

New evidences on the diagnostic value of indirect immunofluorescence test and delayed hypersensitivity skin test in human infection by *Leishmania (L.) infantum chagasi* in the Amazon, Brazil

Novas evidências sobre o valor diagnóstico da reação de imunofluorescência indireta e reação intradérmica de hipersensibilidade tardia na infecção humana por *Leishmania (L.) infantum chagasi* na Amazônia, Brasil

Nueva evidencia sobre el valor diagnóstico de la prueba de inmunofluorescencia indirecta y la reacción intradérmica de hipersensibilidad retardada en la infección humana por *Leishmania (L.) infantum chagasi* en la Amazonia brasileña

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ABSTRACT

This is a prospective study on a cohort of 1099 individuals of both genders, aged 1-84 years (mean 24.4 years), living in an endemic area of American visceral leishmaniasis (AVL) in the Municipality of Cametá, Brazil, from May 2006 to September 2008. It aimed to analyze the prevalence and incidence rates of human infection by *Leishmania (L.) infantum chagasi*, as well as the evolutionary process of its previously defined clinical and immunological profiles: 1. Asymptomatic infection (AI); 2. Symptomatic infection (SI = AVL); 3. Subclinical oligosymptomatic infection (SOI); 4. Subclinical resistant infection (SRI); and 5. Indeterminate initial infection (III). The diagnosis was based on the simultaneous use of indirect immunofluorescence assay (IFA) and delayed hypersensitivity skin test. A total of 304 cases of infection were diagnosed during the period studied (187 for prevalence and 117 for incidence), generating an accumulated prevalence rate of 27.6%. The distribution regarding their clinical and immunological profiles presented the following order: AI 51.6%; III 22.4%; SRI 20.1%; SOI 4.3%; and SI (= AVL) 1.6%. Based on the dynamics of the infection, the main discovery was about the III profile, which had an instrumental role in its evolution, directing it either to the resistant immunological pole – SRI (21 cases - 30.8%) and AI (30 cases - 44.1%) profiles – or to the susceptible immunological pole – SI (1 case - 1.5%) profile. In addition, 16 cases remained within the III profile until the end of the study. It was concluded that this diagnostic approach can help monitor the infection in endemic areas, aiming mainly at preventing morbidity caused by AVL, and reducing the treatment time and expenses.

Keywords: *Leishmania (L.) infantum chagasi*; Infection; Immunologic Tests; Hypersensitivity, Delayed; Fluorescent Antibody Technique, Indirect.

INTRODUCTION

Currently one of the most important aspects related to the interaction between *Leishmania (L.) infantum chagasi* Shaw 2002 (= *Leishmania chagasi* Cunha and Chagas 1937), the etiological agent of American visceral leishmaniasis (AVL), and the immune response in humans is the clinical and immunological spectrum that may result from this interaction. It seems clear that a good understanding of this spectrum, especially with respect to the repertoire of human immune system responses against infection with this agent, may be of crucial importance in treating clinical cases. Until recently, the clinical spectrum of infection was thought to vary between asymptomatic infection, found in resistant individuals with a strong cellular immune response (consisting of delayed hypersensitivity, lymphocyte proliferation, and the production of gamma-interferon), and a symptomatic form found in susceptible individuals in whom suppression of this cellular immune response may lead to classical AVL^{15,33}. However, in addition to these extreme forms of infection, individuals may present with an intermediate, "borderline" form known as subclinical oligosymptomatic infection, whose clinical and immunological characteristics are still not entirely clear^{27,10}.

There are some studies in Brazil that have attempted to characterize the clinical and immunological spectrum of human infection with *L. (L.) i. chagasi*. However, these studies have been based mainly on i) the IgG antibody response (humoral response = susceptibility) or ii) the delayed hypersensitivity skin response (cellular immune response = resistance) of infected individuals. This has made it more difficult to gain a broader understanding of the human immune response against infection^{7,14,19}. In other words, these studies have generally used either serological methods such as the immunoenzymatic ELISA assay or cellular immunity ones such as the delayed-type hypersensitivity skin test in order to obtain a diagnosis of

active *L. (L.) i. chagasi* infection. However, it is known that these types of diagnostic methods underestimate the possibility that certain individuals who reside in an endemic area could simultaneously exhibit both types of immune responses (humoral and cellular) against *L. (L.) i. chagasi* infection. Hence, these types of approaches do not provide a realistic view of infections in an endemic area.

More recently, however, it has been demonstrated that simultaneous use of an indirect immunofluorescence test (IFT) and a delayed hypersensitivity skin reaction (=Montenegro skin test – MST) can provide an immunodiagnosis of symptomatic and asymptomatic human infection with *L. (L.) i. chagasi* in an endemic area. The high specificity of this diagnostic approach, based on the use of species-specific antigens from amastigote (for IFT) and promastigote (for MST) forms of *L. (L.) i. chagasi*, has permitted the identification of a broader clinical and immunological spectrum of human infection with *L. (L.) i. chagasi* in the Brazilian Amazon; the following clinical-immunological profiles of infection were thus defined: Asymptomatic Infection (AI), Symptomatic Infection (SI) (=AVL), Subclinical Oligosymptomatic Infection (SOI), Subclinical Resistant Infection (SRI) and Indeterminate Initial Infection (III)^{11,29}. Although, as previously mentioned, the first three profiles (AI, SI and SOI) have already been presented in the literature, the last two (SRI and III) represent new profiles of the clinical-immunological spectrum of infection.

Based on the above-mentioned observations, it is important to present new evidence on the diagnostic value of the combined use of IFT and MST for human infection with *L. (L.) i. chagasi*, as obtained from a prospective study conducted in an AVL-endemic area in the Municipality of Cametá, Pará State, Brazil. This study has reinforced previous findings about this diagnostic approach for human infection with *L. (L.) i. chagasi*, used primarily for the early diagnosis of infection; it consisted of recognizing

recently infected individuals with the potential to develop an active form of the infection, the AVL. This study discusses the relevance of a diagnostic tool for recently infected cases in an endemic area – those represented by the clinical-immunological profile III – to prevent the morbidity of severe AVL and decrease the time and costs of treatment.

MATERIALS AND METHODS

STUDY AREA

This study was conducted in four small towns (Ajó, Vacaria, Vacajó and Enseada) located in the Municipality of Cametá (01° 56' S: 54° 45' W), which is situated on the banks of the Tocantins River in the northeast region of Pará State, Brazil. The climate is typically equatorial, with an average temperature of 28° C and high humidity. The rainy season in this region, from January to June, has a rainfall of around 2,500 mm or more. The primary forest has almost totally been destroyed, and there are some plantations left in the midst of secondary forests. Approximately 70% of the inhabitants reside in wooden houses constructed on dry land, whereas the other part of the population live in varzea areas (floodplains) covered mainly by low vegetation. The land is flooded twice a day by the Tocantins River. Thus, the climate and the environmental conditions in this area are very similar to those described in another study conducted in the Municipality of Barcarena, Pará State, located approximately 150 km from this area, where the dynamics of human *L. (L.) i. chagasi* transmission was previously studied²⁸.

POPULATION AND STUDY DESIGN

The population analyzed by this study consisted of a cohort of 1,099 individuals (92.2% of the total population, 596 males and 503 females) between the ages of 1 and 84 (average 24.4 years) - a relatively young population. When the study started, there were a total of 1,192 inhabitants in the area¹⁶.

Considering that this study was designed to analyze the prevalence and incidence of human infection with *L. (L.) i. chagasi*, as well as the evolutionary dynamics of its clinical-immunological profiles (AI, SI=AVL, SOI, SRI, and III), it was necessary to plan a prospective study to follow up the cohort (1,099 individuals) over a two-year period (May 2006-September 2008). Thus, IFT and MST were used simultaneously to determine both the prevalence and the incidence. In other words, all individuals were previously selected for prevalence investigation, and the incidence was investigated after 12 and 24 months. Thereafter, these tests were only conducted on individuals who were found to be negative in the prevalence study and in the first incidence study (at 12 months). Therefore, individuals presenting reactivity only to MST, which represents a genetic characteristic of immunological resistance against the infection¹⁸, were removed from the subsequent assessments with this technique similarly to what was done in a longitudinal study conducted in Sudan³⁴. Individuals presenting reactivity in both tests were tested again only for IFT, since it is unnecessary to inject new antigen loads into them, as would be the case with MST. In cases of reactivity

only with IFT, which, in contrast to MST, represents an immunological state of susceptibility to infection, the individuals remained in the cohort for both tests in order to analyze the evolution of humoral and cellular immune responses. Almost 5% (54 individuals) of our original cohort were excluded from our study during follow-up period due to several reasons, such as holidays or trips.

Finally, the population was stratified into three age groups, 1-10 (303 individuals), 11-20 (252), and 21 years of age (544 to analyze the distribution of infection according to age.

We designed the current study in a similar manner as our two previous studies; one has already been published²⁸ and the other was accepted for publication²⁹.

CLINICAL ASSESSMENT OF INFECTED INDIVIDUALS

All the individuals who presented with some type of immunological response for IFT and/or MSR were clinically examined (mainly physically) to identify some sign or symptom recognized as a classical clinical pattern of AVL or a subclinical oligosymptomatic infection. However, it is important to highlight that only the typical cases of AVL underwent conventional therapy with pentavalent antimony, as recommended by the Brazilian National Program to Control AVL²⁴. The cases that presented a diagnosis of subclinical oligosymptomatic infection were followed for a period of up to three months to confirm spontaneous clinical resolution, as observed in another study in Maranhão State, Brazil¹⁴.

CRITERIA FOR THE IDENTIFICATION OF HUMAN INFECTION

IFT-reactivity is indicative of a humoral response (susceptibility) and MST-reactivity is indicative of a cellular immune response (resistance)³, therefore a human case of *L. (L.) i. chagasi* infection was assumed as the presence of reactivity for one or both immunological tests. However, considering that co-infection with human immunodeficiency virus (HIV) can interfere with this diagnostic approach, we should note that there were no human cases of HIV co-infection within the community at the beginning of the study, as recorded by the Health Department of the Municipality of Cametá.

Considering the importance of establishing the specificity of both immunological tests (IFT and MST), we used a semi-quantitative scale scoring results from + to ++++ in the following manner: for IFT, serological titers (IgG) from 80-160 and from 320-640 received + and ++, and those from 1,280-2,560 and from 5,120-10,240 received +++ and ++++, respectively. For MST, weak skin reactions (5-8 mm) received +, moderate reactions (9-12 mm) ++, strong reactions (13-15 mm) +++, and exacerbated reactions (16 mm) ++++. Thus, we assumed that serological reactions from a titer designation of 80 (IgG) and skin reactions forming indurations 5 mm in diameter were considered positive cut-off points for IFT and MST, respectively^{23,31,28}. Therefore, when combining the clinical state of the infected individuals with the semi-quantitative scale of the IFT and MST results,

we could identify the following immunological profiles in the clinical infection groups: AI (MST+/++++ and IFT-), SI (=AVL) and SOI with the same immunological profile (MST- and IFT+++/++++), SRI (MST+/++++, and IFT+/++) and III (MST- and IFT+/++)^{11,29}.

IMMUNOLOGICAL TESTING PROCEDURES

The implementation of MST followed the same technical steps described in other studies for the diagnosis of American cutaneous leishmaniasis (ACL)^{31,28}. Notwithstanding, considering that this study area has similar epidemiological characteristics that suggest the possibility of ACL transmission, we used a highly specific antigen for AVL, generated from promastigote forms of a stationary phase culture (RPMI 1640 medium) of a regional strain of *L. (L.) i. chagasi* (MCAO/BR/2003/M22697/Barcarena, Pará State) isolated from a dog infected with visceral leishmaniasis in the Municipality of Barcarena. The promastigote forms of the parasite were fixed with a merthiolate solution (1/10,000), at a final concentration of approximately 10×10^6 parasites/mL. As a control antigen, we used an equal dose of 0.1 mL of merthiolate solution (1/10,000) injected intradermally into the opposite forearm of each individual. It should be noted that since the Instituto Evandro Chagas (IEC) is a laboratory connected to the Secretariat of Health Surveillance of Brazil's Ministry of Health (MS, Brazil), all reagents destined for human research were previously assessed by a quality control program before use in humans.

The implementation of IFT was based on a previous study²³, which demonstrated that antigens from the amastigote forms of *L. (L.) i. chagasi* have a greater sensitivity and specificity than antigens from the promastigote forms of the same parasite and from *Leishmania (L.) major*-like (Bio-Manguinhos/FIOCRUZ, Brazil), as well as those from the amastigote forms of *L. (L.) amazonensis*. Briefly, the antigens from the amastigote forms were fixed onto the surface of IFT slides by applying small fragments from the liver, spleen (*L. i. chagasi*) and skin (*L. amazonensis*) of a hamster (*Mesocricetus auratus*) infected with these parasites. When used for the laboratory

diagnosis of canine visceral leishmaniasis, this procedure was also shown to be more specific than commercial kits for IFT and ELISA from Bio-Manguinhos, Brazil²⁰.

These procedures for conducting the two immunological tests, MST and IFT, have been previously published^{11,28}.

STATISTICAL ANALYSIS

The results were analyzed using the program Bio-Stat 4.0⁴ and χ^2 , and binomial tests were used to determine the significance of the differences between the clinical-immunological profiles of the infection, with a confidence interval of 95%.

APPROVAL BY THE ETHICS COMMITTEE

This work was approved by the Ethics Committee in Human Research of the IEC, with the protocol number: CEP/IEC 16/2003.

RESULTS

DISTRIBUTION OF THE CLINICAL-IMMUNOLOGICAL PROFILES OF HUMAN INFECTION WITH *L. (L.) i. CHAGASI*: PREVALENCE

The prevalence rate of infection was 17% (187 cases/1,099 individuals). This accounts for 90 cases diagnosed only by MST (AI clinical-immunological profile), 54 diagnosed only by IFT (four cases with a SI=AVL profile, nine with a SOI profile, and 41 with a III profile), and 43 diagnosed by both tests (SRI profile). Thus, the distribution of these profiles showed a higher frequency ($P < 0.05$) for the AI profile (48.1%) than the others: SRI (23%), III (22%), SOI (4.8%), and SI=AVL (2.1%) (Table 1). The results also showed that the frequencies of the SRI and III profiles were higher ($P < 0.05$) than those of the SOI and SI profiles; however, no statistically significant difference was observed ($P > 0.05$) between the SRI and III profiles, and between the SOI and SI profiles. In addition, these results demonstrated that the vast majority of infected individuals (93%, 174) were asymptomatic (AI, SRI and III profiles).

Table 1 – Frequency of clinical-immunological profiles of human infection with *L. (L.) i. chagasi*: prevalence, incidence, and cumulative prevalence and final evolution in the Municipality of Cametá, Pará State, Brazil

Evaluations	Clinical-immunological profiles				
	AI	SI	SOI	SRI	III
Prevalence (n = 187 cases)	90 (48.1)	4 (2.1)	9 (4.8)	43 (23.0)	41 (22.0)
Incidence (12 months) (n = 64 cases)	28 (43.7)	–	4 (6.3)	15 (23.4)	17 (26.6)
Incidence (24 months) (n = 53 cases)	39 (73.6)	1 (1.8)	1 (1.8)	3 (5.7)	10 (18.9)
Final incidence (n = 117 cases)	67 (57.3)	1 (0.8)	1 (0.8)	18 (15.4)	27 (23.1)
Cumulative prevalence (n = 304 cases)	157 (51.6)	5 (1.6)	5 (1.6)	61 (20.1)	68 (22.4)
Final evolution (n = 304 cases)	238 (78.3)	1 (0.3)	1 (0.3)	46 (15.1)	16 (5.3)

AI: Asymptomatic Infection; SI: (=AVL) Symptomatic Infection; SOI: Subclinical Oligosymptomatic Infection; SRI: Subclinical Resistant Infection; and III: Indeterminate Initial Infection;
Conventional sign used: – Numerical data not equal to zero due to rounding.

DISTRIBUTION OF CLINICAL-IMMUNOLOGICAL PROFILES OF HUMAN INFECTION WITH *L. (L.) I. CHAGASI*: INCIDENCE

The first incidence rate, estimated 12 months after the beginning of the study, was 7.2% (64 new cases / 892 uninfected individuals in the initial prevalence study). This accounted for 28 new cases were diagnosed only by MST (AI profile), 21 only by IFT (four cases with a SOI profile and 17 with a III profile), and 15 by both tests (SRI profile). The distribution of clinical-immunological profiles showed that the AI profile once again had a higher frequency (43.7%) ($P < 0.05$) than the other profiles, III (26.6%), SRI (23.4%) and SOI (6.3%) (Table 1). We note, therefore, that there were no cases of AVL (=SI profile) among the new cases of infection (first year of the study). These results also demonstrated that the frequencies of the III and SRI profiles were higher ($P < 0.05$) than that of the SOI profile; no statistically significant difference was observed ($P > 0.05$) between the frequencies of the III and SRI profiles.

The second incidence measurement, calculated 24 months after the beginning of the study, was 6.6% (53 new cases / 802 uninfected individuals in the first incidence study). Overall, 39 new cases of infection were diagnosed only by MST (AI profile); 11 were diagnosed only by IFT (one case with a SI profile=AVL and ten cases with a III profile) and three were positive for both tests (SRI profile). The distribution of clinical-immunological profiles demonstrated, once again, that the AI profile (73.6%) was more frequent ($P < 0.05$) than the others: III (18.9%), SRI (5.7%), and SI (1.8%) (Table 1). This time, the SOI profile was not present among the new cases of infection (second year of the study). These results showed that the III profile (18.9%) was more frequent ($P < 0.05$) than the SRI (5.7%) and SI (1.8%) profiles, and, finally, that the SRI profile was more frequent ($P < 0.05$) than the SI profile.

As an explanation for the number of individuals who were assessed for incidence compared to the number expected, it is important to highlight that, based on the initial prevalence measurement, we expected to examine 912 uninfected individuals for the first incidence study. However, only 892 (97.8%) were examined, signifying a loss of 20 (2.2%) individuals. In the second incidence measurement, based on the outcomes of the first incidence study, we expected to examine 828 uninfected individuals. However, we examined only 802 (96.8%), with a loss of 26 (3.2%) individuals. Thus, during the follow-up of the cohort (1,099 individuals) over the two years of the study, there was a total loss of 46 (5.4%) individuals; we considered this to be a small, non-significant loss.

In summary, the two measurements of incidences recorded a total of 117 new cases during the two years of study; the AI profile had the highest frequency rate (57.3%), followed by the III (23.1%), SRI (15.4%), SOI (3.4%) and SI=AVL (0.8%) profiles (Table 1). Therefore, we observed that the vast majority of new cases of infection (95.7%) were also asymptomatic (AI, III and SRI profiles).

DISTRIBUTION OF CLINICAL-IMMUNOLOGICAL PROFILES OF HUMAN INFECTION WITH *L. (L.) I. CHAGASI*: CUMULATIVE PREVALENCE

After three assessments (one for prevalence and two for incidence), a total of 304 cases of human infection with *L. (L.) i. chagasi* were recorded, with an cumulative prevalence rate of 27.6%; the AI profile was the most prevalent (51.6%), followed by III (22.4%), SRI (20.1%), SOI (4.3%), and, finally, SI=AVL (1.6%) (Table 1).

DISTRIBUTION OF CLINICAL-IMMUNOLOGICAL PROFILES OF HUMAN INFECTION WITH *L. (L.) I. CHAGASI* BY AGE: PREVALENCE AND INCIDENCE

When considering the age distribution of clinical-immunological profiles of infection in the prevalence study (187 cases), we observed that, in the 1-10 age group (23%, 43 cases), there was no difference ($P > 0.05$) between the frequency rates of the AI (30.2%, 13), SRI (30.2%, 13) and III (25.6%, 11) profiles, which were higher ($P < 0.05$) than those of the SI (9.3%, 4) and SOI (4.7%, 2) profiles. In the 11-20 age group (23.5%, 44 cases), there was no difference ($P > 0.05$) between the frequencies of the AI (47.7%, 21) and III (34.1%, 15) profiles, which were higher ($P > 0.05$) than those of the SRI (13.6%, 6) and SOI (4.6%, 2) profiles. Finally, in the 21 age group (53.5%, 100 cases), we noted a higher frequency ($P < 0.05$) of the AI profile (56%, 56) than the other profiles: SRI (24%, 24), III (15%, 15), and SOI (5%, 5). On the other hand, when comparing the age distribution of cases of infection within the same profile, three findings stood out in the prevalence study: i) we noted an increasing frequency of AI-profile cases with age, with 13 cases (14.5%) in the 1-10 age group, 21 cases (23.3%) in the 11-20 age group, and 56 cases (62.2%) in the 21 age group, which suggests an increasing trend of the AI profile with age; ii) the four cases with a SI (=AVL) profile were diagnosed in individuals in the youngest age group, 1-10 years; and iii) among the cases with the III profile (44 cases), there was no difference ($P > 0.05$) in the frequency between age groups, with 11 cases (26.8%) in the 1-10 age group, 15 cases (36.6%) in the 11-20 age group, and 15 cases (36.6%) in the 21 age group (Table 2).

Table 2 – Distribution of clinical-immunological profiles of human infection with *L. (L.) i. chagasi* by age: prevalence and incidence in the Municipality of Cametá, Pará State, Brazil

Assessments n (%)		Clinical-immunological profiles				
		AI	SI	SOI	SRI	III
Prevalence (n = 187 cases)						
1 - 10*	43 (23.0)	90 (48.1)	4 (2.1)	9 (4.8)	43 (23.0)	41 (22.0)
11 - 20	44 (23.5)	28 (43.7)	–	4 (6.3)	15 (23.4)	17 (26.6)
21	100 (53.5)	39 (73.6)	1 (1.8)	1 (1.8)	3 (5.7)	10 (18.9)
Final incidence (n = 117 cases)						
1 - 10	51 (43.6)	67 (57.3)	1 (0.8)	1 (0.8)	18 (15.4)	27 (23.1)
11 - 20	44 (37.6)	157 (51.6)	5 (1.6)	5 (1.6)	61 (20.1)	68 (22.4)
21	22 (18.8)	238 (78.3)	1 (0.3)	1 (0.3)	46 (15.1)	16 (5.3)

AI: Asymptomatic Infection; SI: (=AVL) Symptomatic Infection; SOI: Subclinical Oligosymptomatic Infection; SRI: Subclinical Resistant Infection; and III: Indeterminate initial infection;

*Age groups (years);

Conventional sign used: – Numerical data not equal to zero due to rounding.

As for the two incidence measurements (117 cases), we note that the highest frequency of cases occurred in the two lowest age groups: i) in the 1-10 age group (43.6%, 51 cases), the AI profile was more frequent (58.8%, 30 cases) ($P < 0.05$) than the III (27.5%, 14 cases), SRI (5.9%, three cases), SOI (5.9%, three cases) and SI (1.9%, one case) profiles; ii) in the 11-20 age group (37.6%, 44 cases), the AI profile was more frequent (68.2%, 30 cases) ($P < 0.05$) than the SRI (18.2%, eight cases) and III (13.6%, six cases) profiles. The 21 age group (18.8%, 22 cases) showed not only the lowest infection frequency, but also equal rates ($P < 0.05$) for the AI (31.8%, seven cases), SRI (31.8%, seven cases) and III (31.8%, seven cases) profiles, with the lowest frequency for the SOI profile (4.6%, one case). On the other hand, when the infection frequency was compared for the same profiles, two findings stood out: i) for both the AI and III profiles, the highest frequency of cases, 89.5% (60 cases) and 74% (20 cases) respectively, occurred in the lowest age groups (1-10 and 11-20 years), which indicates a higher frequency of new infections in individuals under 21 years of age; and ii) the only disease case (SI=AVL profile) occurred, once again, in the lowest age group, 1-10 years (Table 2).

DYNAMICS OF THE CLINICAL AND IMMUNOLOGICAL PROGRESSION OF HUMAN INFECTION WITH *L. (L.) i. CHAGASI*

The results related to the clinical and immunological progression of the infection are shown in table 1 and figure 1 (final evolution of the infection). However, to clarify this process, we decided to evaluate the results obtained for the cumulative prevalence (total number of cases diagnosed in the prevalence and incidence studies) in order to eventually elucidate the final evolution of the cases of infection. Thus, starting with the recent cases of infection with the III profile (IFT+/++ and MST-), we observed that, of the 68 (22.4%) diagnosed cases (41 in the prevalence and 27 in the

incidence studies), 21 (30.9%) evolved to the SRI profile (converted to MST+/++++), 30 (44.1%) to the AI profile (converted to MST+/++++ and IFT-), 1 (1.5%) to the SI (=AVL) profile (amplified to IFT+/++++), and 16 (23.5%) retained the same profile until the end of the study. Next, of the 61 (20.1%) cases (43 in the prevalence and 18 in the incidence studies) displaying the SRI profile (IFT+/++ and MST+/++++), 47 (77%) evolved to the AI profile (converted to IFT-) and 14 (23%) maintained the same profile. As 21 cases of the III profile evolved to the SRI profile, and other 8 of the SOI profile and 3 of the SI profile also evolved to the SRI profile, the final evolution of the SRI profile was of 15.1% (46 cases). With respect to the SOI profile (IFT+/++++ and MST-), of the 13 (4.3%) diagnosed cases (nine in the prevalence and four in the incidence studies), eight (61.5%) evolved to the SRI profile (converted to MST+/++++ and IFT reaction decreased to +/+), two (15.4%) evolved to the AI profile (converted to MST+/++++ and IFT-), and 3 (23.1%) maintained the same profile. Of the five (1.6%) cases of AVL (IFT+/++++ e MST-), four were diagnosed in the prevalence study and one in the incidence studies; clinical treatment with pentavalent antimony resulted in three (60%) conversions to the SRI profile (converted to MST+/++++ and IFT reaction decreased to +/+), one (20%) to the AI profile (converted to MST+/++++ and IFT-), and one (20%) case maintained the initial immune response profile until the end of the study but was asymptomatic (clinically cured). Finally, regarding the 157 cases (51.6%) of the AI profile (MST+/++++ and IFT-) (90 diagnosed in the prevalence study and 67 in the incidence studies) they did not evolve to other profiles because they already represent the pool of individuals with genetic resistance to infection. However, 30 cases were added from the III profile, 47 from the SRI profile, two from the SOI profile, and one from the SI (=AVL) profile, yielding a final frequency of 78.3% (238 cases).

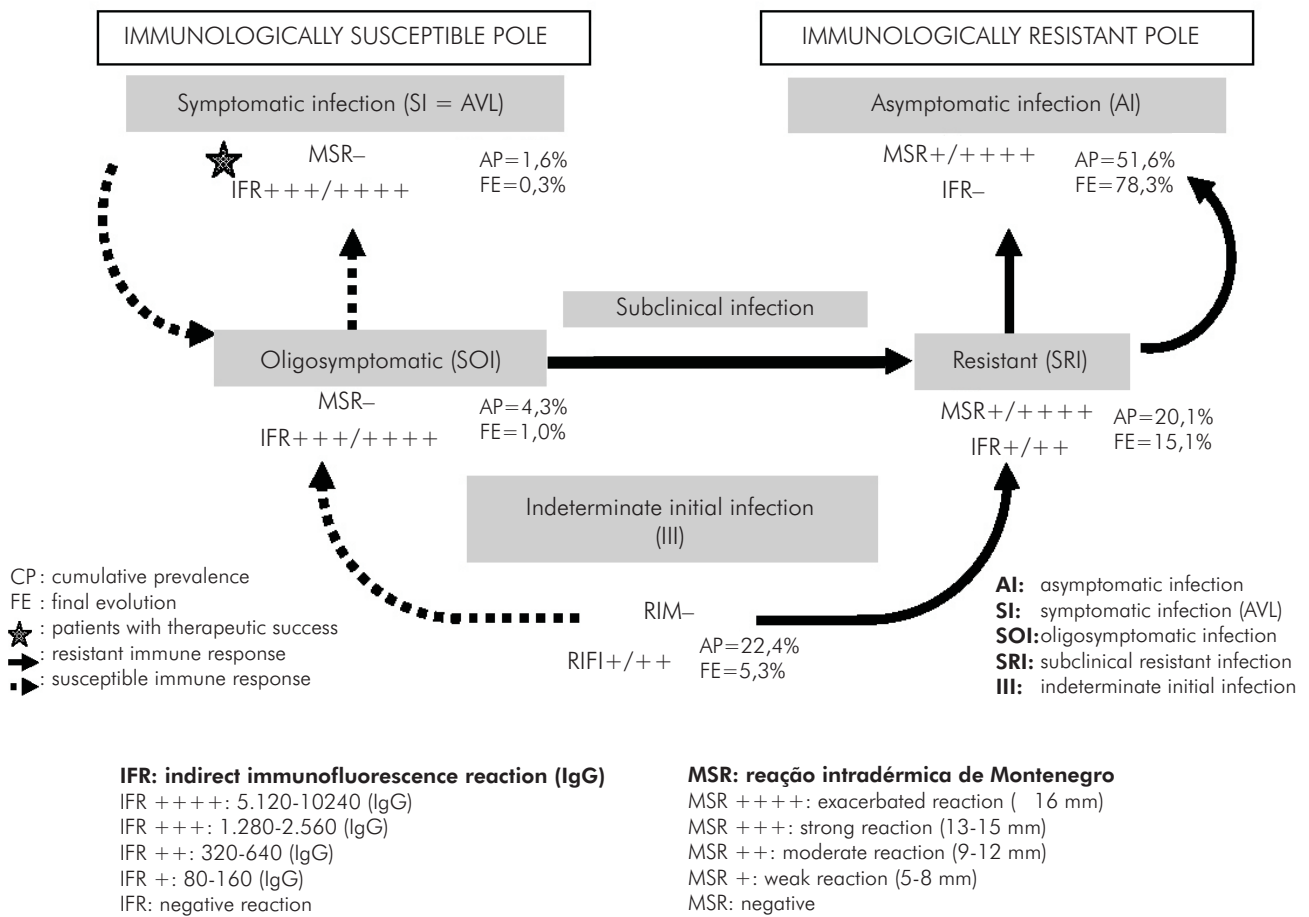


Figure 1 – Dynamics of the clinical and immunological evolution of human infection with *Leishmania (L.) infantum chagasi* in the Amazon Region, Brazil

In summary, the only profile that became more frequent over the progression of the clinical and immunological response was the AI profile (51.6% - 78.3%); the frequency of the other profiles decreased: SI (1.6% - 0.3%), SOI (4.3% - 1%), SRI (20.1% - 15.1%), and, with the most significant decrease, III (22.4% - 5.3%).

Finally, it is important to point out that, throughout the study period, no cases of cutaneous leishmaniasis were detected among MST- and IFT-reactive individuals. This confirmed the specificity of the MST and IFT immunological reactions in diagnosing cases of human infection with *L. (L.) i. chagasi*. Similarly, there were no known cases of co-infection with HIV among the individuals who took part in the study.

DISCUSSION

There is no doubt as to the importance of the AI profile in the clinical-immunological spectrum of human infection with *L. (L.) i. chagasi*. The results of this study demonstrate, once again, that the AI was the most frequent of all the profiles diagnosed, especially in the cumulative prevalence analysis (old and new cases of infection); it represented 51.6% of all diagnosed cases, followed by the III (22.4%), SRI (20.1%), SOI (4.3%), and SI (=AVL) (1.6%) profiles (Table 1). Thus, the high frequency of the AI profile may be interpreted as a short period of time that elapses

between the initial stage of infection (III profile) and its final stage (AI profile). This indicates that the majority of the III profile cases present with a short period of humoral response (IFT+), followed by rapid conversion of the delayed-type hypersensitivity response (SRI profile) and, lastly, a negative conversion of IFT-.

In a previous study²⁹, a similar situation was observed in an AVL-endemic area in the Municipality of Barcarena, Pará, around 150 km from the location of the present study; in that study, the AI profile corresponded to 73.2% of all cases of infection regarding their cumulative prevalence. Therefore, considering that delayed-type hypersensitivity represents a strong expression¹⁸ of cellular immune resistance^{33,10} against infection with *L. (L.) i. chagasi*, these results confirm that the vast majority (final distribution of the AI profile, 78.3%) of infected individuals in an endemic area are genetically resistant to infection. Also, considering that the SRI profile (final distribution, 15.1%) represents an evolutionary stage of infection with respect to the resistant pole (AI profile), the AI frequency could reach 90% in an endemic area.

Accordingly, if delayed hypersensitivity can be considered a definitive genetic characteristic of cellular immune resistance against infection, it is probable that the loss of this response (delayed hypersensitivity) may result in immunosuppression² or in low specificity of response to

Leishmania antigens used to stimulate delayed hypersensitivity¹³. In the Brazilian Amazon, however, a much greater specificity was observed for delayed hypersensitivity reactions (16 x 12 mm) using *L. (L.) i. chagasi* species-specific antigen from this study than the reactions (11 x 6 mm) produced by the *L. (L.) amazonensis* antigen, a causative agent of ACL, in two adult individuals (30 years of age) native to an AVL-endemic area (Municipality of Igarapé-Miri) in Pará State, Brazil. This not only suggests that cellular immunity has a long duration (immunological memory), but also that it results from a species-specific immune response³⁰.

As for the SRI profile, which represents a new stage of infection for this diagnostic approach, its performance was not very different from that observed in previous studies²⁹. Its frequency rate in the prevalence study (23%) was relatively higher than in the final incidence (15.4%), suggesting that it is a stage more often found among older cases of infection and, consequently, with a higher probability of evolving towards infection resistance (AI profile). This fact was confirmed in 47 (77%) of the 61 SRI cases diagnosed in this study through the negative conversion of the humoral response and the maintenance of delayed hypersensitivity (IFT- and MST+/++++). Thus, since the expression of delayed hypersensitivity is genetically controlled, from the moment that a recently infected individual (III profile) converts the delayed hypersensitivity (SRI profile), the infection will naturally progress to the resistance profile (AI profile).

With respect to the III profile, which not only constitutes the other new stage of infection, but also the most recent stage of infection in this diagnostic context, we found it interesting that, in contrast to a previous study carried out in the Municipality of Barcarena (Pará State, Brazil), where the prevalence rate of infection was 12.6%, the recent cases of infection varied little throughout the phases of this study: initial prevalence, 22%; incidence at 12 months, 26.6%; incidence at 24 months, 18.9%; final incidence, 23.1%; and cumulative prevalence, 22.4%. This is indicative of stable transmission at a higher level in the current study area, in the Municipality of Cametá, where the prevalence of infection was also higher (17%). In addition, considering that the final incidence of the III profile of 23.1%, we expected that at least 20-23% of the new cases of infection in this area would require clinical monitoring, since approximately 5-6% of these cases presented with the potential to develop into susceptible clinical forms of the infection, SOI and SI (=AVL). Thus, these findings should be taken into consideration when developing new programs to control AVL.

The SOI and SI (=AVL) profiles presented the lowest frequency rates in all phases of this study, although the former had a cumulative prevalence (4.3%) almost three times higher than the latter (1.6%). Nonetheless, similarly to the first study²⁹, both profiles combined (cumulative prevalence of 5.9%) did not surpass the range of 6% of the total number of cases in the endemic area of the Brazilian

Amazon. This finding appears to be very different from the data collected in the northeast of Brazil. In Bahia State, the subclinical oligosymptomatic form (SOI) was detected in 60% of 86 infected children under the age of 15⁶. In Maranhão State, this form was diagnosed in 17.4% of infected children in the same age group¹⁴. We should stress, however, that in both cases the final diagnosis of disease (SOI) was based on clinical parameters and supported only by a serological test (ELISA). Therefore, it is possible that the differences observed in the two studies from the northeast region of Brazil are due to the age group restriction (up to 15 years) of the individual participants (the children presented with a greater susceptibility to symptomatic infection), although in this study we observed that seven (53.8%) of the 13 SOI profile cases were in the youngest age groups (1-10 and 11-20 years). On the other hand, the incidence of the SI (=AVL) profile was almost negligible (0.2 / 1,000 inhabitants) when compared to the much higher rates recorded in the northeast region of Brazil^{16,9,25}. This indicates a more intense transmission of infection and/or an increased susceptibility of infected individuals to develop active disease.

When the distribution of the clinical-immunological profiles was considered with respect to age, it was demonstrated once more that the AI profile was more frequent than the other profiles in almost all the groups analyzed (1-10, 11-20 and > 21 years), in terms of both prevalence and incidence (Table 2), with exception of the prevalence in the 1-10 age group (30.2%), which was equal to the frequency rate of the SRI profile, and the incidence in the > 21 age group (31.8%), which was also equal to the frequency of the SRI and III profiles. Thus, the AI profile was the most frequent across age groups and phases of the study (prevalence and incidence measurements).

On the other hand, when the frequency of infection was compared within the same profiles, the following notable findings were observed. First, the prevalence of the AI profile increased with age. Although this may be interpreted as clear evidence that delayed hypersensitivity (acquired cellular immunity) increases with age^{6,13,26,1}, in fact it only reflects the fact that older individuals (> 21 years of age) have had a longer exposure than younger individuals (1-10 and 11-20 years of age). In contrast, the incidence of the AI profile decreased with age, with a significant reduction of the number of cases from the two youngest groups (1-10 and 11-20 years of age: 30 cases each, 44.8%) to the oldest one (> 21 years: seven cases, 10.4%). This demonstrates that children and adolescents constitute the majority of the AI profile among new cases of infection. Second, the four cases of AVL (SI profile) occurred in the youngest age group (1-10 years), which confirms that AVL is a disease typical of young children. Third, among the SOI profile cases, we noted an almost equal distribution between the age groups, with seven (53.8%) cases in the two youngest groups (1-10 and 11-20

years of age) and six (46.2%) cases in the older group (21 years). Fourth, we noted that the prevalence of the SRI profile was greater in the 21 age group (55.8%) compared to the 1-10 and 11-20 age groups (44.2%). This suggests a greater frequency of the AI profile among individuals who have been infected longer in the older age group. However, this tendency was inverted when examining the incidence, with a greater frequency in the 1-10 and 11-20 age groups (61.1%) compared to the older group (38.9%). Finally, with respect to the III profile, a significantly greater frequency of cases in the 1-10 and 11-20 age groups (74%) compared to the 21 age group (26%) was only evident in the incidence measurements (new cases of infection). Again, this suggests that the transmission of infection occurs mainly in or close to the home, where children and adolescents are infected^{21,22,32}.

With respect to the dynamics of the evolution of infection, the importance of the findings related to the III profile cannot be overlooked because, for this diagnostic approach this clinical-immunological profile plays a fundamental role in the transition to other profiles of human infection. Thus, this study shows that, of the 68 diagnosed cases, 30 (44.1%) evolved to the AI profile and 21 (30.8%) into the SRI profile. This accounts for almost 75% (74.9%) of the cases that progressed to the infection resistance (AI profile) pool, which constituted 78.3% of the final distribution of profiles. Additionally, one (1.5%) case progressed to the infection susceptibility pool (SI-AVL profile) and 16 (23.5%) did not change over the course of the study. Thus, these results appear to agree completely with the rationale behind this diagnostic approach²⁹, in that the progression of infection from the III profile to the resistance pool (AI profile) or to the susceptibility pool (SI profile) may depend on the genetic profile of the individual's cellular immune response^{8,17} (Figure 1).

Based on the above premise, we consider the progression of the susceptible profiles (SOI and SI) to be a priority. Although these groups show the same immunological profile (MST- and IFT+ + + / + + + +), they can be distinguished by the fact that the former (SOI) presented with spontaneous clinical progression towards cure in ten (77%) of the 13 diagnosed cases (eight evolved to the SRI profile, two into the AI profile, and three remained unaltered until the end of the study). For the latter group (AVL), pentavalent antimony therapy was needed in order to favorably progress towards cure (three progressed to the SRI profile, one into the AI profile, and one maintained the initial immunological profile but remained

asymptomatic). In this respect, it is important to mention the results obtained on the evolution of the SOI form from the states of Maranhão¹⁴ and Ceará¹⁵: 33 cases in Maranhão and 12 cases in Ceará showed a similar progression to the evolution pattern observed in the present study. In addition, another study conducted in Bahia State⁵ reported that individuals with the SOI form were able to produce more interferon-gamma (IFN- γ) in peripheral blood mononuclear cell cultures than individuals with AVL, which can help to better understand the performance of these symptomatic forms of human infection with *L. (L.) i. chagasi*.

With respect to the profiles showing resistance to infection (SRI and AI), our impression was that SRI appears to represent a developmental stage towards the AI profile, since the majority (77%) of cases converted to IFT-, evolving to the immune status of the AI profile. This may help to explain the high frequency of the AI profile found in this study and in a previous study²⁹. On the other hand, a few cases with the AI profile in the prevalence study exhibited transitory sero-conversion to IFT+ at low levels (+/+ +), which was followed by negative reconversion; this finding is interpreted as resulting from a possible antigen impulse of short duration caused by a reinfection aborted by the cellular immune response of these individuals. However, the vast majority of AI cases retained an unaltered immune profile, suggesting that the AI profile represents the end of the developmental process of the infection (Figure 1).

Finally, the infection dynamics showed that only the AI profile increased in frequency over the course of infection, rising from cumulative prevalence of 51.6% to a final distribution of 78.3%, whereas the frequency of the other profiles decreased significantly: SI (=AVL) from 1.6% to 0.3%; SOI from 4.3% to 1.0%; SRI from 20.1% to 15.1%; and III from 22.4% to 5.3%. Therefore, considering the role of the SRI and III profiles in the context of this diagnostic approach, the importance of these new clinical-immunological stages in promoting the evolution of the infection, especially the III profile, appears irrefutable. This fact may be of great value in preventing AVL morbidity, as well as in reducing the time and costs required for treatment.

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Novas evidências sobre o valor diagnóstico da reação de imunofluorescência indireta e reação intradérmica de hipersensibilidade tardia na infecção humana por *Leishmania (L.) infantum chagasi* na Amazônia, Brasil

RESUMO

Estudo prospectivo realizado no período de maio/2006-setembro/2008, numa coorte de 1.099 indivíduos, ambos os sexos, com idades de 1 a 84 anos (média 24,4 anos), residente em área endêmica de leishmaniose visceral americana (LVA) no Município de Cametá, Pará, Brasil, objetivando analisar a prevalência e a incidência da infecção humana por *Leishmania (L.) infantum chagasi*, assim como a dinâmica da evolução dos seus perfis clínico-imunológicos previamente definidos: 1. Infecção assintomática (IA); 2. Infecção sintomática (IS=LVA); 3. Infecção subclínica oligossintomática (ISO); 4. Infecção subclínica resistente (ISR); e 5. Infecção inicial indeterminada (III). O diagnóstico da infecção baseou-se no uso simultâneo da reação de imunofluorescência indireta (RIFI) e reação intradérmica de hipersensibilidade tardia. Um total de 304 casos da infecção foi diagnosticado no período do estudo (187 na prevalência e 117 na incidência), gerando prevalência acumulada de 27,6%, cuja distribuição no âmbito dos perfis clínico-imunológicos foi da seguinte ordem: IA 51,6%, III 22,4%, ISR 20,1%, ISO 4,3% e IS (=LVA) 1,6%. Com base na dinâmica da infecção, o principal achado recaiu no perfil III, que teve papel fundamental na evolução da infecção, dirigindo-a ora para o pólo imunológico de resistência, perfis ISR (21 casos - 30,8%) e IA (30 casos - 44,1%), ora para o polo imunológico de susceptibilidade, perfil IS (um caso - 1,5%); além destes, 16 casos mantiveram o perfil III até o fim do estudo. Concluiu-se que esta abordagem diagnóstica pode ajudar no monitoramento da infecção na área endêmica, visando, principalmente, prevenir a morbidade da LVA, assim como reduzir o tempo e despesas com o tratamento.

Palavras-chave: *Leishmania (L.) infantum chagasi*; Infecção; Imunodiagnóstico; Hipersensibilidade Tardia; Técnica Indireta de Fluorescência para Anticorpo.

Nueva evidencia sobre el valor diagnóstico de la prueba de inmunofluorescencia indirecta y la reacción intradérmica de hipersensibilidad retardada en la infección humana por *Leishmania (L.) infantum chagasi* en la Amazonia brasileña

RESUMEN

Estudio prospectivo realizado a partir de mayo de 2006 hasta septiembre de 2008 con una muestra de 1.099 individuos de ambos sexos, con edades de 1 a 84 años (media 24,4 años), residentes en un área endémica de leishmaniasis visceral americana (LVA) en el Municipio de Cametá (Estado de Pará, Brasil). Su objetivo es examinar la prevalencia y la incidencia de la infección humana por *Leishmania (L.) infantum chagasi*, así como la dinámica de la evolución de los perfiles clínicos e inmunológicos definidos previamente: 1. Infección asintomática (IA); 2. Infección sintomática (IS = LVA); 3. Infección subclínica oligosintomática (ISO); 4. Infección subclínica resistente (ISR); y 5. Infección inicial indeterminada (III). El diagnóstico de la infección, se basa en la utilización simultánea de la reacción de inmunofluorescencia indirecta (RIFI) y reacción intradérmica de hipersensibilidad retardada. Se diagnosticaron un total de 304 casos de la infección durante el periodo de estudio (187 en la prevalencia y 117 en la incidencia), dando una prevalencia acumulada de 27,6%, cuya distribución, dentro de los perfiles clínicos e inmunológicos, fue del siguiente orden: IA 51,6%, III 22,4%, ISR 20,1%, ISO 4,3% e IS (= AVL) 1,6%. Con base en la dinámica de la infección, el principal hallazgo recae en el perfil III, que tuvo un papel decisivo en la evolución de la infección, dirigiéndola ora hacia el polo inmunológico de resistencia, perfiles ISR (21 casos, 30,8%) e IA (30 casos, 44,1%), ora hacia el polo inmunológico de susceptibilidad, perfil IS (1 caso, 1,5%); además, de estos casos, 16 mantuvieron el perfil III hasta el final del estudio. Se concluyó que este método de diagnóstico puede contribuir al monitoreo de la infección en las zonas endémicas, con el objetivo principal de prevenir la morbilidad de la LVA, así como reducir el tiempo y los gastos con el tratamiento.

Palabras clave: *Leishmania (L.) infantum chagasi*; Infección; Pruebas Inmunológicas; Hipersensibilidad Retardada; Técnica del Anticuerpo Fluorescente Indirecta.



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