basin, Amazon, Venezuela)

Country (Reference)	Malaria endemicity	No. of isolates investigated	h*				Percentage individuals with multiple infection
			MSP1	MSP2	GLURP	H*	
Venezuela Padamo 1995/6†	Mesoendemic	41	29.8 (5)	22.7 (3)	9.5 (2)	20.3	2.5
French Guiana (Ariey <i>et al.</i> 1999)	Hypoendemic	142	62.5 (4)	30.1 (2)	55.8 (3)	49.5	10.6
Brazil (Creasey <i>et al.</i> 1990)	–	20	64.2 (4)	47 (3)	–	55.6	30
Thailand (Paul <i>et al.</i> 1998)	Hypoendemic	56	84.4 (9)	83.9 (12)	79.5 (7)	82.6	70.4
Sudan (Babiker <i>et al.</i> 1995)	Mesoendemic	39–40	75 (8)	90.3 (15)	–	82.7	–
PNG (Paul <i>et al.</i> 1995)	Hyperendemic	116–221	77 (9)	90.3 (14)	87.8 (9)	85	72
Senegal (Konaté <i>et al.</i> 1999)	Holoendemic	135	92 (33)	94 (31)	–	93	90

* Parameters *h* and *H* have been multiplied by 100 and have been calculated from the literature for the regions different to the Padamo. The number of alleles of each gene is shown in parenthesis.

† This study.

French Guiana despite the higher level of malaria transmission in Padamo. In our study, we have examined the totality of the *P. falciparum* infections detected in humans (thus minimizing sampling error) in the complete population survey in a spatially and temporally defined area of S. America. Selection bias can be excluded considering that sampling was comprehensive and genotyping was done directly on the field samples and not after *in vitro* culture.

Despite recombination taking place in the mosquito phase (Walliker *et al.* 1987; Wellems *et al.* 1990), the presence of fewer allelic variants circulating in the Padamo population may have diminished the opportunity for frequent emergence of new genotypes while favouring self-fertilization and the predominance of a few successful genotypes. In fact, the limited diversity and the highly homogeneous pattern of genotypes in the Padamo do not seem to be a transient phenomenon, but a characteristic of the natural *P. falciparum* population of this area. Two pieces of evidence support this premise (i) in the hosts, the Ab responses are directed mostly to a few, predominant, antigen variants of MSP1 and MSP2, and (ii) the results are similar for 2 surveys carried out a year apart on the same communities of the Padamo. The relatively high prevalence of Ab in the non-infected people offered the opportunity to extend indirectly the information on the prevalence of genotypes interpreting the Ab as specific 'footprints' left by past or chronic infections in the host population. Since it has been previously shown that the humoral response to MSP1 and MSP2 is family-specific, the presence of antibodies directed to more than 1 allelic family in the same individual would

indicate multiple exposure rather than cross-reaction (Taylor *et al.* 1995; Cavanagh & McBride, 1997; Cavanagh *et al.* 1998). The low diversity of human Ab responses correlated with the restricted diversity identified by DNA typing. The presence of this restricted repertoire of antibodies, especially in slide-negative individuals, is an indication that the low diversity is not a 'point-in-time' event. Secondly, the genetic data from the 1997 survey, albeit the small number of *P. falciparum* isolates, showed a similar limited diversity with the same dominant strain as in the previous year. Interestingly, some novel MLG were found in 1997 that were not detected among the isolates collected in 1995/6. These could be genotypes newly introduced into the communities, new genotypes produced by recombination, or genotypes that were present in the previous survey but not detected.

Other ecological and evolutionary factors, such as random genetic drift and migration may contribute to the maintenance of such a population structure (Hartl, 1988; Dye, 1991). *P. falciparum* samples obtained from communities surrounding the Padamo region also comprised single infections and similar allelic types and frequencies to those seen in the communities of our study (data not shown). Since migrants come primarily from nearby populations and these appear to have similar allele frequencies, the effects of migration in the Padamo population might not be sufficient to generate the higher degree of diversity found in other endemic regions of the world.

A second striking feature of the Padamo *P. falciparum* population was the finding of a pre-

dominant multilocus genotype and of significant linkage disequilibrium among all genetic markers tested. Linkage disequilibrium has recently been described in other S. American locations, including Venezuela (Urdaneta *et al.* 2001) and the Brazilian Amazon (Anderson *et al.* 2000; Da Silveira *et al.* 2001). Although the use of PCR-derived genotyping data may lead to underestimation of genetic differences (Arnot, 1998), this is unlikely to be an explanation for our results for several reasons. Firstly, the *MSP1*/Block2 region of 9 isolates carrying MLG type 'A' was identical in sequence, strongly suggesting that isolates containing MLG type 'A' have at least the same *MSP1* allele. Secondly, microsatellite typing of the multicopy repetitive *rif* element correlated with MLG generated with *MSP1*, *MSP2* and *GLURP*. Finally, the frequency distribution of the MLG was not confounded by other epidemiological variables such as geographical location (Wahlund effect), time, age, sex or ethnic group, and therefore appears to reflect true structuring of the parasite population that is co-circulating among the Yanomami and Yekwana people in our study area.

The demonstration of significant linkage disequilibrium in the Padamo parasite population could be considered as circumstantial evidence that *P. falciparum* propagates clonally in this area (Maynard-Smith *et al.* 1993; Tibayrenc *et al.* 1990). Analyses of bacterial and protozoan organisms have shown that population structures can vary from highly sexual to strictly clonal (Selander *et al.* 1986; Tibayrenc *et al.* 1990; Maynard-Smith *et al.* 1993; Paul *et al.* 1995). A clonal mode of reproduction in *P. falciparum* is possible since the capability of self-fertilization exists (Walliker *et al.* 1987; Wellem's *et al.* 1990). Alternatively, our results may reflect temporary disequilibrium in the Padamo parasite population. Such a scenario, termed an *epidemic population* structure, can occur in an essentially sexual species when a significant association between loci arises due to the emergence and rapid expansion of a highly successful individual genotype (Maynard-Smith *et al.* 1993). Our data give clear indications that the population under survey may undergo clonal propagation. However, the picture obtained here differs from the results obtained with other parasites which undergo long-term clonal evolution, like *Trypanosoma cruzi*, where natural populations appear to be distributed into clear-cut genetic subdivisions or 'discrete typing units' (Tibayrenc, 1998). The failure to find a significant association among loci when the f^* test was applied to the Padamo *P. falciparum* population is consistent with the hypothesis of an epidemic population structure. A statistical type II error (lack of power) could also explain this non-significant result since only 9 individual MLG were found in the population of study.

An acute outbreak has been one postulated

mechanism by which a clonal population of *P. falciparum* could arise (Babiker *et al.* 1997; Paul & Day, 1998; Laserson *et al.* 1999; Arez *et al.* 1999). A particular genotype may not only be maintained but may rapidly spread if it has an advantage of, for example, carrying drug resistance markers in an area where drug pressure exists (Paul & Day, 1998). In the Padamo, chloroquine and pyrimethamine/sulfadoxine are still the drugs of choice for malaria treatment. Although *P. falciparum* chloroquine-resistance *in vivo* is high (Tami *et al.* 1999), it was not linked to any particular MLG; nor were any specific genetic polymorphisms associated with drug resistance that could have generated the observed linkage disequilibrium, since all isolates carried 'resistant' alleles (Tami *et al.* 1999). A second mechanism that may contribute to a clonal population structure is the introduction of a novel genotype among hosts with no previous immunity to antigens coded by this particular strain (Gupta & Day, 1994*a, b*; Greenwood *et al.* 1991). A 'clonal' outbreak of *P. falciparum* has been reported previously in another Yanomami community (Laserson *et al.* 1999) adding weight to a tentative hypothesis that *P. falciparum* may at times propagate in 'clonal' bouts in the Amazon forest.

We have shown that the genetic structure of a natural *P. falciparum* population of the Venezuelan Amazon differs markedly from the genetic structure of populations in other areas experiencing a similar malaria endemicity. We suggest that the low genetic diversity in this American population of *P. falciparum* may not only relate to the level of transmission, but to other influences like a 'bottleneck effect'. Such a bottleneck effect could be the consequence of (i) the relatively recent introduction of the species into this continent from the Old World since European colonization; (ii) the possibility that American anophelines may not transmit all parasite lines that were/are introduced, and/or (iii) the reduction of *P. falciparum* prevalence and geographical distribution after the Venezuelan malaria eradication campaign (Gabaldon). In the Venezuelan Amazon region and, we would like to suggest, in South America, *P. falciparum* displays a population structure that may differ from that in other endemic areas of the world. In Africa and South-East Asia, the high proportion of multi-clone infections have prevented the characterization of clearly distinguishable MLG and thus obscured any linkage disequilibrium, despite the finding that inbreeding coefficients are high (Babiker *et al.* 1994; Paul *et al.* 1995, 1998). It may, therefore, be that *P. falciparum* frequently propagates with an epidemic or clonal population structure in S. America, but this characteristic may be best detected when examining a non-biased and representative sample of strains within a spatially and temporally defined location. This form of spread has important implications for the de-

ployment of new interventions such as novel chemotherapies and malaria vaccines. The presence of single infections in the majority of individuals in our study population offers a unique setting for longitudinal studies to look into the population dynamics of *P. falciparum* at a micro-epidemiological level. This will greatly facilitate the investigation of the emergence and spread of drug-resistant strains and provide an opportunity to elucidate relevant questions related to host-parasite interactions and anti-malarial immunity. These questions cannot be easily tackled in endemic areas with highly diverse parasite populations and a high proportion of multiply infected individuals.

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