FAMILY ANALYSIS OF MALARIA INFECTION IN DIENGA, GABON

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Abstract. Fifty children from 9 families were enrolled in a longitudinal study of 8 months to evaluate individual levels of *Plasmodium falciparum* density in blood during asymptomatic infections. Individual parasite densities were adjusted for age and date of blood intake. The arithmetic means of these adjusted parasite densities (MAPD) were not influenced by sickle cell trait nor by G6PD enzyme activity. On the contrary, family analysis revealed the presence of similar MAPD values according to the sibships. Moreover, sibships frequently infected with *P. malariae* exhibited the highest *P. falciparum* MAPDs. The difference in aggressiveness of malaria vectors between the northern and southern halves of the village did not explain the distribution of MAPD, nor did it explain the differences in mean frequency of *P. malariae* infection among the sibships. We conclude that the familial characteristic of susceptibility to both *P. falciparum* and *P. malariae* infections is more likely influenced by the host's genetic background than by differences in the levels of malaria transmission.

INTRODUCTION

Plasmodium falciparum malaria related morbidity and mortality depend on several factors, including host defense (controlled by the genetic background of the individual), cumulative number and seasonality of exposures to infected bites, and, probably, virulence of the infective parasitic strains. Population studies identified several genes associated with resistance to malaria, mainly against severe malaria.[1] In most instances, the onset of the febrile episode corresponds to an increase of the parasite density above the pyrogenic threshold.[2] Several red cell disorders affect parasite growth, and consequently are associated with reduced parasite blood density.[3] Nevertheless, genes controlling the *P. falciparum* blood infection levels still remain to be precisely identified. Segregation analysis has allowed detection of a predominant recessive gene or a polygenic complex of factors controlling infection level in the blood.[4–6] These family studies are the only tools available to date for studying inherited characters within nuclear families. When compared to population studies involving important and uncontrolled genetic polymorphism, studies investigating families or twins are more likely to select for a reduced number of genes and alleles.

We previously reported on a treatment-reinfection study of 61 children from 10 families in the village of Dienga, located in Gabon.[7] The study allowed a measure of *P. falciparum* parasite densities during asymptomatic episodes, and a computation of individual values of parasite densities adjusted for age and date of sampling (MAPD). Nine sibships (defined as a group of siblings) comprising 4 children or more were successfully followed and enrolled into the present study. Parasitological data including MAPDs were significantly correlated to the familial links between children. Using genetic traits and geographical informations related to the variation of exposition to the vectors, we tried to explain the family distribution of parasitological phenotypes.

MATERIALS AND METHODS

Study area. The study was conducted in Dienga, a village located in the Ogooué-Lolo province, southeast of Gabon.

The climate is equatorial with a long (June–September) and a short (January–February) dry season separated by two rainy seasons. *P. falciparum* and *P. malariae* malaria are highly endemic, with seasonal variations in vector density and transmission. The annual mean of infective bites is about 100/man (Elissa N., unpublished data). The population of the village belongs to the Banzabi ethnic group.[8] Self-medication is highly reduced probably thanks to the permanent CIRMF (Centre International de Recherches Médicales de Franceville) team members who perform routine microscopic diagnostics of malaria and give treatment free of charge.

Subjects. Children were selected from those enrolled in a previous study of treatment-design with their consent and those of their parents.[7] During the follow-up, subjects who showed signs of parasites and fever were treated for malaria and slides from the 2 following weeks were excluded from the analysis. Sibships comprising at least 4 children, for whom at least 5 thick blood films were recorded, were enrolled into the present study. Fifty children from 9 distinct families fulfilled these conditions. Family structures were defined from a previous census (Renaut A., unpublished data). Maternal filiation of the children was a much more reliable information than paternal filiation. Thus, we defined sibships on the basis of being issued by the same mother. The mean age was 9.7 years ranging from 1–17 years. The protocol of the study was approved by the local Ethics Committee.

Parasitological measurements. The parasitological data from our previous study of the individual delay of reinfection after radical parasite cure have been analyzed without consideration of the filiation of the children.[7] The delay of reinfection was defined as the period separating the end of a curative treatment and the first reappearance of blood stages of *P. falciparum*, independently of the presence or not of symptoms. Blood smears obtained all along this 30 weeks follow-up were used to calculate the MAPDs for *P. falciparum*. To avoid the impact of the anti-malarial treatment performed at the beginning of this study, MAPDs were computed from slides collected from the 9th week following treatment, when the *P. falciparum* prevalence rate had reached again its initial level before treatment (67%). Consequently, we included in the present analysis the slides from

	n	MAPD		P
Host genetic factors				
Gender (M/F)	50	−0.01 (−0.28; 0.61)	−0.19 (0.45; 0.10)	0.21
Blood group (O/non O)	48	−0.05 (−0.41; 0.63)	−0.01 (−0.41; 0.27)	0.62
Hb phenotype (AA/AS)	42	0.00 (−0.27; 0.48)	−0.18 (−0.58; 0.20)	0.29
G6PD A-allele				
males (normal/hemizygote)	15/4	0.16 (−0.12; 0.43)	−0.11 (−0.52; 0.38)	0.42
females (normal/heterozygote)	11/7	−0.16 (−0.38; 0.24)	−0.19 (−0.80; 0.04)	0.44
Parasitological factors				
Delay of reinfection with *P. falciparum*	50	R = −0.655		<0.0001
Frequency of *P. malariae* infection	50	R = 0.610		<0.0001

Differences between groups were assessed by regression analysis for delay of reinfection and frequency of infection with *P. malariae,* and by the non-parametric Mann-Whitney *U*-test for the other factors.

Values are medians (25th percentile—75th percentile) except for parasitological factors.

day 0 as well as those obtained between the 9th and the 30th week, corresponding to 12 visits between May and December 1998, with a minimum of 5 visits per child. Slides obtained during febrile episodes, as well as those collected 2 weeks after an additional anti-malarial treatment were excluded. Thick blood smears were stained by Giemsa and the *P. falciparum* parasite density was defined as the number of parasites per microliter of blood, estimated from the number of *P. falciparum* parasites/1000 leukocytes and a mean leukocyte count of 6500/μl, as determined by the mean leukocyte count of this cohort (data not shown). The threshold of sensitivity was therefore around 7 parasites per microliter of blood. Parasite densities were normalized by a log-transformation (Log(PD+1)). As age and season are known to influence parasite densities, the log-transformed parasite densities were adjusted for the date of blood intake and for age (age was split into 1–2, 3–5, 6–9, 10–13, and 14–17 age groups in years). For adjustment, the mean of Log(PD+1) of the age and date-groups were subtracted to each value of subjects constituting these groups. MAPDs are the arithmetic mean of the adjusted values (5 to 12 slides per child being available). Infections with other species, essentially *P. malariae,* were detected, and the individual frequency of *P. malariae* infection was calculated as the ratio between the number of slides positive for *P. malariae* and the whole number of slides. MAPD and frequency of *P. malariae* infection for the sibships were calculated using the arithmetic means of the corresponding values for all children belonging to a same sibship.

Haematological assays. Blood group and presence of sickle cell trait were determined from blood samples taken at the beginning of the study, using serologic and electrophoretic methods respectively, as previously described.[7] G6PD genotype was typed by polymerase chain reaction followed by enzymatic digestion to detect the mutations at positions 202 and 376.[9] Results were analyzed as dichotomous values determined by the presence or not of the G6PD A-allele in male and female groups.

Geographical variation of exposure to malaria vectors within the village. Vector captures were conducted during nightly sessions from January to December 1996 and from March 1998 to March 1999 in a total of 21 houses spread throughout the entire village. During this period, 24 captures were carried out simultaneously in 2 or 3 houses by human-bait volunteers who caught mosquitoes and placed them into tubes for subsequent examination in the laboratory. The number of malaria vectors (*Anopheles gambiae s.l., An. hancocki* and *An. funestus*) containing sporozoites in their salivary glands was not sufficient to allow a reliable statistical analysis (30 infected mosquitoes caught during 7 captures; and 333 *Anopheles* caught during 24 captures). Consequently, analysis was conducted considering the anopheline aggressiveness and not the entomological inoculation rate. For each capture, the rate of mosquitoes caught in each house was calculated. A mean rate was then calculated for each house, representing the specific home-going of mosquitoes. As captures were not performed in the houses concerned by the present study, the anopheline aggressiveness was compared between geographic zones defined inside the village, and not between these houses.

Statistical analysis. Statistical analysis was first performed to identify relations between MAPD and other factors without considering sibships, and was then carried on with sibships. *P* values <0.05 were considered significant.

RESULTS

Parasitological and host genetic data. For the 50 children enrolled, the individual mean parasite densities ranged from 0 to 9,217 parasites/μl during asymptomatic episodes. Twenty-four subjects presented at least once with *P. malariae* infection, with a frequency ranging from 8 to 56% of the blood films. Sickle cell trait, G6PD A- allele, and O blood group occurred in 38, 28, and 33% of subjects, respectively.

At first, MAPD were analyzed regardless of family relationships between subjects. MAPD were correlated negatively to the delay of reinfection with *P. falciparum* and positively to the frequency of *P. malariae* infection, but were not related to gender, blood group or sickle cell trait (Table 1). No association between MAPDs and the G6PD A- allele was found when comparing hemizygote males and heterozygote females to normal groups (Table 1). Because of the sample sizes, MAPDs were compared in two separate analyses, performed between individuals presenting or not the G6PD A- allele, and between individuals presenting or not a G6PD deficient phenotype (as determined in 7), but no association was found (*P* = 0.15 for genotype and *P* = 0.96 for phenotype, Mann-Whitney *U*-test). When data were analyzed at the familial

		sibships									
		S10	S21	S51	S73	S80	S116	S149	S178	S278	P
Number of children		6	7	5	6	5	4	5	6	6	
Age (years)	median	8	13	10	13	11	8.5	11	7	8.5	0.35
	range	3-12	2-17	7-15	7-16	3-14	5-11	9-15	1-15	1-13	
Host genetic factors											
Gender (M/F)		4/2	5/2	3/2	2/4	2/3	2/2	3/2	3/3	5/1	NA
Blood group (O/nonO)		0/5 §	2/5	2/3	0/5 §	3/2	2/2	3/2	1/5	3/3	NA
Hemoglobin (AA/AS)		5/1	4/3	3/2	3/2 §	4/0 §	4/0	3/2	0/3 §	0/3 §	NA
G6PD (non A-/A-)		3/1 §	3/2 §	5/0	4/1 §	3/1 §	3/0 §	2/3	1/2 §	2/1 §	NA
Parasitological factors											
Delay of reinfection (weeks)	median	17.5	28	11	21	nd	8	30	30	15	0.67
	range	3-32	5-32	3-28	5-32		6-9	5-30	3-32	4-25	
Rate of P. malariae infection (%)	median	21	0	17	0	0	31	0	0	19	0.02
	range	0-40	0-17	0-33	0-27	0-33	13-56	0-10	0-9	0-33	

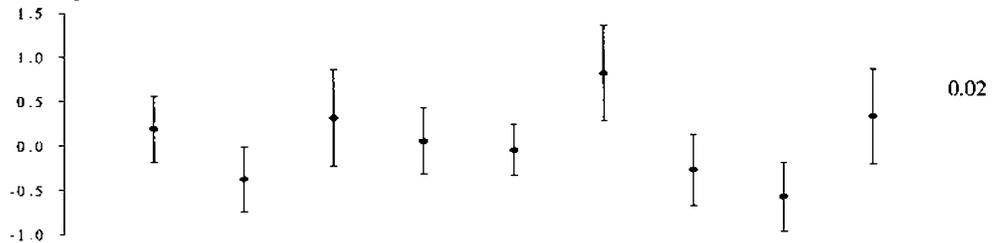MAPD (*P. falciparum*) ... 0.02

FIGURE 1.   Parasitological and genetic characteristics of 9 sibs from Dienga, Gabon. Age and parasitological factors were analyzed using the non-parametric Kruskal-Wallis test. For the delay to reinfection, subjects who were not reinfected until the 30th week of follow-up were given the value 32. See [7]. nd: not determined: children of sibship S80 were partially lost during the follow-up before they became reinfected. NA: not applicable. MAPD values were expressed as mean ± standard error. § One or several subjects were not tested.

level, MAPD as well as the frequency of *P. malariae* infection differed between sibships (Kruskal-Wallis test; $P = 0.02$ for MAPD and frequency of *P. malariae* infection), suggesting a familial character for MAPD and *P. malariae* infection rate (Figure 1). The median values of the delay to reinfection were
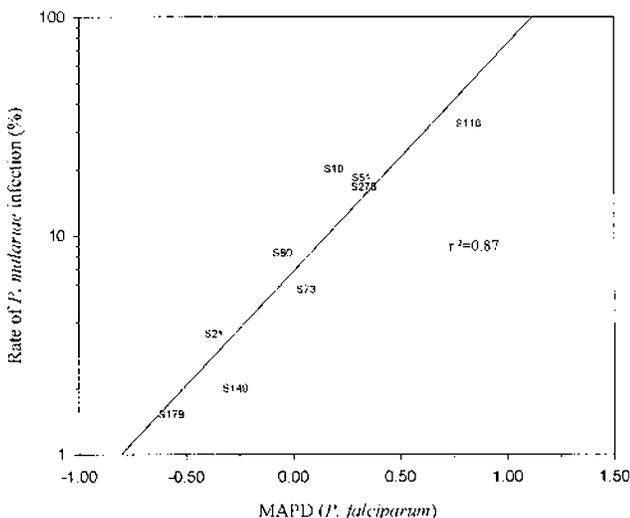

FIGURE 2.   Correlation between mean *P. falciparum* MAPD and the mean rate of *P. malariae* infection of sibships. Each point represents the mean MAPD values and the mean rate of *P. malariae* infection for each sibship, and bars represent corresponding standard errors. $P < 0.001$.

shorter in sibships with the highest rates of *P. malariae* infection associated with the highest MAPD values but the *P* value was not significant because of a high variance of the delay of reinfection in sibships (Figure 1). The number of subjects in each group was too small to analyze the host genetic factors within sibships using the chi square test. For each sibship, the MAPD were compared to the frequency of *P. malariae* infection. *P. malariae* infection occurred a least once in each family and we observed a strong correlation between both variables; this association therefore may constitute a family characteristic (Figure 2). Multivariate analysis could not be applied to the analysis of sibships, MAPD and *P. malariae* infection rate because of the small numbers of individuals in each group. Then, we adjusted individual MAPD to the frequency of *P. malariae* infection and found that MAPD were not related to sibships ($P = 0.30$). This result suggests that *P. falciparum* and *P. malariae* infections are indissociable and that the sensitivity to one species implies sensitivity to the other.

Comparison between parasitological characteristics of the sibships and geographical variations of exposition to the malaria vectors. Geographical location of sibships' houses within the village is illustrated in Figure 3A. Houses were distributed all along the main road of the village. The MAPD and the frequency of *P. malariae* infection of the sibships were homogeneously distributed within the village (Figure 3B and 3C). For each house selected for mosquitoes capture, the mean rates of mosquitoes caught (*An. gambiae*, *An. hancocki* and *An. funestus*) is illustrated in Figure 3D. These values
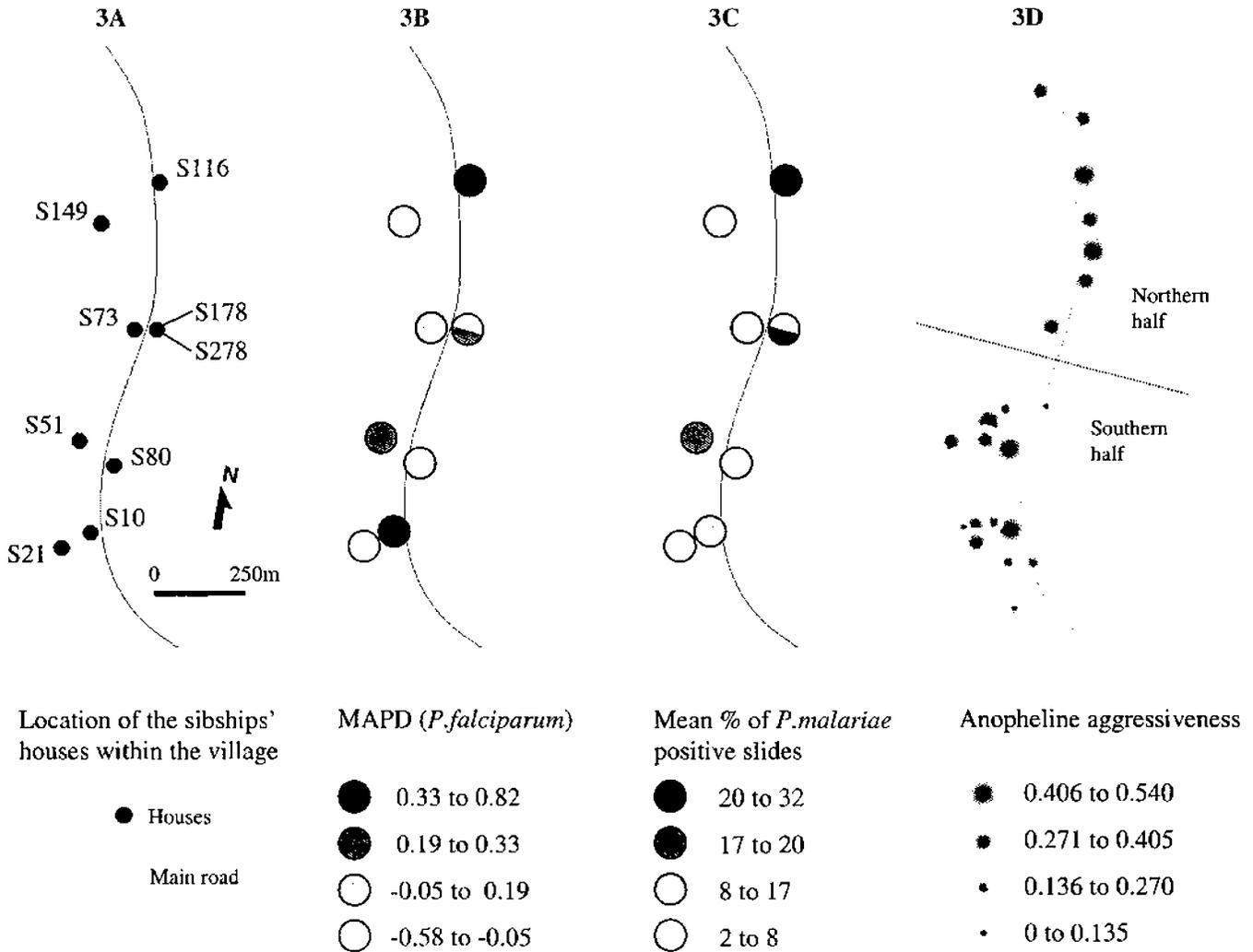
FIGURE 3. Distribution of the sibships' houses in the village of Dienga and corresponding parasitological characteristics and anopheline agressivity. The furthest homes are separated by around 1 km. S178 and S278 live in the same house (3A). Circles in gray scale represent the ranges of MAPD per sibship for the (3B) and frequency of *P. malariae* infection (3C). The average nightly exposure rate of houses enrolled in mosquitoes captures are represented in figure 3D.

were not significantly different between houses (Mann-Whitney *U*-test, $P = 0.46$). Nevertheless, the Welch test (comparison of two means with significantly different variances) revealed a higher aggression rate of mosquitoes in the northern half of Dienga compared to the southern half ($P = 0.04$; Pilkington H., unpublished data). Among sibships enrolled in the present study, houses of sibships S10, S21, S51 and S80, were located in the southern half of the village while sibships S73, S178, S278, S149 and S116 lived in the northern half. However, using Kruskal-Wallis test, no significant difference appeared between the geographical location of the sibships' houses and either MAPD values, the delay to reinfection nor the frequency of *P. malariae* infection.

DISCUSSION

The individual parasite densities were obtained from a longitudinal parasitological study and were adjusted for age and date of blood sampling. Individual MAPD were correlated positively to the frequency of *P. malariae* infection and

negatively to the delay of reinfection. Family analysis revealed that *P. falciparum* MAPD and the frequency of *P. malariae* infection differed significantly from one sibship to the other. On the other hand, the relationship between sibships, MAPD and *P. malariae* infection rate did not appear to be related to the variation of the vector density within the village.

Presence of a relation between sibships and *P. falciparum* parasite densities was already observed in segregation analysis of two familial studies, which demonstrated the inheritance of a complex of genes controlling parasite densities.[5,6] According to these results and to another longitudinal study,[10] we did not find any association between lower MAPD and any of the red blood cell factors under study. In Dienga, previous studies showed that sickle cell trait carriers had higher adjusted parasite densities and higher frequencies of multiple infections compared to normal subjects.[9,11] These studies concerned 300 school children over 6 years of age and followed-up during 1 year. In the present report, the survey involved a larger age range (1–21 years) than school

children and it was mainly performed during the dry season of low malaria transmission. As the influence of the sickle cell trait on malaria infection is known to be related to the age and to the season of transmission, the divergent results obtained by the different studies performed in the same village of Dienga were not surprising.[12,13] Sickle cell trait and G6PD deficiency are usually associated with protection against acute malaria, but their role on asymptomatic infections remains controversial.[3,12,14–17] We previously observed a relation between the G6PD phenotype and *P. falciparum* prevalence rate before treatment, suggesting a role for this deficient variant against blood stage parasites in asymptomatic infections.[7] Such a relationship was not observed with MAPD in this report when examining both G6PD genotypes and phenotypes.

More surprisingly, MAPD for *P. falciparum* were highly correlated with the frequency of infection with *P. malariae.* We previously demonstrated that *P. malariae* infected subjects were usually co-infected with *P. falciparum,* and were also more rapidly reinfected with *P. falciparum* as compared to those without *P. malariae* infection.[7] Furthermore, the mean frequency of *P. malariae* infection was significantly related to the sibships, and sibships with higher frequencies of *P. malariae* infections were those with higher *P. falciparum* MAPD values. When MAPD were adjusted to the frequency of *P. malariae* infection, the distribution of the variable was no more associated to the sibship suggesting that both infections are indissociable. Consequently, susceptibilities to both *P. falciparum* and *P. malariae* species were found to be closely related, and seemed to be under the control of factors linked to the family. An individual susceptibility common to both *Plasmodium* species has previously been documented by studies conducted in Garki, Nigeria,[18] which rather concluded to deficient specific immune responses to malarial antigens in co-infected subjects than to higher levels of transmission or vector exposure. In accordance to this hypothesis, subjects with protective humoral response against one of these two species would be protected against the other species due to the demonstrated cross-reactivity of antibodies directed against both *P. falciparum* and *P. malariae.*[19] We therefore suggest that the correlation observed in our study between *P. falciparum* MAPD and the frequency of *P. malariae* infection in sibships was rather related to the individual ability, under genetic control, to mount an effective immune response, than to a similar level of transmission of both species. A recent study conducted in Sri Lanka, in an endemic area for *P. falciparum* and *P. vivax,* seems to show that real differences exist in the genetic mechanisms that control the two species.[20] In accordance with several studies, the authors have observed a marked deficit in mixed-species infections.[21–24] In contrast to *P. falciparum* and *P. vivax* co-infections, mixed-species infections with *P. falciparum* and *P. malariae* exhibited a marked excess of prevalence than expected in the case of non-related infections.[7,18,25] Consequently, the genetic control of common genes to both species can not be excluded.

In order to assess whether differences in mosquitoes may be related to the level of infection, we investigated the relation between transmission and prevalence of infection by a retrospective analysis of data of vector captures. Geographical analysis revealed a greater level of exposure to malaria vectors in the northern half of Dienga than in the southern half. Nevertheless, none of the parasitological factors (MAPD, delay of reinfection and *P. malariae* infection) were related to the geographical position of the sibships' houses in the village, suggesting that the geographical variations of the malaria transmission at the village scale were not sufficient to explain the distribution of the parasitological characteristics of the sibships. Although we cannot thoroughly rule out the influence of variations in exposure between houses at a micro-scale level, the distribution of the parasitological status among inhabitants of different houses appears to be more likely related to some genetic or sociocultural factors, as people living in the same house frequently share family links. The genetic control of the parasite densities has already been demonstrated by studies on twins and by segregation analysis of familial studies.[4–6,26] The genetic regions involved in the expression of the MAPD phenotype have been localized in the 5q31-q33 portion of chromosome 5, which includes genes encoding several cytokines contributing to the Th1/Th2 cell type imbalance.[27] Moreover, a recent study has demonstrated the inheritance of the humoral response against blood stages of *P. falciparum.*[28]

In conclusion, the individual level of susceptibility to *P. falciparum* and *P. malariae* in symptomless infections appears to depend on family characteristics. Neither the sickle cell trait nor the G6PD deficiency could explain this observation, and the immune responses against blood stages of the parasite are more likely to be involved. Previous studies conducted in the population of the village of Dienga revealed genetic factors related to cellular and humoral immune responses.[9,29] The contribution of the host genetic diversity on the severity and distribution of malaria infections among sibships remains to be explored.

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