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Age-Dependent Acquisition of Protective Immunity to Malaria in Riverine Populations of the Amazon Basin of Brazil

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Abstract. Five community-based cross-sectional surveys of malaria morbidity and associated risk factors in remote riverine populations in northwestern Brazil showed average parasite rates of 4.2% (thick-smear microscopy) and 14.4% (polymerase chain reaction [PCR]) in the overall population, with a spleen rate of 13.9% among children 2–9 years of age. *Plasmodium vivax* was 2.8 times more prevalent than *P. falciparum*, with rare instances of *P. malariae* and mixed-species infections confirmed by PCR; 9.6% of asymptomatic subjects had parasitemias detected by PCR. Low-grade parasitemia detected by PCR only was a risk factor for anemia, after controlling for age and other covariates. Although clinical and subclinical infections occurred in all age groups, the risk of infection and disease decreased significantly with increasing age, after adjustment for several covariates in multilevel logistic regression models. These findings suggest that the continuous exposure to hypo- or mesoendemic malaria may induce significant anti-parasite and anti-disease immunity in native Amazonians.

INTRODUCTION

The incidence of malaria has increased > 10-fold in Brazil since the 1970s, with nearly 600,000 clinical cases recorded in 2005 (Ministry of Health of Brazil, unpublished data). Human migration and the establishment of new frontier agricultural settlements and open mining enclaves in the rainforest are the main factors associated with increased malaria transmission in the areas of endemicity across the Amazon Basin.[1] Frontier malaria, which is typically hypo- or mesoendemic, affects mostly migrants from malaria-free regions who became involved in agriculture, cattle ranching, lumber extraction, or mining in the Amazon[1,2] and rarely leads to the acquisition of clinical immunity that characterizes adults continuously exposed to holoendemic malaria in rural Africa. All age groups are assumed to be similarly vulnerable to infection and disease in these frontier areas.[3–5]

However, the assumption that clinical immunity is rarely acquired in areas of relatively low malaria endemicity[6] is challenged by recent studies of native Amazonians. Despite the relatively low levels of local malaria transmission, residents in isolated communities scattered along the margins of the Amazonian rivers often harbor subclinical infections with very low parasite loads, most of them detected only by polymerase chain reaction (PCR).[7–9] Although both clinical and subclinical infections are seen in all age groups, the proportion of infections that are asymptomatic tends to increase with age, suggesting that the continuous exposure to relatively stable but hypo- or mesoendemic malaria may induce significant protection against the disease.[7] Here we show that the epidemiology of malaria in traditional riverine populations of the Amazon Basin of Brazil demonstrates some features that are typical of holoendemic Africa, such as the frequent occurrence of subclinical infections with subpatent parasitemias and the finding of the heaviest burden of infection and disease among young children, arguing for an age-related acquisition of both anti-parasite and anti-disease immunity in this area of relatively low malaria transmission.

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MATERIALS AND METHODS

Study area and population. Jaú National Park, located within the Negro river watershed, covers an area of 2.27 million hectares in northwestern Brazil that is equivalent in size to the territory of Israel (Figure 1). This is the largest continuous area of protected tropical rainforest in the world, with an average annual rainfall of 2,000–2,250 mm and an average annual temperature of 26–27°C. At the beginning of the field study, we enumerated 180 dwellings (typically with wooden walls, unscreened windows, and thatched, wooden, or zinc roof) in the area, with 885 inhabitants living on subsistence farming, hunting, and fishing along the Unini and Paunini rivers (660 people, 140 dwellings) and the Jaú river (225 people, 40 dwellings). The population grouped into 13 relatively structured communities (16–114 people each) and 17 scattered localities (1–57 people each). Both *Plasmodium vivax* and *P. falciparum* are transmitted year-round, with increased incidence at the beginning (August–September) and the end (February–March) of the rainy season. The eligible study population comprised inhabitants in 11 communities (7 along the Unini and Paunini rivers and 4 along the Jaú river) and 3 localities (1 along the Unini river and 2 along the Jaú river). Two communities (total, 144 inhabitants) located close to the mouth of the Unini river were excluded because their inhabitants often traveled to other rural and urban areas outside the park. We also excluded 14 isolated localities (total,147 inhabitants) because of the long distance from one another and their small population.

Cross-sectional parasite prevalence surveys. Three cross-sectional surveys (A, B, and C) were performed during the first travel of the field team to the park, between November 9, 2002 and January 15, 2003. Surveys A (N = 275 subjects enrolled) and B (N = 287) comprised six communities and one locality visited during the upstream and downstream journeys along the Unini river. Survey C (N = 48) comprised two communities and one locality visited during the downstream journey along the Jaú river. During the second travel to the park, two

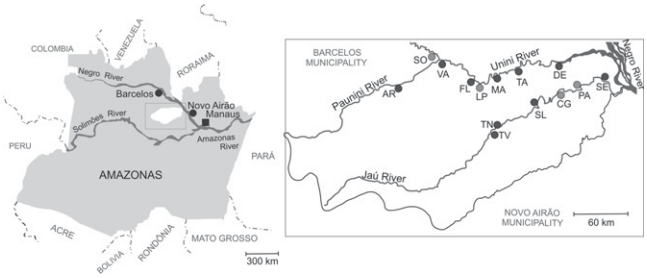

FIGURE 1. Map of the state of Amazonas, northwestern Brazil, showing the location of Jaú National Park (left panel) and the communities and localities studied within the Park (right panel). DE, Democracia; TA, Tapiíra; MA, Manapana; FL, Floresta; VA, Vista Alegre; AR, Aracu; SE, Seringalzinho; SL, São Lázaro; TN, Tambor Novo; TV, Tambor Velho; LP, Lago das Pombas; SO, Solimõezinho; PA, Patauá; CG, Capoeira Grande.

cross-sectional surveys (D and E) were performed between May 16 and July 19, 2003. Survey D (N = 325) comprised six communities visited during the upstream journey along the Unini river, one community along the Paunini river (tributary of the Unini), and one locality along the Unini river. Finally, Survey E (N = 166) comprised four communities and two localities visited during the upstream journey along the Jaú river. Of 594 residents in the selected communities and localities, 540 (90.9%) participated in one or more surveys, and 296 (49.8%) participated in at least one survey performed during each travel; 6 subjects (1.0%) refused to participate and 24 (4%) were excluded because they were not permanent residents in the area, could not be located during the visits, or were not eligible for blood collection (age < 3 months). All study participants provided finger-prick (Survey A) or venous blood samples (Surveys B, C, D, and E) for malaria diagnosis, irrespective of any clinical symptoms, and were physically examined by the same field physician. Spleen sizes were recorded according to the classification of Hackett.[10] A detailed structured questionnaire was applied to all study participants to obtain demographic, clinical, and behavioral information. Most (67.8%) study participants were born in Jaú National Park; only 13 subjects (0.6%) were migrants from extra-Amazonian states, where malaria is not endemic.

Laboratory diagnosis of malaria. A total of 1,095 Giemsa-stained thick smears were examined under ×1,000 magnification for malaria parasites during the cross-sectional surveys (minimum of 100 microscopic fields examined). Parasitemias were estimated by counting parasites per 200 leukocytes, assuming a white blood cell count of 8,000/μL. Three sets of slides were sent for review by an expert microscopist at the National Reference Laboratory of the Ministry of Health of Brazil, in Brasília: 1) all positive slides, 2) all negative slides from patients with malaria-positive PCR (see below), and 3) a random sample of 10% of the remaining negative slides. Nested PCR amplification of a species-specific segment of the *18S rRNA* gene of human malaria parasites[11] was performed on 1,042 blood samples. Briefly, parasite DNA was extracted from venous blood samples using the Wizard Genomic DNA purification kit (Promega, Madison, WI), following the manufacturer's instructions; DNA from finger-prick blood samples spotted on filter paper was extracted using the protocol described by Plowe and others.[12] The first round of PCR amplification (35 cycles) was carried out with 1 μL of parasite DNA solution with the universal oligonucleotide primers P1-Up (forward) (5′-TCC ATT AAT CAA GAA CGA AAG TTA AG-3′) and P2 (reverse) (5′-GAA CCC AAA GAC TTT GAT TTC TCA T-3′), which amplify a 130-bp fragment of the *18S rRNA* gene of human malaria parasites. The second round of PCR amplification (18 cycles) was carried out with 1 μL of the first PCR product (diluted 1:50 in distilled water), the universal forward primer P1 (5′-ACG ATC AGA TAC CGT CGT AAT CTT-3′), and one of the following species-specific reverse primers: F2 (5′-CAA TCT AAA AGT CAC CTC GAA AGA TG-3′), V1 (5′-CAA TCT AAG ATT AAA CTC CGA AGA GAA A-3′), or M1 (5′-GGA AGC TAT CTA AAA GAA ACA CTC ATA T-3′). The length of the second PCR product is ~100 bp. The assay protocols used in both rounds of amplification were exactly as described by Win and others.[11]

Laboratory diagnosis of anemia. Subjects who provided venous blood samples (Surveys B, C, D, and E) had their packed cell volume (PCV) determined using standard methods; anemia was defined by applying the age-specific PCV cut-off values suggested by the World Health Organization.[13] The PCV values below which anemia was considered to be present are as follows: 0.33 for children 6–59 months of age, 0.34 for children 5–11 years of age, 0.36 for children 12–14 years of age and non-pregnant women > 15 years of age, 0.33 for pregnant women, and 0.39 for men > 15 years of age.

Malarial antibody studies. To infer the occurrence of malarial infections between cross-sectional surveys, we compared titers of IgG antibodies to *P. falciparum* and *P. vivax,* measured by indirect fluorescent antibody test, in paired samples collected from the same subjects during Travel 1 and Travel 2. The assay protocol is described in detail elsewhere,[14] with *P. falciparum* and *P. vivax* antigens prepared directly from the blood of infected subjects.[15] Titers ≥ 16 were considered positive, because this cut-off value resulted in the best combination of sensitivity and specificity (i.e., highest diagnostic efficiency) when 108 negative control sera, 45 *P. falciparum*–positive control sera, and 48 *P. vivax*–positive control sera were analyzed.[14] By applying this cut-off titer, our indirect fluorescent antibody test had a sensitivity of 96% and a specificity of 98% for anti-*P. falciparum* antibodies and a sensitivity of 93% and a specificity of 96% for anti-*P. vivax* antibodies (data from Carvalho and others[14]). When comparing paired samples, we defined exposure to malaria between blood draws to have occurred if 1) IgG antibodies were undetectable (below the cut-off titer) in the first sample and detectable in the second sample, or 2) there was an increase of 4-fold greater IgG antibody titers between the first and second blood draw.

Clinical definitions. Clinical malaria was defined, in the main analysis, when 1) malaria parasites were detected by conventional microscopy (confirmed after expert slide review), PCR, or both, and 2) subjects reported a history of fever in the preceding 30 days or an axillary temperature > 37.8°C measured during the physical examination or 3) subjects had any of the symptoms listed below up to 15 days after the blood sample was collected for malaria diagnosis. Infected subjects with other apparent causes of fever were excluded from further analysis. Subclinical infections were defined when malaria parasites were detected, by any laboratory method, in patients who remained free of fever or any other symptom suggestive of malaria (chills, headache, profuse sweating, weakness, myalgia, arthralgia, abdominal pain, nausea, vomiting, dizziness, or diarrhea) for at least 15 days after sample collection. Symptomless subjects who were followed for < 15 days

and those who had received anti-malarials in the preceding 30 days or herbal remedies for fever in the preceding 3 days could not be classified and were also excluded from further analysis. Subjects enrolled during the upstream journey were revisited during the downstream journey (2–43 days later), irrespective of the results of microscopy, to determine whether their clinical status (presence or absence of symptoms) had changed. Those with subclinical infections diagnosed by microscopy onsite were reassessed daily by the field physician and a local health worker and treated at the end of the travel (1–39 days after the first visit) or whenever they became symptomatic; symptomatic infections were treated as soon as the microscopic diagnosis was made. Because PCR results were not available in a timely fashion, infections detected by PCR only were left untreated. To confirm the consistency of our main conclusions regarding the association between age and symptoms, we reanalyzed the data with an alternative definition of clinical disease that considers a shorter time window: 1) detection of malaria parasites by conventional microscopy (confirmed after expert slide review), PCR, or both and 2) reported or measured fever during 3 days preceding enrollment.

Statistical analysis. A database was created with SPSS 13.0 (SPSS, Chicago, IL). Proportions were compared with χ^2 tests, with Yates correction of continuity when appropriate, whereas continuous variables were compared with non-parametric Mann-Whitney *U* tests. Multiple logistic regression models with stepwise backward deletion were built to describe independent associations between potential risk factors and selected outcomes. Because of the nested structure of the data (there are one, two, or three observations per individual), we used two-level logistic models with robust SE, with Level 1 variables corresponding to each observation (one or more per individual) and Level 2 variables corresponding to each individual. Covariates were selected for inclusion in logistic models if they were associated with the outcome, at the level of significance of 20%, in exploratory unadjusted analysis. The first outcome variable was malarial infection (irrespective of any symptom) diagnosed during any cross-sectional survey. Level 1 covariates were age at the time of the survey (continuous variable), use of anti-malarials in the preceding 30 days, and date of the cross-sectional survey (either first or second travel). The Level 2 covariates were sex, place of residence (Unini or Jaú river [residents along the Paunini river were included in the Unini area] and either upper or lower part of the river), use of bednets, sleeping habits (sleeping and wake-up time), and bedroom characteristics (presence or absence of complete walls). The second outcome variable was the occurrence of clinical symptoms among subjects with laboratory-confirmed malarial infection. Level 1 variables were age at the time of the survey (< 5 or ≥ 5 years), approximate date of the survey (either Travel 1 or Travel 2), and parasite load (either patent or subpatent parasitemia, detected by PCR only); Level 2 covariates were sex, place of residence (Unini or Jaú river and either upper or lower part of the river), sleeping habits (sleeping and wake-up time), and bedroom characteristics (presence or absence of complete walls). To determine the clinical impact of subpatent malarial infection, we built a two-level multiple logistic regression model, with anemia as the outcome variable, that included as covariates age, sex, presence of subpatent parasitemia (detected by PCR only), history of previous slide-confirmed malarial infection, and all other factors found to be significantly associated with the risk of malarial infection. Subjects with patent parasitemia (i.e., positive Giemsa-stained thick smears) were excluded from the models. The HML software package (version 6.03; Scientific Software International, Lincolnwood, IL) was used for all multilevel analyses. Only variables associated with statistical significance at the 5% level or those that altered the parameter estimate (odds ratio [OR]) by ≥ 10% were retained in the final models.

Ethical considerations. The study protocol was approved by the Ethical Committee of the Oswaldo Cruz Institute (157/2002), Rio de Janeiro, Brazil, in June 21, 2004. All study participants or their legal guardians provided written informed consent.

RESULTS

Prevalence of malarial infection and disease. Conventional microscopy detected parasites in 46 of 1,095 (4.2%) thick smears analyzed, with average parasite rates of 4.9% for residents along the Unini river and 1.4% for residents along the Jaú river ($\chi^2 = 4.30$, $P = 0.038$). PCR gave positive results in 150 of 1,042 samples analyzed (14.4%), being on average 3.3 times more sensitive than conventional microscopy in detecting malarial infections. Overall, 158 of 1,095 (14.4%) samples were positive by either PCR or microscopy; of 1,042 samples tested by both methods, malaria infections were detected by both methods in 39 instances, by PCR only in 112 instances, and by microscopy only (confirmed by expert slide review) in 7 instances.

Although the parasite prevalence detected by PCR was slightly higher on the Unini compared with the Jaú (15.4% versus 10.4%), the difference did not reach statistical significance ($\chi^2 = 3.09$, $P = 0.079$). The prevalence of infection detected by microscopy, PCR, or both was higher in the more remote communities scattered along the upper part of the Unini and Jaú rivers (19.9%) compared with the more accessible communities in the lower part of these rivers (8.5%; $\chi^2 = 27.64$, $P < 0.0001$); similar differences are found when analyses are made separately for each river ($P < 0.0001$ for Unini and $P = 0.004$ for Jaú). *P. vivax* predominated over *P. falciparum* in most surveys (overall ratio, 2.8:1; considering results of microscopy and PCR); mixed-species infections and those with *P. malariae* corresponded to only 3.3% and 2.0%, respectively, of all infections detected by PCR (Table 1).

According to the traditional stratification of malaria endemicity based on spleen rates in children 2–9 years of age,[16] the study area is characterized as mesoendemic; if parasite rates estimated by conventional microscopy are considered, the area is characterized as hypoendemic (Table 2), but these figures may have been affected by seasonal variation in malaria transmission and therefore in parasite point-prevalences.

The overall prevalence of malarial infection, measured during cross-sectional surveys by combining microscopy and PCR, decreased steadily with age (Figure 2, top panel). The decrease was significant for all malaria parasite species ($P < 0.0001$), for *P. vivax* ($P < 0.0001$), and for *P. falciparum* ($P = 0.003$) by χ^2 tests for linear trend. The age-related decrease in the prevalence of malaria remained significant when infections detected by microscopy and by PCR only were analyzed separately ($P < 0.0001$ for both, χ^2 tests for linear trend). Low parasitemias detected by PCR only were found in 28 (50.0%) of 56 malaria infections diagnosed in children < 5 years of age.

TABLE 1
Malarial infections by microscopy and PCR and overall malaria prevalence in riverine populations of Jaú National Park, Brazil, 2002–2003

Survey	Method	*N*	Pf	Pv	Pm	Mixed	Prevalence
A	Microscopy	275	4	8	0	0	12 (4.4%)
	PCR	233	7*	13*	0	1	19 (8.2%)
B	Microscopy	286	2	7	0	0	9 (3.1%)
	PCR	277	16†	42†‡	3‡	4	57 (20.6%)
C	Microscopy	47	1	0	0	0	1 (2.1%)
	PCR	47	5	1	0	0	6 (12.8)
D	Microscopy	321	4	18	0	0	22 (6.9%)
	PCR	320	11	41	0	0	52 (16.3%)
E	Microscopy	166	0	2	0	0	2 (1.2%)
	PCR	165	1	15	0	0	16 (9.7%)
All surveys combined	Microscopy	1,095	11	35	0	0	46 (4.2%)
	PCR	1,042	40§	112‡§	3	5	150 (14.4%)

PCR-positive samples comprise both microscopy-positive and microscopy-negative samples.
* Includes one *P. vivax–P. falciparum* mixed-species infection.
† Includes three *P. vivax–P. falciparum* mixed-species infections.
‡ Includes one *P. vivax–P. malariae* mixed-species infection.
§ Includes four *P. vivax–P. falciparum* mixed-species infections
N = number of subjects examined; Pf = number of infections with *P. falciparum*; Pv = number of infections with *P. vivax*; Pm = number of infections with *P. malariae*; Mixed = number of mixed-species infections.

In the older age groups, however, the proportions of infections that were subpatent (detected by PCR only) were substantially higher, between 69.2% and 92.8% of all infections diagnosed.

Of 158 laboratory-confirmed malarial infections, we were able to classify 121 episodes (76.6%) as either symptomatic (58 or 47.9%) or asymptomatic (63 or 52.1%). Of 37 malaria episodes that could not be classified as asymptomatic or symptomatic, 30 (81.1%) were diagnosed by PCR only and 7 by both PCR and conventional microscopy. Only 28.3% of parasite carriers < 5 years of age were classified as asymptomatic, using the more strict definition (malaria parasites detected by conventional microscopy [confirmed after expert slide review], PCR, or both; absence of reported fever in the preceding 30 days; axillary temperature < 37.8°C measured during the physical examination; and absence of symptoms up to 15 days after the blood sample was collected for malaria diagnosis), with an increasing proportion of subclinical infections in the older age groups (χ^2 test for linear trend, $P = 0.012$; Figure 2, middle panel). A similar age pattern was observed when asymptomatic infections were defined using less strict criteria (malaria parasites detected by conventional microscopy [confirmed after expert slide review], PCR, or both; absence of reported fever in the preceding 3 days; and axillary temperature < 37.8°C measured during the physical examination), again with an increasing proportion of subclinical infections in the older age groups (χ^2 test for linear trend, $P = 0.0004$; Figure 2, bottom panel). These findings suggest that the observed association between increasing age and clinical immunity does not result from the misclassification of febrile illnesses of nonmalarial origin as symptomatic malaria in young children. In fact, only 19.2% of the children < 5 years of age who were classified as symptomatic using the more strict criteria would be defined as asymptomatic using the more relaxed criteria.

More than nine percent of subjects who remained symptomless for at least 15 days of follow-up were shown to carry malaria parasites (Table 3). Not surprisingly, most (57 of 63, 90.5%) of these subclinical infections were subpatent, being detected only by PCR. Of 46 infections detected by thick-smear microscopy onsite, 25 (54.3%) were symptomatic at the time of blood draw, 7 (15.2%) were initially symptomless but became symptomatic after 1–15 days of follow-up, 6 (13.0%) remained asymptomatic, and 8 infected subjects (17.4%) were lost for follow-up. Of 112 infections detected by PCR only, 20 (17.9%) were symptomatic at the time of blood draw, 63 (56.2%) remained asymptomatic over the next 15 days of follow-up, and 29 (25.9%) were followed for < 15 days. Few subjects with parasitemias detected by PCR only had symptoms after 15 days of follow-up (e.g., only five had symptoms between 15 and 30 days of follow-up).

Risk factors for malarial infection. Multilevel logistic regression analysis showed that young age, residence along the Unini river, residence in the upper part of either Unini or Jaú rivers, wake-up time after 6:00 AM, and absence of complete walls in the bedroom were significant independent predictors of infection with malaria parasites, irrespective of the species, detected by either microscopy, PCR, or both (Table 4). The risk of *P. vivax* infection was independently associated with young age, male sex, residence along the Unini river, residence in the upper part of either Unini or Jaú rivers, sleep time before 9:00 PM, and absence of complete walls in the bedroom. The independent predictors of *P. falciparum* infection were young age,

TABLE 2
Indices of malaria endemicity (parasite rate and spleen rate in children 2–9 years of age) measured during consecutive cross-sectional surveys in riverine populations of Jaú National Park, Brazil, 2002–2003

Index	Survey					All surveys combined
	A	B	C	D	E	
Parasite rate	10.0% (7/70)*	8.1% (6/74)	6.3% (1/16)	10.2% (9/88)	1.9% (1/54)	7.9% (24/302)
Spleen rate	15.7% (11/70)†	14.9% (11/74)	12.5% (2/16)	10.1% (9/89)	16.7% (9/54)	13.9% (42/303)

* Percent (slide positive/total individuals examined by thick smear microscopy).
† Percent (palpable spleen/total individuals physically examined).

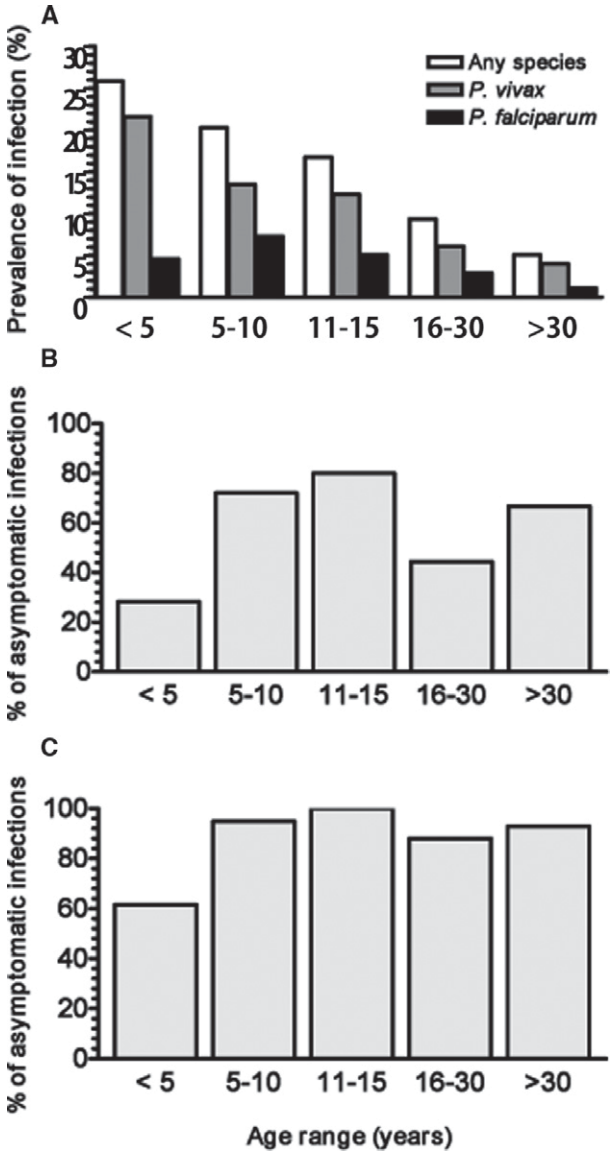

FIGURE 2. Overall age-specific prevalence of malarial infection diagnosed by conventional microscopy, PCR, or both (top panel) and age-specific proportion of infections, irrespective of the malaria parasite species, which were asymptomatic among inhabitants of Jaú National Park, Brazil, 2002–2003 (middle and bottom panels). In the top panel, the numbers of subjects in each age group are as follows: < 5 years, 218; 5–10 years, 192; 11–15 years, 138; 16–30 years, 278; > 30 years, 275. The proportions of infections detected by PCR only in each age group were as follows: < 5 years, 50.0%; 5–10 years, 84.6%; 11–15 years, 86.9%; 16–30 years, 69.2%; > 30 years, 92.9% (subjects not tested by PCR were excluded from this analysis). Two sets of criteria were used to define asymptomatic infections (data shown in the middle and bottom panels). In the middle panel, asymptomatic malaria was defined using the following criteria: 1) malaria parasites detected by conventional microscopy (confirmed after expert slide review), PCR, or both; 2) absence of reported fever in the preceding 30 days; 3) axillary temperature < 37.8°C measured during the physical examination; and 4) absence of symptoms up to 15 days after the blood sample was collected for malaria diagnosis. The numbers of subjects in each age group are as follows: < 5 years, 46; 5–10 years, 25; 11–15 years, 20; 16–30 years, 18; > 30 years, 12. In the bottom panel, asymptomatic malaria was defined using more relaxed criteria: 1) malaria parasites detected by conventional microscopy (confirmed after expert slide review), PCR, or both; 2) absence of reported fever in the preceding 3 days; and 3) axillary temperature < 37.8°C measured during the physical examination. The numbers of subjects in each age group are as follows: < 5 years, 54; 5–10 years, 39; 11–15 years, 23; 16–30 years, 25; > 30 years, 14.

female sex, date of the survey (lower prevalence in Travel 2), residence in the upper part of each river, and absence of complete walls in the bedroom. Note that female sex was significantly associated with reduced risk of *P. vivax* infection but increased risk of *P. falciparum* infection; when combining both species, no significant association between malarial risk and sex was found. We have no clear-cut explanation for the observed sex-related differences of risk for these malaria parasite species, which co-circulate in the same areas and are believed to be transmitted by the same local mosquito vectors.

The association of parasite prevalence with age could be caused either by 1) an age-related variation in exposure to malaria or 2) differences in the ability to control new infections. To explore the first hypothesis, we compared titers of IgG antibodies to *P. vivax* and *P. falciparum* in paired samples collected during the first and second travel to infer age-related levels of exposure to these species at the end of the 2003 rainy season (February–March).[17] Data shown in Figure 3 suggest that exposure to malaria during the high-transmission season, as inferred by seroconversion or increase in antibody titers, does not vary substantially with age in the study area age (χ^2 test, $P = 0.94$ and 0.64 for *P. vivax* and *P. falciparum,* respectively), indicating that the association between age and risk of malarial infection cannot be explained by differences in exposure to the parasite. Therefore, the observed age-related differences in the prevalence of infection and disease are likely to result, at least in part, from different levels of acquired immunity. Although *P. vivax* infections predominated during the cross-sectional surveys, seroconversion rates between surveys were higher for anti-*P. falciparum* than anti-*P. vivax* antibodies. Because the sensitivity of the method used for antibody detection may vary according to the species,[14] we suggest that between-species comparisons of seroconversion rates may be inappropriate. We also tested whether the exposure to *P. vivax* and *P. falciparum,* as inferred from the analysis of paired antibody titers, differed between males and females in the study area. No significant sex-related difference was found in the proportion of subjects with serologic evidence of recent exposure to *P. vivax* (χ^2 test, $P = 0.56$) and *P. falciparum* (χ^2 test, $P = 0.15$).

Risk factors for clinical disease. The outcome variable (clinical disease) used in the first multilevel logistic regression analysis considered the presence of symptoms during a relatively large period of time (30 days preceding the enrollment and 15 days after the enrollment) to define symptomatic disease. This analysis showed that age ≥ 5 years was associated with a reduced risk of symptoms (adjusted OR [aOR], 0.30; 95% confidence interval [CI]: 0.12–0.76; $P = 0.012$), whereas residence along the Jaú river (aOR, 9.98; 95% CI: 1.69–58.83; $P = 0.012$) and the presence of patent parasitemia (aOR, 11.06; 95% CI: 3.96–30.91; $P < 0.0001$) were significant independent predictors of the presence of clinical symptoms among infected subjects. There was a trend toward a decreased risk of symptomatic disease in females (aOR, 0.68; 95% CI: 0.28–1.69), but without statistical significance ($P = 0.410$). Among patients with patent parasitemia detected by conventional thick-smear microscopy, parasite counts were quite similar in asymptomatic (geometric mean, 447.27/μL; $N = 6$) and symptomatic subjects (geometric mean, 495.17/μL; $N = 32$; Mann-Whitney U test, $P = 0.94$).

Children < 5 years of age may be more likely than older subjects to have fever of nonmalarial origin during the relatively

TABLE 3
Malarial infections diagnosed by microscopy and PCR in symptomless subjects with at least 15 days of follow-up and overall prevalence of malarial infections in asymptomatic subjects in Jaú National Park, Brazil, 2002–2003

Survey	Method	*N*	Pf	Pv	Pm	Mixed	Prevalence
A	Microscopy	226	0	1	0	0	1 (0.4%)
	PCR	187	3	2	0	0	5 (2.7%)
B	Microscopy	214	0	0	0	0	0
	PCR	209	10*	20*†	2†	2	30 (14.4%)
C	Microscopy	37	0	0	0	0	0
	PCR	37	1	0	0	0	1 (2.7%)
D	Microscopy	173	1	3	0	0	4 (2.3%)
	PCR	173	8	17	0	0	25 (14.5%)
E	Microscopy	38	0	1	0	0	1 (2.6%)
	PCR	38	0	1	0	0	1 (2.6%)
All surveys combined	Microscopy	688	1	5	0	0	6 (0.9%)
	PCR	644	22*	40*†	2†	2	62 (9.6%)

N = number of asymptomatic subjects examined during the cross-sectional survey who had no symptoms in the preceding 30 days and remained asymptomatic after a follow-up of at least 15 days; Pf = number of infections with *P. falciparum*; Pv = number of infections with *P. vivax*; Pm = number of infections with *P. malariae*; Mixed = number of mixed-species infections.
* Includes one *P. vivax–P. falciparum* mixed-species infection.
† Includes one *P. vivax–P. malariae* mixed-species infection.

large period of time (30 days preceding the enrollment and 15 days after the enrollment) considered in the definition of clinical malaria used in the main analysis. This could lead to a classification bias, because young children with malaria parasites detected by either microscopy or PCR would be more likely to be classified as symptomatic because of any febrile illness not associated with malaria. To minimize this potential bias, which may have affected our main conclusions regarding the association between age and acquired immunity, we reanalyzed the data using an alternative definition of clinical malaria that considers a shorter observation period: 1) detection of malaria parasites by conventional microscopy (confirmed after expert slide review), PCR, or both and 2) reported or measured fever during 3 days preceding the enrollment. Age > 5 years remained significantly associated with reduced risk of symptomatic disease (aOR, 0.14; 95% CI: 0.05–0.58; $P < 0.0001$) in a subsequent multilevel model with the alternative definition of clinical disease. The presence of patent parasitemia (but not the place of residence) remained a significant predictor of the presence of clinical symptoms among infected subjects (aOR, 10.23; 95% CI: 3.58–29.20; $P < 0.0001$) in this alternative model.

Subpatent parasitemia as a risk factor for anemia. Although the association between slide-positive malarial infection and anemia is well known,[18] there is less abundant evidence supporting subpatent malarial infection as a risk factor for anemia. Of 815 PCV determinations made during cross-sectional surveys in our study population, 142 (17.4%) were diagnostic of anemia, with similar proportions of anemic males (18.3%) and females (16.3%) (χ^2 test, $P = 0.50$). The prevalence of anemia decreased with age (χ^2 test for linear trend, $P < 0.0001$); 33.1% of samples from children < 5 years but only 13.4% of those from adults > 30 years had low PCV. We built two-level multiple logistic regression models with anemia as the outcome variable and all variables listed in Table 4 as covariates; the presence of subpatent parasitemia was included as a further covariate, and subjects with patent infection detected by microscopy (irrespective of the PCR results) were excluded from the analysis. The presence of low-level parasitemia was found to be a significant predictor of anemia (aOR, 1.92; 95% CI: 1.14–3.23; $P = 0.015$) after controlling for age, sex, date of the survey, place of residence, sleeping habits, and type of bedroom ($N = 777$ observations). We did not study other possible causes of anemia (such as geohelminth infection, iron

TABLE 4
Results of the final two-level logistic regression models ($N = 1{,}095$) including covariates significantly associated with malarial infection in consecutive cross-sectional surveys of human populations of Jaú National Park, Brazil, 2002–2003

Outcome variable and covariates	Adjusted OR	(95% CI)	*P*
Malarial infection (any species)*			
Place of residence (Jaú vs. Unini river)	0.43	(0.26–0.73)	0.002
Place of residence (upper vs. lower river)	1.85	(1.19–2.89)	0.007
Bedroom walls (incomplete versus complete)	2.17	(1.39–3.40)	0.001
Wake-up time (at of after 6:00 AM vs. earlier)	1.80	(1.06–3.07)	0.030
Age in years (continuous variable)	0.96	(0.94–0.97)	< 0.0001
Plasmodium falciparum infection*			
Place of residence (upper vs. lower river)	2.87	(1.42–5.78)	0.004
Sex (female vs. male)	2.17	(1.25–3.76)	0.006
Bedroom walls (incomplete vs. complete)	2.62	(1.34–5.10)	0.005
Date of survey (second vs. first travel)	0.50	(0.25–0.98)	0.042
Age in years (continuous variable)	0.96	(0.95–0.98)	0.001
Plasmodium vivax infection*			
Place of residence (Jaú vs. Unini river)	0.41	(0.25–0.69)	0.001
Place of residence (upper vs. lower river)	1.75	(1.07–2.84)	0.025
Sex (female vs. male)	0.62	(0.41–0.94)	0.023
Bedroom walls (incomplete vs. complete)	1.81	(1.10–2.98)	0.020
Sleep time (before or at 9:00 PM vs. later)	2.09	(1.06–4.15)	0.035
Age in years (continuous variable)	0.96	(0.94–0.97)	< 0.0001

* Outcome variable.

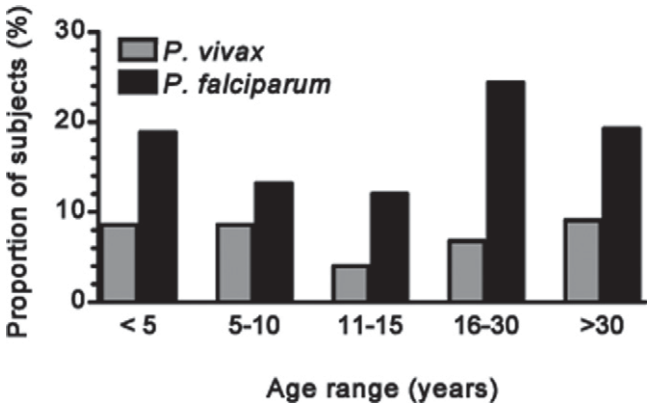

FIGURE 3. Age-specific proportions of subjects acquiring IgG antibodies to *P. vivax* and *P. falciparum,* as determined by indirect fluorescent antibody test, at the end of the rainy season (February–March) of 2003. The number of paired samples analyzed was 194 and 202 for antibodies to *P. vivax* and *P. falciparum,* respectively.

deficiency, and hemoglobinopathies) in our population, but we consider it unlikely that these factors would be major confounders of the association between low-level parasitemia and anemia, because they would similarly affect PCR-positive and PCR-negative subjects.

DISCUSSION

Several findings from this large study of malaria morbidity in remote Amazonian populations have major biological and epidemiologic implications. First, because the prevalence of infection and the proportion of infections that are symptomatic both decrease with age, most malaria morbidity in the study population occurs in children. This contrasts with the classic descriptions of frontier malaria across the Amazon Basin[1–4] and of forest malaria in Southeast Asia,[19] which affect mostly adult men with regular forest-related activities. Because no apparent difference was found in the levels of exposure to the parasite across age groups, as inferred by antibody reactivity patterns, we suggest that the age-related decrease in the risk of infection and disease reflects some degree of immunity acquired by native Amazonians after a few years of continuous exposure to relatively stable but hypo- or mesoendemic malaria. Residents along the Unini river had higher parasite rates measured by microscopy during the cross-sectional surveys, suggesting that they are exposed to higher levels of malaria transmission; accordingly, the proportion of infections among them that are symptomatic is lower than that observed among residents on the Jaú river.

The scattered native populations living in traditional style along the Amazonian rivers are able to acquire anti-disease immunity[7,9] and, most notably, anti-parasite immunity (this paper) after a few years of exposure to hypo- or mesoendemic malaria. One of the factors contributing to the fast acquisition of immunity might be the relatively low level of overall genetic diversity in local *P. falciparum* populations,[20] with a rather limited repertoire of variant antigens,[21] although no comparable data are available for the most prevalent malaria parasite species, *P. vivax*. We can hypothesize that, at least for *P. falciparum*, exposure to the whole repertoire of variant surface antigens that circulate in the area may occur within a few years and result in substantial levels of acquired, variant-specific immunity.[22]

The high prevalence of malarial infection in asymptomatic subjects has clear public health implications. We found malaria parasites by PCR in 9.6% of subjects with no recent or current malaria symptoms. Because the active and passive case detection (ACD and PCD) strategies currently used to diagnose and treat malaria in Brazil rely on a history of recent fever, these subclinical infections would remain undetected and untreated, representing a potential source of gametocytes for local vectors. Aggressive active case detection (AACD), which consists in examining the whole population of a given endemic area irrespective of any clinical symptoms, is the only available strategy to diagnose subclinical infections and possibly the most cost-effective measure to control malaria in some settings.[23] Nevertheless, traditional AACD strategies based on conventional microscopy may still miss a substantial proportion of subclinical infections with subpatent parasitemias (Table 3).[7,9] The use of PCR-based diagnosis as a public health tool for AACD in such contexts has been advocated.[9] Although we have shown that the carriage of subpatent parasitemias, most of them subclinical, by native Amazonians may lead to clinical consequences, such as the increased risk of anemia, we believe that the widespread use of PCR-based diagnosis remains severely constrained by its high cost and complexity. Vector control (rational use of insecticides) and prevention of vector–man contact (use of impregnated bed nets) would possibly be more cost-effective for controlling malaria in Jaú National Park than PCR-based surveillance. The potential contribution of microscopy-based AACD remains to be determined in this and similar settings; some asymptomatic infections may surely be detected and treated, possibly reducing the risk of mosquito infection.

The low prevalence of *P. malariae* (2.0% of all malaria infections detected by PCR) in our population contrasts with the high proportion of infections with this species found in area of frontier malaria in Rondônia (9.4%)[24] and Mato Grosso (11.9%).[24] The proportion of mixed-species infections among subjects with PCR-detected parasites was also lower (3.3%) among residents in Jaú National Park than in other native (5.4–36.1%)[7] or migrant populations (25.7–30.2%)[24,25] across the Amazon Basin of Brazil. The reasons for these differences are unclear, because similar PCR protocols were used in all studies.

This study has four major limitations. First, we were able to perform sequential cross-sectional surveys, but no reliable estimates of malaria incidence between the surveys could be obtained and analyzed. As a result, the putative association between risk factors and outcomes could not be assessed prospectively. Second, the cross-sectional surveys could not be carried out at the peaks of malaria transmission in the area (February–March and August–September) and may have yielded underestimated parasite rates. The difficult access to the riverine communities by boat during part of the year determined the dates of the cross-sectional surveys and limited the duration of each stay in the area. Third, defining clinical malaria in populations exposed to low-level malaria transmission remains a major challenge. For example, a recent febrile illness of nonmalarial origin, which often affects young children, may cause an episode of asymptomatic parasite carriage to be misclassified as clinical malaria. To circumvent this limitation, we reanalyzed our data by using a less strict definition of asymptomatic malaria, which considered only the absence of fever in the preceding three days, and obtained rather similar results. Fourth, only limited entomologic data

have been obtained during the surveys, and entomologic inoculation rates could not be estimated. Despite these limitations, we found a clear association between age and risk of malarial infection and disease that is unlikely to have been affected by the timing or design of the study. We are now outlining further studies of native Amazonian populations to investigate the patterns and mechanisms of acquired immunity to malaria and the relative contribution of several parasite- and host-related factors to this process.

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REFERENCES

1. Marques AC, 1987. Human migration and the spread of malaria in Brazil. *Parasitol Today 3:* 166–170.
2. Castro MC, Monte-Mór L, Sawyer DO, Singer BH, 2006. Malaria risk on the Amazon frontier. *Proc Natl Acad Sci USA 103:* 2452–2457.
3. Camargo LMA, dal Colletto GM, Ferreira M, Gurgel SM, Escobar AL, Marques A, Krieger H, Camargo EP, Silva LHP, 1996. Hypoendemic malaria in Rondonia (Brazil, Western Amazon Region): seasonal variation and risk groups in an urban locality. *Am J Trop Med Hyg 55:* 32–38.
4. Camargo LMA, Ferreira MU, Krieger H, Camargo EP, Silva LHP, 1994. Unstable Hypoendemic malaria in Rondonia (Western Amazon Region, Brazil): epidemic outbreaks and work-associated incidence in an agro-industrial rural settlement. *Am J Trop Med Hyg 51:* 16–25.
5. Prata A, Urdaneta M, McGreevy PB, Tada MS, 1988. Infrequency of asymptomatic malaria in an endemic area in Amazonas, Brazil. *Rev Soc Bras Med Trop 23:* 51–54.
6. Macdonald G, 1957. *The Epidemiology and Control of Malaria*. London: Oxford University Press.
7. Alves FP, Durlacher RR, Menezes MJ, Krieger H, Silva LHP, Camargo EP, 2002. High prevalence of asymptomatic *Plasmodium vivax* and *Plasmodium falciparum* infections in native Amazonian populations. *Am J Trop Med Hyg 66:* 641–648.
8. Branch OL, Casapia WP, Gamboa DV, Hernandez JN, Alava FF, Roncal N, Alvarez E, Perez EK, Gotuzzo E, 2005. Clustered local transmission and asymptomatic *Plasmodium falciparum* and *Plasmodium vivax* malarial infections in a recently emerged, hypoendemic Peruvian Amazon community. *Malar J 4:* 27.
9. Roshanravan B, Kari E, Gilman RH, Cabrera L, Lee E, Metcalfe J, Calderón M, Lescano AG, Montenegro SH, Calampa C, Vinetz JM, 2003. Endemic malaria in the Peruvian Amazon region of Iquitos. *Am J Trop Med Hyg 69:* 45–52.
10. Hackett LW, 1944. Spleen measurement in malaria. *J Natl Malar Soc 3:* 121–133.
11. Win TT, Lin K, Mizuno S, Zhou M, Liu Q, Ferreira MU, Tantular IS, Kojima S, Ishii A, Kawamoto F, 2002. Wide distribution of *Plasmodium ovale* in Myanmar. *Trop Med Int Health 7:* 231–239.
12. Plowe CV, Djimde A, Bouare M, Duombo O, Wellems TM, 1995. Pyrimethamine and proguanil resistance conferring mutations in *Plasmodium falciparum* dihydrofolate reductase: polymerase chain reaction methods for surveillance in Africa. *Am J Trop Med Hyg 52:* 565–568.
13. World Health Organization, 2001. *Iron Deficiency Anemia: Assessment, Prevention and Control*. Geneva: World Health Organization.
14. Carvalho ME, Ferreira MU, Souza MRD, Ninomia RT, Matos GF, Camargo LMA, Ferreira CS, 1992. Malaria seroepidemiology: comparison between indirect fluorescent antibody test and enzyme immunoassay using bloodspot eluates. *Mem Inst Oswaldo Cruz 87:* 205–208.
15. Sulzer AJ, Wilson M, 1967. The use of thick-smear antigen slides in the malaria indirect fluorescent antibody test. *J Parasitol 53:* 1110–1111.
16. Snow RW, Gilles HM, 2002. The epidemiology of malaria. Warrell DA, Gilles HM, eds. *Bruce Chwatt's Essential Malariology*. Fourth edition. London: Edward Arnold, 85–106.
17. Giha HA, Rosthoj S, Dodoo D, Hviid L, Satti GMH, Scheike T, Arnot DE, Theander TG, 2000. The epidemiology of febrile malaria episodes in an area of unstable and seasonal transmission. *Trans R Soc Trop Med Hyg 94:* 645–651.
18. Ekvall H, 2003. Malaria and anemia. *Curr Opin Hematol 10:* 108–114.
19. Erhart A, Thang ND, Hung NQ, Toi LV, Hung LX, Tuy TQ, Cong LD, Speybroeck N, Coosemans M, D'Alessandro U, 2005. Forest malaria in Vietnam: a challenge for control. *Am J Trop Med Hyg 70:* 110–118.
20. Anderson TJC, Haubold B, Williams JT, Estrada-Franco JG, Richardson L, Mollinedo R, Bockarie M, Mokili J, Mharakurwa S, French N, Whitworth J, Velez ID, Brockman A, Nosten F, Ferreira MU, Day KP, 2000. Microsatellite markers reveal a spectrum of population structures in the malaria parasite *Plasmodium falciparum*. *Mol Biol Evol 17:* 1467–1482.
21. Albrecht L, Merino EF, Hoffmann EHE, Ferreira MU, de Mattos Ferreira RG, Osakabe AL, Dalla Martha RC, Ramharter M, Durham AM, Ferreira JE, del Portillo HA, Wunderlich G, 2006. Extense variant gene family repertoire overlap in Western Amazon *Plasmodium falciparum* isolates. *Mol Biochem Parasitol 150:* 157–165.
22. Bull PC, Marsh K, 2002. The role of antibodies to *Plasmodium falciparum*-infected-erythrocyte surface antigens in naturally acquired immunity to malaria. *Trends Microbiol 10:* 55–58.
23. Macauley C, 2005. Aggressive active case detection: a malaria control strategy based on the Brazilian model. *Soc Sci Med 60:* 563–573.
24. Cavasini MTV, Ribeiro WL, Kawamoto F, Ferreira MU, 2000. How prevalent is *Plasmodium malariae* in Rondônia, Western Brazilian Amazon? *Rev Soc Bras Med Trop 33:* 489–492.
25. Scopel KKG, Fontes CJF, Nunes AC, Horta MF, Braga EM, 2004. High prevalence of *Plamodium malariae* infections in a Brazilian Amazon endemic area (Apiacás–Mato Grosso State) as detected by polymerase chain reaction. *Acta Trop 90:* 61–64.