Evaluation of the Naturally Acquired Antibody Immune Response to the Pv200L N-terminal Fragment of *Plasmodium vivax* Merozoite Surface Protein-1 in Four Areas of the Amazon Region of Brazil

Luciane M. Storti-Melo, Wanessa C. Souza-Neiras, Gustavo C. Cassiano, Leonardo C. Taveira, Antônio J. Cordeiro, Vanja S. C. A. Couto, Marinete M. Póvoa, Maristela G. Cunha, Diana M. Echeverry, Andréa R. B. Rossit, Myriam Arévalo-Herrera, Sócrates Herrera, and Ricardo L. D. Machado*

University of São Paulo State Júlio Mesquita Filho, São José do Rio Preto, São Paulo State, Brazil; Faculty of Medicine of São José do Rio Preto, São Paulo State, Brazil; SEAMA Faculty, Macapá, Amapá State, Brazil; Evandro Chagas Institute, Belém, Pará State, Brazil; Federal University of Pará, Institute of Biologic Science, Belém, Pará State, Brazil; Instituto de Immunología, Universidad del Valle, Cali, Colombia; Malaria Vaccine and Drug Development Center, Cali, Colombia; Faculty of Medicine Foundation of São José do Rio Preto, São Paulo State, Brazil; Depto de Microbiologia e Parasitologia - Instituto Biomédico, Universidade Federal Fluminense, São Domingos, Rio de Janeiro, Brazil

Abstract. Frequency and levels of IgG antibodies to an N-terminal fragment of the *Plasmodium vivax* MSP-1 (Pv200L) protein, in individuals naturally exposed to malaria in four endemic areas of Brazil, were evaluated by enzyme-linked immunosorbent assay. Plasma samples of 261 *P. vivax*-infected individuals from communities of Macapá, Novo Repartimento, Porto Velho, and Plácido de Castro in the Amazonian region with different malaria transmission intensities. A high mean number of studied individuals (89.3%) presented with antibodies to the Pv200L that correlated with the number of previous malaria infections; there were significant differences in the frequency of the responders (71.9–98.7) and in the antibody levels (1:200–1:51,200) among the four study areas. Results of this study provide evidence that Pv200L is a naturally immunogenic fragment of the PvMSP-1 and is associated with the degree of exposure to parasites. The fine specificity of antibodies to Pv200L is currently being assessed.

INTRODUCTION

Plasmodium vivax is the main species responsible for malaria infection in the American continent and in vast areas of the Middle East, East and Southeast Asia, Oceania, and the Eastern Mediterranean region.^{1,2} In America, Brazil and Colombia are the most malaria-endemic countries with *P. vivax* accounting for more than 70% of the malaria clinical cases in the region with a high percentage of cases originating in the Amazon region.^{3,4} Vaccines against malaria are considered a potentially cost-effective measure for malaria control and elimination, therefore, significant effort is currently being invested in vaccine development.⁵ In addition to the recent progress toward the development of a vaccine against *P. falciparum*, further development of a functional vaccine for most endemic areas of the world where both *P. falciparum* and *P. vivax* co-exist requires a better understanding of the protective immune responses induced by both malaria parasites. Individuals permanently exposed to malaria infection in endemic areas eventually develop clinical immunity; analyses of such immune responses may contribute to the identification of target antigens with vaccine potential.^{6–9}

The merozoite surface protein 1 (MSP-1), a glycoprotein of ~195 kDa expressed in all *Plasmodium* species studied, is considered to have great vaccine potential.¹⁰ It has been shown that in *P. falciparum*, MSP-1 is synthesized as a large precursor polypeptide that is then proteolytically processed into smaller fragments during late schizont development.¹¹ Its molecular weight varies slightly among parasite isolates caused by antigen size polymorphism,^{12,13} however the processed MSP-1 polypeptides contain remarkably conserved sequences¹⁴; one such

sequence is localized in proximity of the amino terminus of the protein within a 80 kDa processing fragment that includes a region termed 190L (Pf190L).^{15,16} The latter peptide region has an elevated number of B- and T-cell epitopes¹⁷ and has shown to be highly immunogenic and partially protective in monkey and human trials.^{18,19} *Plasmodium vivax* MSP-1 has a region with significant sequence homology to the Pf190L fragment that has been termed Pv200L.²⁰ Although most studies in the PvMSP-1 have concentrated on the carboxyl region, a few have been designed to analyze the antigenicity of the amino terminus of the protein that appears to be associated with clinical protection.⁷ A longitudinal study conducted in individuals from Rondonia State, Brazil indicated that more than 90% of the sera from patients having experienced more than three previous malaria infections contained antibodies to the ICB2-5 fragment, located on the amino terminus of the PvMSP-1 protein, which comprises the Pv200L fragment.²¹ Additionally, the Pv200L fragment was first tested in a seroepidemiological study conducted in Colombia and indicated that the majority of individuals previously exposed to *P. vivax* malaria infection had antibodies to rPv200L.²⁰ Moreover, immunizations of BALB/c mice and *Aotus* monkeys with the Pv200L fragment indicated great immunogenicity and partial protection against experimental challenge with *P. vivax* blood stages.²⁰

Herein, we have evaluated the frequency and levels of anti-Pv200L antibodies in naturally acquired *P. vivax* infections in four malaria-endemic areas of the Brazilian Amazon region with different *P. vivax* transmission intensities. These findings have allowed us to better define the immunological relevance of this protein fragment and its potential as a vaccine target.

MATERIAL AND METHODS

Study areas. In the Brazilian Amazon region malaria predominates in Mesoendemic conditions with wide variations in transmission, as can be observed by the non-immune or semi-immune status of the adult population and by presence

*Address correspondence to Ricardo L. D. Machado, Centro de Investigação de Microrganismos, Faculdade de Medicina de São José do Rio Preto, Avenida Brigadeiro Faria Lima 5416, Vila São Pedro, Zipcode: 15090-000, São José do Rio Preto, São Paulo Brazil. E-mail: ricardomachado@famerp.br

of the asymptomatic carriers.^{22,23} Because of this, the samples were collected in four different sites of the Amazon region of Brazil: Macapá, (Amapá State); Novo Repartimento (Pará State); Porto Velho (Rondônia State); and Plácido de Castro (Acre State). Macapá is the capital of the state of Amapá and is located on the Amazon River with a tropical forest. Its estimated population is 366,486 inhabitants and its annual parasitic index (API) was 6.0 in 2009. Porto Velho is the capital of the State of Rondônia in the upper Amazon River basin, with 383,425 inhabitants and an API of 53.7 last year. Plácido de Castro is located at the border of Rondônia and Amazonas states, it has a population of 18,235 inhabitants and its API was 20.6 in 2009. Novo Repartimento is a gold mining area in southeastern Pará State. Its population is estimated as 55,759 habitants and had an API of 15.4 last year. The climate in these areas is characterized as tropical with no dry season; the mean monthly precipitation level is at least 60 mm.

Study subjects and blood samples. A total of 261 adult individuals were evaluated in this study following a protocol approved by the Institutional Review Board (IRB) of the Faculty of Medicine, São José do Rio Preto. All participants were enrolled according to the following criteria: sought medical assistance for clinical malaria symptoms, were > 18 years of age, and had a positive *P. vivax* malaria diagnosis by thick blood film. We excluded from the study pregnant women, patients < 18 years of age, and no other concomitant illness. Participants were asked to sign a written consent form before blood samples were drawn. Epidemiological data such as age, gender, past history of malaria, and current infection information were obtained from a specific interview and also from medical records. Five mL of peripheral blood was collected by venipuncture from each of the individuals using tubes containing EDTA. Approximately 3 mL of plasma was then stored at -80°C until use, and the remaining cell volume was used for malaria diagnose confirmation by using a polymerase chain reaction (PCR) technique.

Parasite detection. Patients were examined to confirm the *P. vivax* malaria diagnose by using Giemsa stained thick blood smear. Slides were kept at room temperature and parasite density was quantified after examination of 200 microscopic fields at 1,000× magnification under oil-immersion. All slides were examined by two independent well-trained microscopists who were unaware of each result according to the Ministry of Health in Brazil recommended procedures.²⁴ Additionally, diagnose was confirmed in all samples by using a PCR technique.²⁵ For the PCR technique DNA samples were extracted from frozen pellets of infected erythrocytes using the Easy-DNATM extraction kit (Invitrogen, Carlsbad, CA), and a semi-nested PCR using specific small-subunit (SSU) ribosomal RNA (rRNA) primers.²⁵ Briefly, two rounds of amplification were performed with oligonucleotide primers that target small sub-unit rRNA genes of *Plasmodium*. The first round (35 cycles) used a pair of universal (i.e., genus-specific) primers, P1 and P2, whereas the second round (18 cycles) used the species-specific reverse primers F2, V1 and M1 combined with the universal forward primer P1. Oligonucleotide primer sequences and detailed PCR protocols are given elsewhere.

Pv200L protein. The Pv200L protein was expressed as a recombinant protein in *Escherichia coli* as described previously.²⁰ The protein encodes the fragment containing amino acid residues 121–416 of the *P. vivax* MSP-1. The fragment was amplified, cloned, and expressed

in *E. coli* according to standard methods,²⁰ and purified by immobilized metal ion affinity chromatography (IMAC) with Ni-nitrilotriacetic acid matrix (Qiagen, Brazil), dialyzed against phosphate-buffered saline (PBS) at 4°C, and reprocessed several times to reduce *E. coli* contaminants. Final batches of rPv200L were analyzed for homogeneity and purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analytical reverse-phase high-performance liquid chromatography (HPLC).

Assessment of the antibody response. Human plasma samples were evaluated using an enzyme-linked immunosorbent assay (ELISA) for the presence of naturally acquired antibodies to rPv200L, as described elsewhere.²⁰ Briefly, flat-bottomed, 96-well microplates (Nunc Maxisorp, Rochester, NY) were coated with 100 mM of rPv200L per well and incubated overnight at 4°C. Microplates were blocked for 2 hours with PBS containing 0.05% Tween 20 and 1% bovine serum albumin (Sigma, St. Louis, MO). After one wash with T-PBS, samples diluted 1:200 were added and incubated for 1 hour at room temperature. Plates were washed five times with PBS containing 0.05% Tween 20, and secondary antihuman immunoglobulin G (IgG) antibody labeled with alkaline phosphatase (Sigma) was added at a final dilution of 1:4000. Plates were incubated for 1 hour at room temperature, washed with T-PBS, and developed with *p*-nitrophenylphosphate (Sigma). The reaction was stopped after 15 minutes with 10 uL of 1N NaOH and read at 405 nm. Every sample was tested in triplicate. The ELISA IgG cutoff was defined as the average of negative control samples plus three standard deviations. The results were expressed as an index of reactivity ($IR = OD_{405}$ values of tested sample divided by the value of the cutoff). Values of $IR < 1.0$ were considered negative, values of $IR \geq 1.0$ and < 10 were considered positive (antibody titers estimated to vary from 1:200 until 1:3,200), whereas values of $IR \geq 10$ were considered highly positive (antibody titers estimated to vary between 1:3,200–51,200).

Statistical analysis. Analyses were performed using R version 2.8.1 statistical software (The R Foundation for Statistical Computing, Vienna, Austria [http://www.rproject.org]). Differences among the frequencies of responders were analyzed using Pearson's χ^2 or, alternatively, the Fisher's exact test. Comparisons of antibody level (IR) were performed using the Kruskal-Wallis test with *post hoc* Bonferroni's pairwise comparisons. Relationship between the antibody responders and previous malaria episodes was analyzed by binary logistic regression. Differences were considered significant when P value ≤ 0.05 , and when Bonferroni's correction was performed P value ≤ 0.017 or P value ≤ 0.008 , respectively, to the number of compared groups (3 or 4).

RESULTS

Frequency and level of antibodies to Pv200L in the four Amazonian communities. An average of 65 individuals per study area was evaluated for a total of 261 subjects. The overall frequency of antibodies that recognized the Pv200L in ELISA was 89.3%. The average IR was 5.5 and 17.6% of samples showed high antibody titers ($IR \geq 10$). Significant differences ($P = 0.000001$, Fisher's exact test) in responder frequency among the four study sites were observed (Table 1).

In Macapá, the lowest percentage of positive Pv200L antibodies in individuals was detected (71.9%), whereas Novo

TABLE 1

Frequency and antibody levels of *Pv200L* in sera of infected individuals with *Plasmodium vivax* in four areas from Brazilian Amazon region

Area	Samples (n)	Antibody frequency (%) [*]	Index of reactivity (IR) – antibody level		
			Average	> 1 (%)	> 10 (%)
Macapá	64	71.9	4.4	59.4	12.5
Novo Repartimento	76	98.7 [†]	6	80.3	18.4
Porto Velho	55	96.4 [‡]	5.7	78.2	18.2
Plácido de Castro	66	89.4	6.2	68.2	21.2
Total	261	89.3	5.5	71.7	17.6

^{*} Fisher's exact test ($P = 0.000001$) analyzing the four areas.

[†] Macapá vs. Novo Repartimento ($P = 0.000002$).

[‡] Macapá vs. Porto Velho ($P = 0.0004$), Bonferroni's correction P value ≤ 0.008 .

Repartimento had the highest frequency of responders (98.7%). In the *post hoc* analysis by Bonferroni's pairwise comparisons (significant P value ≤ 0.008), Macapá frequencies were statistically lower compared with Novo Repartimento ($P = 0.000002$) and Porto Velho ($P = 0.0004$).

Taken together, the four study areas were statistically different ($P = 0.0008$, Kruskal-Wallis test) in terms of antibody levels (estimated using IR values). The lowest antibody titers were detected in Macapá subjects (average of IR = 4.4) and the lowest frequency of high responders ($IR \geq 10$; 12.5%). On the other hand, the highest average IR (6.2) was found in Plácido de Castro with a frequency of 21.2% of high responders (Table 1). Figure 1 indicates each individual IR value plotted by endemic area. Individuals from Macapá were the only ones presenting bottom line antibody concentrations. The IR values were used to construct a boxplot by area that can also be seen in Figure 1, Macapá presented lower median and confidence interval values than others. Statistical pairwise comparisons (Bonferroni's correction, P value ≤ 0.008) confirmed that the lowest antibody responses occurred in Macapá; Macapá versus Porto Velho, $P = 0.0011$; Macapá versus Plácido de Castro, $P = 0.0039$; Macapá versus Novo Repartimento, $P = 0.0000$.

Relationship between frequency and level of antibodies with previous malaria episodes. Findings showed a correlation between the frequency and antibody levels and number of previous malaria episodes. For this, plasma samples from 200 individuals were separated and categorized according to the number of previous malaria episodes and then further divided into three groups: (0) primary infected, (1 or 2) individuals

FIGURE 1. Antibody levels to *Pv200L* in individuals from four areas of the Brazilian Amazon region. Individual value plot of index of reactivity (IR) and boxplot of the median values of IR in each area with confidence intervals: CI (Macapá, median = 1.5 and CI = 1.1–2.4; Porto Velho, median = 3.8 and CI = 2.6–5.3; Plácido de Castro, median = 2.7 and CI = 2.1–4.7; and Novo Repartimento, median = 4.1 and CI = 3.4–5.5). The * represents outliers.

FIGURE 2. Correlation between the frequency of samples for IR values and the number of previous malaria episodes. Individuals ($N = 200$) were grouped according to the number of previous malaria infections. The number of individuals of each group was 10, 82, and 108 for zero, 1 or 2, and 3 or more previous malaria groups, respectively. The index of reactivity (IR) values are: $IR < 1$ (negative), $IR > 1$ (positive), and $IR > 10$ (high positive). The frequency of sample for each IR value was statistically different between the three groups ($P = 0.006$ Pearson χ^2 test).

with one or two previous malaria episodes, and (3 or more) individuals with at least three previous malaria episodes. We found that the frequency of *Pv200L* antibody-positive samples was statistically different among these three groups (Figure 2; $P = 0.006$, Pearson χ^2). The percentage of individuals that did not recognize *Pv200L* ($IR < 1$ or negative) was significantly higher in the (0) group (40%) as compared with Group 1 (1 or 2 episodes; 12.2%) and to Group 3 (3 or more; 7.4%) ($P = 0.042$ and $P = 0.010$, respectively, Pearson χ^2). None of the individuals of the primary-infection group presented high antibody titer ($IR > 10$) (Figure 2). This tendency was also confirmed by binary logistic regression analysis. The percentage of responders in the primary-infected group was compared with the other groups. The odds ratio (OR) for groups with 1 or 2 and 3 or more previous malaria infections was OR = 4.8 ($P = 0.031$) and OR = 8.3 ($P = 0.004$), respectively.

Correlation between *Pv200L* antibody levels and the number of previous malaria infections showed that all primary infected (0) had lower IR values (Figure 3) with average of IR ($IR = 2.0$) lower than average of IR of all patients (other three

FIGURE 3. Correlation between antibody levels to *Pv200L* and number of previous malaria episodes. Individual value plot of index of reactivity (IR) for primary infected (0), with 1 or 2, and 3 or more previous malaria groups and boxplot of the median of IR for number of previous malaria groups and the confidence intervals: CI (0, median = 1.46 and CI = 0.3–4.7; 1 or 2, median = 3.8 and CI = 2.7–4.5; and 3 or more, median = 3.1 and CI = 2.5–5.1). The * represents outliers.

groups; IR = 5.4). However, this difference was significant only between primary infected (0) and the three or more malaria episode groups ($P = 0.010$, Kruskal-Wallis test, Bonferroni's correction P value ≤ 0.017).

DISCUSSION

In this study, we confirmed the high prevalence of individuals in malaria-endemic areas of Brazil who develop antibodies specific to the Pv200L fragment of the *P. vivax* MSP-1 upon exposure to natural infection. The mean number of individuals presenting antibodies to the Pv200L in this study was higher (89.3%) than that previously reported in Colombia (72.8%) where transmission intensity is lower than the Amazon region. There were significant differences in the frequency of responders and in antibody levels among the four study sites. Although we could not reliably record the number of previous episodes in individuals with multiple previous infections, the surprisingly high difference in antibody titers, which ranged from negative (IR < 1 = 10.7%) to highly positive (IR > 10 = 17.7%), indicates that some of the individuals may have been exposed to numerous *P. vivax* infections, certainly more than in Colombian individuals who presented significantly lower titers (10^3 – 5×10^5)^{20,26}. In the latter study, individuals were not only exposed to lower transmission intensity but a proportion was Fy- volunteers with only a transient exposure to limited amounts of circulating merozoites.²⁶

Several studies in Brazil^{7,9,27,28} and other countries^{29–32} have demonstrated the high antigenicity of different regions of the *P. vivax* MSP-1. The previously described antibody specificity, particularly to the C-terminal region (42-kD and 19-kD sub-fragments), correlates with inhibition of blocking parasite invasion *in vitro*^{33,34} and induction of protective immunity in animal models.^{19,35} We have focused considerable efforts on the genetic and immunological characterization of the N-terminal (Pv200L) fragment of *P. vivax* MSP-1²⁰ and have found that it has not only significant antigenicity both in human and animals, but it displays high immunogenicity in mice and monkeys and induces partial protection against experimental parasite challenge in *Aotus* monkeys vaccinated with the same protein tested here.

These results are in agreement with those of other seroepidemiologic studies that used longer fragments from N-terminal of PvMSP-1.^{7,21,36} Although the C-terminal region of MSP-1 was referred to in Brazil as the most immunogenic,^{9,28} high antibody levels (IR > 10) and IR average corroborates Pv200L high antigenicity in naturally acquired malaria. Moreover, reduced risk of *P. vivax* infection and clinical protection against malaria were, in fact, associated with antibodies to a specific N-terminal fragment from MSP-1.⁷ We must point out that maybe the same protection may not occur with other antigens from the N-terminal region, however, it is an extrapolation that should be considered. Inhibitory antibodies specific for the 19-kD C-terminal of *P. falciparum* MSP-1 were not correlated with delayed appearance of infection but, actually, declined significantly in 2 months after treatment, which might have contributed to the risk of reinfection.^{27,37} Herein, all individuals were infected at the time of sampling.

In Brazil, the antibody response profile was variable in the different areas, whereas Macapá showed the lowest frequency and antibody levels among studied areas; Novo Repartimento displayed the highest frequency of responders (98.7%). Several reasons, not mutually exclusive, may account for the

discrepant differences observed. Macapá had the lowest API (6.0 in 2009) that can justify the low frequency and the low level of antibody that was found. On the other hand, Porto Velho presented the highest API (53.7) followed by Plácido de Castro (20.6) and Novo Repartimento (15.8). Interestingly, Novo Repartimento but not Porto Velho showed the highest frequency of responders. Another possible explanation is the characteristics of *P. vivax* and *P. falciparum* transmission in each site. Although individuals with patent *P. falciparum* infection do not recognize the N-terminal recombinant protein of *P. vivax* MSP-1,³⁶ a combination of previous *P. vivax* and *P. falciparum* infections may have an unpredictable outcome in terms of the human antibody response.⁹

For that reason, we evaluated whether there was a correlation between the frequency and antibody levels and the number of past malaria infections. To answer this question, the samples were classified according to the number of previous malaria in three groups: (0), (1 or 2), and (3 or more) past malaria episodes. As expected, the analysis showed that the frequency of responders and the antibody levels to Pv200L fragment was higher in those individuals with a greater number of previous malaria infections. Although two previous Brazilian studies did not show this tendency for the N-terminal of the *P. vivax* MSP-1,^{9,28} our data are in agreement with another study in which the proportion of responders to PvMSP-1 variants increased during subsequent infections.³⁸ Moreover, in studies that evaluated the C-terminal of the MSP-1³¹ and other parasite proteins, such as DBP-II^{39,40} and AMA-1^{31,41} a positive correlation was also observed between the increase in the antibody response and the number of past malaria episodes. These data taken together suggest that multiple infections can provide a boost for the production of the specific antibodies. As observed in the initial evaluation of Pv200L in Colombia, the higher antibody responses in infected patients as compared with healthy individuals with previous exposure to malaria may be explained by an immune boosting by parasites after natural infection and may provide advantages for boosting of antibody response induced by vaccination.²⁰

In summary, results of this study provide evidence that Pv200L is an immunogenic fragment of the N-terminal region of the *P. vivax* MSP-1 in naturally exposed individuals from the Brazilian Amazon. Observed differences among the study areas may be explained by the frequency of malaria exposure in each region and that it might also be associated with the wide variations found in the malaria transmission pattern, parasite polymorphisms, and the genetic background of human populations. Additional studies regarding the fine specificity of antibodies to the Pv200L fragment and factors that modulate immune responses against heterologous parasitic proteins, especially in other geographical areas, are warranted to improve the understanding of the immunology of malaria with an eye toward identification of viable malaria vaccine candidates.

Received January 21, 2010. Accepted for publication April 19, 2010.

Acknowledgments: We acknowledge all individuals enrolled in this study. We thank the following people for assistance in obtaining samples: Carlos Eugênio Cavasini, Aline Barroso, Maria Cristina Figueredo, and Mauro Tada; to Luiz Hildebrando Pereira da Silva for facilities at CEPEN; Augusto Valderrama and David Narum for their valuable contribution in production of the Pv200L recombinant protein; and to Claudia L. Garcia for technical support with the manuscript.

Financial support: Work reported in this manuscript was funded in part by Fundação de Amparo à Pesquisa do Estado de São Paulo

(FAPESP), São Paulo State, Brazil (02/09546-1) and in part by the Conselho Nacional para o Desenvolvimento Científico e Tecnológico (CNPq), Brasília, Brazil (302353/03-8). LSM is a PhD student from the Genetic Program of the University of São Paulo State Júlio Mesquita Filho and supported by scholarship from FAPESP. Work in Colombia was jointly sponsored by COLCIENCIAS (grant 1106-04-16489), the Ministry of Social Protection (grant 2304-04-19524) (contract no. 253-2005), and by the National Institute of Allergy and Infectious Diseases (NIAID Grant no. 49486/TMRC) and through an International Center of Excellence for Malaria Research NIAID/ICEMR grant no U 19AI089702.

Authors' addresses: Luciane M. Storti-Melo, Wanessa C. Souza-Neiras, Gustavo C. Cassiano, Leonardo C. Taveira, Antônio J. Cordeiro, Andréa R. B. Rossit, and Ricardo L. D. Machado, Centro de Investigação de Microrganismos, Faculdade de Medicina de São José do Rio Preto, Vila São Pedro, São José do Rio Preto, São Paulo, Brazil, E-mails: stortilu@yahoo.com.br, wanejan@yahoo.com.br, gcapatti@hotmail.com, Leonardocastro@yahoo.com.br, joseantoniocondeiro70@gmail.com, andrea-regina@vm.uff.br, and ricardomachado@famerp.br. Vanja S. C. A. Couto, Faculdade SEAMA, Macapá, Amapá, Brasil, E-mail: vanjacouto@seama.edu.br. Marinete M. Póvoa, Instituto Evandro Chagas, Ananindeua, Belém, Pará, Brasil, E-mail: marinetepovoaa@iec.pa.gov.br. Maristela G. Cunha, Universidade Federal do Pará, Instituto de Ciências Biológicas, Belém, Pará, Brasil, E-mail: mgcunha@ufpa.br. Diana M. Echeverry, Myriam Arévalo-Herrera, and Sócrates Herrera, Instituto de Immunología, Facultad de Salud, Universidad del Valle, Cali, Colombia and Centro Internacional de Vacunas, Cali, Colombia, E-mails: dimeche@gmail.com, marevalo@inmuno.org, and sherrera@inmuno.org.

Reprint requests: Ricardo L. D. Machado, Centro de Investigação de Microrganismos, Faculdade de Medicina de São José do Rio Preto, Avenida Brigadeiro Faria Lima 5416, Vila São Pedro, Zipcode: 15090-000, São José do Rio Preto, São Paulo Brazil, Tel: 55-17-32015736, Fax: 55-17-32015909, E-mail: ricardomachado@famerp.br.

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