A cross-sectional study on the clinical and immunological spectrum of human *Leishmania (L.) infantum chagasi* infection in the Brazilian Amazon region

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Received 23 August 2007; received in revised form 17 June 2009; accepted 17 June 2009
Available online 16 July 2009

KEYWORDS
Leishmania infantum chagasi; Infection; Clinical examination; Immunologic tests; Cross-sectional study; Brazil

Summary The objectives of this study were to identify individuals with symptomatic and/or asymptomatic infection due to *Leishmania (L.) infantum chagasi*; to study the two types of infection, both clinically and immunologically, and to determine the prevalence rate of infection at the beginning of the study. This was a cross-sectional study with a cohort of 946 individuals, of both genders, from the age of 1 year, living in the municipality of Barcarena, PA, Brazil, an area endemic for American visceral leishmaniasis (AVL). The leishmanin skin test (LST) and the indirect fluorescent test (IFAT), were used for the diagnosis of infection. One hundred and twenty cases of infection were diagnosed, with a prevalence rate of 12.6%; eight cases showed high seroreactivity (1280—10 240, IgG) in IFAT and no LST reaction; four of these cases were typical AVL and four had subclinical oligosymptomatic infection. Using two immunological methods with a clinical examination of the infected individuals enabled the identification of five clinical-immunological profiles which may promote a better understanding of the interaction between *L. (L.) i. chagasi* and the human immune response: asymptomatic infection (AI) 73.4%; subclinical resistant infection (SRI) 15%; subclinical oligosymptomatic infection (SOI) 3%; symptomatic infection (AVL) 3% and indeterminate initial infection (III) 5%.

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1. Introduction

The clinical manifestations of visceralizing Leishmania spp. are determined by the interactions of the parasite with the human immune system. To evaluate the human immune response to this disease, two types of assay have mainly been used: the IFAT or ELISA for evaluating the humoral response and the leishmanin skin test (LST) for evaluating the cellular, delayed-type hypersensitivity (DTH) response. However, in most situations these assays have been used separately, which has not given a view of the whole immune response to infection.

To illustrate this situation, some studies carried out in Africa, especially in Sudan,1,2 and Ethiopia,3—5, reported the epidemiological, clinical and immunological features of human infection caused by Leishmania (L.) donovani using either LST to measure DTH and IFAT to measure the antibody response. In some Asian countries, such as India,6 and Nepal,7 which have a high prevalence of visceral leishmaniasis (VL) caused by L. (L.) donovani, similar immunological approaches were used to understand the features of human disease. In European Mediterranean countries, including Italy, Spain and Greece,8 the clinical and immune responses to human infection with Leishmania (L.) infantum were investigated using similar approaches. In South America, where Brazil has the highest incidence of human VL due to Leishmania (L.) infantum chagasi, few studies have reported the epidemiological, clinical and immunological profiles of infection based on the same immunodiagnostic models.9—16

The clarification of the clinical manifestations that occur in the spectrum of human infection with L. (L.) chagasi infection has been the aim of some studies in Brazil.14,17—20 In most of these studies, however, the diagnosis of infection was only based on the antibody response of the infected individuals allowing categorisation of the disease into three clinical forms: asymptomatic infection (AI), subclinical oligosymptomatic infection (SOI) and symptomatic infection [SI = American visceral leishmaniasis (AVL)]. It is quite possible, however, that this type of diagnostic approach may have neglected some clinical or immunological findings associated with the DTH response of infected individuals living in the endemic area.

For these reasons and because of the scarce information on the interaction between the human L. (L.) chagasi infection and the immune response in the Brazilian Amazon region,21 we conducted a cross-sectional study of the prevalence rates and the clinical and immunological spectrum of infection by combining the two types of immune response assays: IFAT for the humoral response and LST for the DTH response aiming to improve our understanding on the clinical and immunological spectrum of the human L. (L.) chagasi infection in the Brazilian Amazon region.

2. Materials and methods

2.1. Study area

This study was carried out during October—November 2003 in Santana do Cafezal village, which is situated on the banks of the river Cafezal, 7 km from the administrative centre of Barcarena municipality (01° 30′ S; 48° 37′ W). It is considered to be within the metropolitan region of Belém, PA, in the north of Brazil. The climate is typically equatorial, with an average temperature of 27°C and high humidity. The annual rainfall is in the region of 2500 mm or more, with the period from January to June forming the principal rainy season. Following extensive destruction of the primary forest, the area now consists mainly of plantations, with occasional patches of developing secondary forest. Approximately 70% of the inhabitants occupy wooden houses in non-flood land, which is surrounded by secondary forest, while the rest lives in the várzea, an area of low vegetation which is flooded twice daily by waters of the Cafezal River.

2.2. Study design and population

The study was designed to identify individuals of all ages, with symptomatic or asymptomatic infection with L. (L.) chagasi; to characterise the infection both clinically and immunologically and to determine the prevalence rate of infection. Specifically, we looked for a better understanding of the transmission dynamics as well as the clinical and immunological features of the different patterns of the disease within the spectrum of human L. (L.) chagasi infection.

The population enrolled in the study consisted of a cohort of 946 individuals (almost 90% of the total population), being 568 males and 378 females aged 1—89 years old with a median of 20 years, suggesting a relatively young population. When the study began, the number of inhabitants in the area was estimated to be 1064.22 In order to obtain a clearer idea of the transmission dynamics of infection, the total population was divided into three age groups: 1—10, 11—20 and ≥21 years. The groups consisted of 260, 218 and 468 individuals, respectively. This stratification considered that people aged ≥21 years have the same ability to develop a specific immune response against infection. For the infection diagnosis, both IFAT and LST were performed in all individuals previously selected for the prevalence study. In addition, all individuals presenting any type of immune reaction were clinically examined in order to identify any signs and/or symptoms that could be associated with the classical features of AVL.

2.3. Criteria for identification of human infection

The definition of a case of human infection with L. (L.) chagasi was the presence of reactivity in either or both immunological tests. IFAT demonstrates humoral immunity associated with the CD4+ Th2 immune response (antibody response = susceptibility) and LST demonstrates cellular immunity associated with the CD4+ Th1 immune response (hypersensitivity = resistance).23 With the objective of expressing the specificity of IFAT and LST, a scale of semi-quantitative results was used with scores varying from + to ++++. For IFAT, serological titres of 80—160 and 320—640 (IgG) received + and ++ and those of 1280—2560 and 5120—10 240 were given +++ and ++++, respectively; for LST, exacerbated intradermal reactions (≥16 mm) were regarded as ++++, strongly positive (13—15 mm) as +++,
moderately positive (9–12 mm) as ++ and weakly positive (5–8 mm) as +. Thus, it was assumed that serological reactions of 80 (IgG) titre and intradermal reactions forming papules or indurations of ≥5 mm in diameter were regarded as the positive cut-off in IFAT and LST, respectively.24–27

2.4. Immunological test procedures

The procedure used for LST was previously described in other studies on American cutaneous leishmaniasis.26,27 The antigen used in Santana do Cafezal, a village situated in an area where cutaneous and visceral leishmaniasis are potentially concomitant, should have a high specificity for the visceral disease. Thus, to promote high specificity in LST, cultured promastigote forms from the stationary phase in RPMI 1640 medium (Sigma-Aldrich, St Louis, MO, USA) of a regional strain of L. (L.) i. chagasi (MCAO/BR/2003/M22697/Barcarena, PA, Brazil) was used. They were fixed in merthiolate solution (1/10 000), with a final concentration of approximately 10 × 10⁶ parasites/ml. As control for the Leishmania antigen, 0.1 ml of the merthiolate solution (1/10 000) was administered intradermally in the opposite forearm of each individual.

IFAT was performed as proposed by Lima et al.24,25 who demonstrated that amastigote antigens of L. (L.) i. chagasi had a higher specificity and sensitivity than those of promastigotes of the same species and L. (L.) major-like (Bio-Manguinhos, RJ, Brazil) and than amastigote antigens of L. (L.) amazonensis. Briefly, amastigote antigens were impregnated in the IFAT slides by imprint of small fragments of spleen and liver from hamster infected with the parasite. Crude L. (L.) i. chagasi amastigote antigen has the best specificity and sensitivity for the serological diagnosis of human infection with L. (L.) i. chagasi using IFAT. For diagnosis of canine VL this method has also proved to be more specific than IFAT and ELISA (Bio-Manguinhos), which are available through the Brazilian AVL control program.28

2.5. Data analysis

The data were analyzed by the Bio-Estat 4.0 program.29 The χ² and binomial tests were used for the significance of differences between the clinical-immunological profiles of infection with a confidence interval of 95%.

3. Results

3.1. Prevalence rates of human Leishmania (L.) infantum chagasi infection

An infection prevalence rate of 11.2% (106/946) was obtained by LST and of 3.4% (32/946) by IFAT (P < 0.001). Among the 106 LST-reactive individuals, 18 (17%) were also IFAT-reactive, and of the 32 IFAT-reactive, the same 18 (56.2%) were also LST-reactive. This combination permitted the identification of an actual prevalence rate of 12.6% (120/946) for the community, using both tests.

3.2. Frequency of human Leishmania (L.) infantum chagasi infection according to sex, age and LST and IFAT specificity

The distribution of the 120 cases of infection showed no difference between males (55.8%) and females (44.2%). The distribution of infection according to age also showed no difference between the younger age groups, with 19.2% of infected cases aged 1–10 years and 23.8% of cases aged 11–20 years. However, when the rates of these groups were compared with that of the older age group both were significantly smaller (P < 0.001), with more than half (55%) of infected individuals being ≥21 years old.

Regarding LST and IFAT response, among the 106 LST-reactive cases, 41.5% presented exacerbated reactivity (+++), 14.1% were strongly positive (++), 19.8% moderately positive (+) and 26.2% weakly positive (+). Thus over half of the cases (55.6%) had marked (++/+++), immunological resistance (hypersensitivity) to infection. Among the 32 IFAT-reactive cases, 21.8% presented low serological reactivity (+), 53.1% were moderately positive (++), 18.8% strongly positive (+++) and 3.6% highly positive (+++++). Thus 25.1% of cases presented a significant humoral response of strongly (+++) or highly positive (++++), showing immunological susceptibility to infection.

3.3. Clinical-immunological evaluation of human Leishmania (L.) infantum chagasi infection

Of the 120 cases of infection, most (73.4%) were clinically asymptomatic and exhibited an immune response (LST+/++++ and IFAT−) consistent with a clinical-immunological profile of resistant AI. A few (6.6%) were represented by an immune response (LST− and IFAT+/+++ or ++++) that could be associated with either of two susceptible clinical-immunological profiles: (SII = AVL) or SOI. The difference was mainly clinical; the profile of SI was applied to four (3.3%) cases of active AVL (two children and two adults), resulting in an AVL prevalence rate of 0.42%, while SOI was used in another four (3.3%) cases (one child and three adults). These cases presented various manifestations, associated or not and of uncertain duration, which were not characteristic of acute AVL, such as: fever; asthenia; pallor; adenopathy and slight hepatomegaly, but without splenomegaly. There were also some haematological alterations such as mild-to-moderate anaemia and leukopenia. A further 15% of cases were asymptomatic with an immune response reaction in both tests (LST+/++ and IFAT+/++), compatible with an intermediate clinical-immunological profile showing a reasonable degree of resistance (LST+/++). This profile was categorized as subclinical resistant infection (SRI). Finally, 5% of cases were asymptomatic and presented a humoral immune response but with low serological titles (LST− and IFAT+/++). These patients were regarded as a very early infected group with the possibility of their disease evolving to either the resistant profiles SRI and AI or to the susceptible ones SOI and SI. This was considered an indeterminate initial infection (III) (Figure 1).

The frequency of the AI profile (73.4%) was higher (P < 0.001) than the other profiles, SRI (15%), III (5%), SI (3%)
A cross-sectional study on the clinical and immunological spectrum of human Leishmania (L.) infantum chagasi

with respect to the study population, Santana do Cafezal village consists of a community established for over a century, whose population selected for this study spanned at least four generations. Such long-term exposure to L. (L.) i. chagasi transmission may well favour the development of some degree of immune protection in the population. Although the general population has low purchasing power, food is provided principally by fishing and subsistence agriculture, providing a satisfactory feeding profile. Even though some recent evidence exists indicating that susceptibility to VL could be strongly controlled by a genetic mechanism, it is possible that environment and/or nutrition could contribute to the promotion of an improved immune response against human L. (L.) i. chagasi infection, given that the prevalence (0.42%) of active AVL was low in this study.

With respect to the transmission dynamics of infection, this received two types of treatment in this study. The first analysed the infection prevalence by LST and IFAT and enabled a specific and comparative evaluation of the two types of immune response, cellular and humoral. The second considered the combined analysis of LST and IFAT results, which allowed the determination of the actual rate of infection, in an attempt to evaluate the true situation of infection transmission in the study area. In the first analysis the infection prevalence determined by LST (11.2%) was higher (P < 0.001) than that determined by IFAT (3.4%). This demonstrated that among naturally infected individuals, a higher number presented immunological resistance (hypersensitivity) to infection, which may help to explain the low rate of prevalence (0.42%) of AVL in the present study.

Moreover, considering that both tests were performed with the same antigen strain of L. (L.) i. chagasi, it is highly unlikely that the difference found in the prevalence rates determined by LST and IFAT could be attributed to the variability of the antigen specificity used in these tests. It should be highlighted that these findings can be influenced by some methodological procedures and/or epidemiological factors. In the Jacobina municipality, Bahia State, northeastern region of Brazil, a soluble extract of L. (L.) i. chagasi antigen was used to study the infection prevalence by LST in children up to 15 years old. By contrast with the present study, these authors found an infection rate of 34.1%. Considering that the immune response to infection is not limited to only one type, i.e. either a T-cell response or a humoral response, it is clear that these results underestimated the true rate of infection in that area. In another study in the municipality of Raposa, Maranhão State, also in the north-

### Table 1 Clinical and immunological spectrum of human Leishmania (L.) infantum chagasi infection in the Amazon region, Brazil

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Figure 1: Frequency distribution of the clinical-immunological profiles of the human Leishmania (L.) infantum chagasi infection, in Santana do Cafezal village, Barcarena municipality, PA, Brazil. AI: asymptomatic infection; SRI: subclinical resistant infection; III: indeterminate initial infection; SI: symptomatic infection (=American visceral leishmaniasis); SOI: subclinical oligosymptomatic infection.

*Figure 1* Frequency distribution of the clinical-immunological profiles of the human Leishmania (L.) infantum chagasi infection, in Santana do Cafezal village, Barcarena municipality, PA, Brazil. AI: asymptomatic infection; SRI: subclinical resistant infection; III: indeterminate initial infection; SI: symptomatic infection (=American visceral leishmaniasis); SOI: subclinical oligosymptomatic infection.

4. Discussion

This is one of the few epidemiological studies carried out in Brazil to evaluate the transmission dynamics of human L. (L.) i. chagasi infection in individuals aged 1 year and over, using two immunological methods, LST and IFAT. Even though some studies have previously reported on the transmission dynamics of infection, they were limited to children up to 15 years old, which neglected the importance of older dynamics of infection.

With respect to the transmission dynamics of infection, some studies have previously reported on the transmission of infection using two immunological methods, LST and IFAT. Even though the low rate of prevalence (0.42%) of AVL in the present study.

The variability of the antigen specificity used in these tests. It should be highlighted that these findings can be influenced by some methodological procedures and/or epidemiological factors. In the Jacobina municipality, Bahia State, northeastern region of Brazil, a soluble extract of L. (L.) i. chagasi antigen was used to study the infection prevalence by LST in children up to 15 years old. By contrast with the present study, these authors found an infection rate of 34.1%. Considering that the immune response to infection is not limited to only one type, i.e. either a T-cell response or a humoral response, it is clear that these results underestimated the true rate of infection in that area. In another study in the municipality of Raposa, Maranhão State, also in the north-

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eastern region of Brazil, two different procedures were used: only children up to 5 years old were selected, representing a greater limitation compared to other studies and two different antigens were used for immune diagnosis; a soluble extract of L. (L.) amazonensis for LST and a soluble extract of L. (L.) i. chagasi for ELISA.12,13 The results revealed infection prevalence rates of 18.6% by LST and 13.5% by ELISA, higher than those found in the present study (11.2% by LST and 3.4% by IFAT), suggesting a higher concentration of susceptible individuals (children up to 5 years old).

In another study also carried out in Maranhão State, municipality of São José de Ribamar, the prevalence rate of infection in children up to 15 years old was estimated using three antigen types: a soluble extract of L. (L.) i. chagasi for LST, and a crude antigen of the same parasite and a recombinant protein (rK39) for ELISA.14 The data revealed the highest infection rates reported, 61.7% by LST and 19.7% (crude antigen) and 19.4% (rK39) by ELISA. These differences must be examined with caution, considering the procedures used and the epidemiological circumstances of each study area.

In Italy, where the disease is caused by L. (L.) i. infantum, a promastigote-antigen suspension of homologous parasite was used to demonstrate a 16.6% prevalence rate of infection in individuals of different ages by LST.8 This most closely resembles the infection rate found in the present study and may reflect similar transmission dynamics of L. (L.) i. infantum and L. (L.) i. chagasi. In Africa, especially in Sudan, where the disease is caused by L. (L.) d. donovani, leishmaniasis represents a severe public health problem, being responsible for more than 100 000 deaths in the 1980s.4 The epidemiological situation of human infection remains variable, as recent reports show prevalence rates ranging from 33% to 56% in two localities, Mushrau Koka and Um-Salala, respectively, using a promastigote-antigen suspension of the same parasite, demonstrating a higher infection transmission level than found in the Brazilian Amazon region.1

The second analysis was based on a combination of LST and IFAT data, which determined that the actual prevalence rate of infection in the study area is 12.6% (120/946). It should be highlighted that this appears to be an unpublished approach in the investigation of human L. (L.) i. chagasi infection epidemiology. Gender was not found to be a significant variable in the distribution of human L. (L.) i. chagasi infection. With respect to age, the younger age groups had similar infection rates, which were lower than that of the older group, demonstrating that infection progressively accumulates with age, similar to the pattern found in human L. (L.) i. infantum infection in Sicily, Italy.8

Regarding LST and IFAT specificity in the context of infection prevalence, the results showed that among the 106 LST-reactive cases, 56.6% presented a significant immunological resistance to infection (hypersensitivity ++++/++++). This could be explained by a reasonable sample of infected individuals that have naturally received repeated antigenic stimulus through the infected phlebotomine sand fly vector (Lutzomyia longipalpis) over a prolonged period. In this context, it is possible that these ‘natural infective doses’ may represent a major strategy for consideration in a future vaccine program against infection. By contrast, it was observed that among the 32 IFAT-reactive cases, only 25% showed a susceptibility profile to AVL, with high serological titres ranging from 1280 (+++) to 10 240 (++++) (IgG). The great majority (75%) exhibited low serological reactivity 80 (+) to 640 (+++) (IgG), indicating that only few cases expressing a humoral immune response (CD4 type 2 immune response) presented some degree of predisposition for developing AVL, which was confirmed in 12.5% of IFAT-reactive individuals and only in 3.3% of all 120 cases of infection. Moreover, considering the infection prevalence (12.6%), a ratio between infection and disease of 1:30 was estimated, while in the Jacobina municipality, Bahia State, this ranged from 1:6.5 to 1:18.5 with a prevalence rate of 34.1% by LST.11 Again, it should be stated that in these locations the prevalence rates were estimated in children up to a maximum of 15 years of age.

In this study we propose a new, broad clinical-immunological spectrum for human L. (L.) i. chagasi infection in the New World using simple, inexpensive and reproducible methods. We propose five different clinical-immunological profiles of infection, adding two additional profiles, SRI and III, to the three already recognized by others: AI, SI (= AVL) and SOI14–19, Pearson et al.20 This proposed spectrum has the advantage of promoting a better view of the clinical and immunological factors in play in the interaction between L. (L.) i. chagasi and the human immune response, as well as providing a suitable tool for control programs of AVL. This spectrum may also reflect the genetic polymorphism behind the immunological mechanisms responsible for resistance to human VL infection.30–32 This experience also showed that the great majority (73.4%) of infected individuals living in the AVL-endemic area have an immune response profile of resistance to infection (LST+/++++ and IFAT−), confirming the significance of the hypersensitivity mechanism (T-cell immune response) in controlling the infection. This finding has also been demonstrated by others.34 As a result, all these individuals were clinically asymptomatic (AI). The new profile SRI, which also showed a significant degree of resistance (LST+/++) to infection, was represented by a reasonable proportion (15%) of infected individuals. The SRI and AI profiles together were at least 88.4% of all infections in the endemic area, suggesting that most infected individuals present an immune resistance profile to the infection.

Another significant finding among infected individuals was the identification of the III profile (LST− and IFAT+/+++), which consisted of individuals with the earliest stage of infection and with an apparent tendency for a predominantly humoral response, but with no definition of the ultimate immune response profile. This was considered as an indeterminate initial infection, which could evolve either to the resistant profiles SRI and AI or to the susceptible profiles SOI and SI, and may be useful in AVL control programs for monitoring recently infected individuals in the endemic area. The finding that age may alter the features of infection was another conclusion of this work. A number of reports that documented a high number of subclinical oligosymptomatic cases, more than acute AVL cases, only included children up to 15 years old17 or 5 years old.18 In the present work, no difference in the prevalence rates between the SI (3.3%) and SOI profiles (3.3%) was identified, probably
because individuals of all ages were evaluated, suggesting an excess concentration of susceptible individuals in the prior reports. Indeed, the influence of age on the outcome of infection was detected between the age of these two profiles, an average age of 33.6 years was observed among individuals with the SOI profile, which was significantly higher ($P<0.001$) than the average of 10.7 years old in the SI profile, indicating that older individuals seem to develop a better T-cell immune response to infection and present fewer conditions for the SI profile.

The feasibility of this approach for evaluating the clinical and immunological manifestations of human $L. (L.)$ $i$. $chagasi$ infection was confirmed in a recent cross-sectional study carried out in another locality (municipality of Cametá) in the northeast region of Pará State, with a higher incidence of AVL than the municipality of Barcarena. The prevalence rate of infection was 18.4% and the frequency rates of the clinical-immunological profiles were as follows: AI: 47.5%, SRI: 22.3%, SI: 0.5%, SOI: 3.9% and III: 25.7%. This suggests that an area with a higher level of transmission, where two profiles, an average age of 33.6 years was observed, will contain a higher number of more recent cases of infection.

Authors’ contributions: JABC, FTS and CEPC designed the study protocol; JABC, FTS, RL, CMCG, MDL and CEPC contributed to the collection and analysis of the data and preparation of the article. All authors read and approved the final manuscript. FTS and CEPC are guarantors of the paper.

Funding: This research was supported by the Instituto Evandro Chagas (Secretaria de Vigilância em Saúde, Ministério da Saúde, Brazil); Instituto de Medicina Tropical (Universidade Federal do Pará, Brazil); Wellcome Trust (London); Laboratório de Investigação Médica (LIM)-50 (Hospital de Clínicas Federal do Pará, Brazil); and Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP: 06/56319-1, Brazil).

Conflicts of interest: None declared.

Ethical approval: This study was approved by the Ethics Committee in Human Research of the Instituto Evandro Chagas (SVS), Brazil, with the protocol number CEP/IEC 16/2003 and CAPPesq/FMUSP 0235/07.

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