

Diagnosing human asymptomatic visceral leishmaniasis in an urban area of the State of Minas Gerais, using serological and molecular biology techniques

Diagnóstico da leishmaniose visceral humana assintomática em uma área urbana do Estado de Minas Gerais, usando métodos sorológicos e biologia molecular

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ABSTRACT

A population-based cross-sectional study was set up in Sabará country, Southeastern Brazil, to identify asymptomatic human visceral leishmaniasis in an urban area of low disease prevalence. Blood was collected on filter paper ($n=1,604$ inhabitants) and examined by indirect immunofluorescent test, enzyme-linked immunosorbent assay and immunochromatographic strip test. The prevalence rates of infection ranged from 2.4 to 5.6% depending on the test used. One year later, venous blood was collected in a subset of 226 participants (102 seropositive and 124 seronegative). The tests performed were IFAT, ELISA, rk39-ELISA, polymerase chain reaction and hybridization with *Leishmania donovani* complex probe. No clinical signs or symptoms of leishmaniasis were observed. Using hybridization as a reference test, the sensitivity and specificity of serology were respectively: 24.8 and 71% (ELISA); 26.3 and 76.3% (rk39-ELISA); 30.1 and 63.4% (IFAT). Due to disagreements, different criteria were tested to define the infection and hybridization should be considered in epidemiological studies.

Key-words: Asymptomatic visceral leishmaniasis. *Leishmania chagasi*. Prevalence rate. Diagnostic. Hybridization.

RESUMO

Um estudo seccional de base populacional foi desenvolvido no município de Sabará, região sudeste do Brasil, para identificar a leishmaniose visceral humana assintomática em uma área urbana de baixa prevalência da doença. Foi coletado sangue em papel filtro ($n=1.604$ moradores), sendo examinados pela reação de imunofluorescência indireta, ensaio imunoenzimático e teste imunocromatográfico (strip test). As taxas de prevalência da infecção variaram de 2,4 a 5,6%, dependendo do teste utilizado. Um ano depois foi coletado sangue venoso de um subgrupo de 226 participantes (102 soropositivos e 124 soronegativos). Os testes realizados foram IFAT, ELISA, rk39-ELISA, reação em cadeia da polimerase e hibridização com sonda específica para o complexo *Leishmania donovani*. Não foi observado nenhum sinal clínico ou sintoma de leishmaniose. Usando a hibridização como teste de referência, a sensibilidade e especificidade dos testes sorológicos foram, respectivamente: 24,8 e 71% (ELISA); 26,3 e 76,3% (rk39-ELISA); 30,1 e 63,4% (IFAT). Devido a discordâncias, diferentes critérios foram testados para definir a presença da infecção e a hibridização deveria ser considerada em estudos epidemiológicos.

Palavras-chaves: Leishmaniose visceral assintomática. *Leishmania chagasi*. Taxa de prevalência. Diagnóstico. Hibridização.

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Visceral leishmaniasis (VL) in Latin America is caused by *Leishmania (L.) chagasi*, a protozoan belonging to the *Leishmania donovani* complex, mainly transmitted by a sand fly, *Lutzomyia longipalpis*. Dogs are considered as the principal parasite reservoir, playing a central role in the transmission cycle to humans. VL is endemic in the rural areas of most Northeastern Brazilian states. It is nowadays considered as a reemerging infection due to its rapid expansion to non-endemic areas. During the last decade, VL clinical cases and deaths have been reported in increasing numbers, in large cities located in different Brazilian states^{31 34}.

The occurrence of individuals harboring asymptomatic or oligosymptomatic *L. chagasi* infections in Brazil without progression to the disease has been reported in investigations conducted in Northeastern Brazil^{3 4 6 11 13 14 16 17 18}. One of the most important drawbacks in these studies is the difficulty of diagnosing patients in this phase, because of the low level of antibodies and reduced parasite numbers^{2 25 27}.

Population-based studies in urban areas are scarce and, when carried out, use immunological tests, mainly the enzyme-linked immunosorbent assay (ELISA), indirect immunofluorescent antibody test (IFAT); tests that present high sensitivity for disease, but do not discriminate between past and current infection; and the skin-test, positive in asymptomatic infection⁶ and in patients with a past history of visceral leishmaniasis. Specific antigens from the *L. donovani* complex have been used in ELISA and in an immunochromatographic strip test for the identification of active, subclinical and asymptomatic VL in specific population groups^{2 24 33}. Molecular biology techniques, such as polymerase chain reaction (PCR) alone or in combination with hybridization have been used, especially in blood, to confirm the VL diagnosis in suspect cases, due to their sensitivity (ranging from 75 to 98%) and specificity (ranging of 97 to 100%) in identifying the infection in individuals with low levels of parasites and antibodies in the absence of symptoms^{12 21 26 27}.

Regarding VL expansion and urbanization in Brazil, more precise estimates of asymptomatic *L. chagasi* infection occurrence in a given population are necessary in order to understand the extension of the transmission. The objective of this study was the identification of asymptomatic VL cases in a population resident in an urban area, assaying different immunological and molecular biology diagnostic techniques.

PATIENTS AND METHODS

Study design and population. This study was conducted to evaluate asymptomatic *L. chagasi* infection in an urban area of General Carneiro district with a population of 19,500 inhabitants, situated in Sabará County, of the Metropolitan Region of Belo Horizonte, capital of the Minas Gerais State, Brazil. For the last 15 years serological canine surveys conducted routinely by the local Public Health Services have revealed prevalence rates ranging from 5 to 10%. Sporadic human cases and deaths have been reported. From 1989 to 2004, 13 cases of human VL have been reported

from General Carneiro, with the occurrence of one fatality. *Additionally, cutaneous leishmaniasis caused by L. (V.) braziliensis, has been reported by the health services.*

A cross-sectional study was conducted in 1998, using serological tests in blood samples collected in filter paper. The sample size (n = 1,328) was calculated based on the following parameters: 1) an estimated human infection prevalence rate of 3.1%, based on a study conducted in the State of Bahia⁴; 2) precision of the estimate ranging from 2.2% to 4%; 3) a error = 0.05 and 4) population in the study area = 19,500 inhabitants. For operational reasons, households were used as sampling units. A simple random sample of city blocks was selected proportionally to the existent blocks, and within each selected block, a random sample of households was drawn, proportionally to the number of existent houses. All inhabitants of the selected houses were eligible and were enrolled in the cross-sectional study. A total of 440 households were visited and 1,604 inhabitants were selected (8.2% of the population of General Carneiro).

The mean age of the 1,604 participants was 26.8 (\pm 18.6) years, the median age was 23 years (25 and 75% inter-quartile range were 13 and 38, respectively); 51.7% were female; 69.7% had received only elementary school education and 9.8% had never gone to school. The mean period of residence in this area was 15 (\pm 11.2) years.

After 12 months, a subset of 128 participants, who proved positive in serological tests, was enrolled to investigate the course of the infection. A random subset of 128 negative participants was also included as a comparison group. The participants were clinically examined for signs and symptoms of VL in an outpatients clinic of the General Carneiro Public Health Services, interviewed, and a sample of blood was collected and tested for infection using serological and molecular methods.

The study was approved by the Ethical Review Board of the Federal University of Minas Gerais. All participants or their legal guardians, in the case of minors, were required to sign an Informed Consent Form before data collection. Medical attendance and treatment, if necessary, were guaranteed for all participants.

Data collection. In the cross-sectional study, blood samples were collected by finger prick onto filter paper (Klabin nr 25, Brazil) dried and stored at 4°C until testing. Spots of 5mm in diameter were eluted in phosphate-buffered saline (PBS) and examined by IFAT; same size spots were eluted in casein-PBS for testing by ELISA using crude antigen and strip test. In the second evaluation carried out after 12 months, blood was collected in a vacuum tube without anticoagulant; the sera were stored at -20°C and later tested by ELISA using both crude and rk39 recombinant antigens and by IFAT. A second vacuum tube with EDTA was used to collect blood for peripheral mononuclear cells (PBMC), later used for DNA extraction.

Laboratory tests. *Indirect immunofluorescent test* the IFAT was performed according to established methodology³⁰; promastigotes of *Leishmania (L.) amazonensis* (MHOM/BR/1960/BH6),

routinely used in the diagnosis of *Leishmania*, grown to stationary phase in LIT (Liver Infusion Tryptose) medium were used. The samples were considered positive when fluorescence was observed at 1:40 dilution.

Enzyme-linked immunosorbent assay: crude antigen was obtained from the same *L. amazonensis* strain used for IFAT, according to established methods³⁵; rk39 recombinant antigen (specific to the *L. donovani* complex) was used, as previously described⁵. Antibodies were detected with goat conjugated peroxidase-labeled anti-human IgG (Sigma Co, US). Reactions were read at 492nm. The cut-off values established for each reaction (ELISA in filter paper and serum, and rk39-ELISA in serum) were the mean absorbancy value + 2 standard deviations from 20 known negative sera at a 1:80 dilution).

Immunochromatographic strip test: this test used the rk39 antigen and was conducted as previously described, using blood eluted from filter paper³³. Three trained observers read the strips independently; the test was only considered to be positive when the results obtained by all three readers were in agreement.

Anti-Trypanosoma cruzi antibodies: due to cross-reactivity in serological tests, serum samples were also assayed for *T. cruzi* antibodies using indirect hemagglutination (Chagas Kit -FUNED-SES/MG, Brazil) and IFAT (Chagas Kit – BioManguinhos – FIOCRUZ, Brazil).

DNA isolation: PBMC were isolated from 8ml peripheral blood collected in EDTA-coated tube after centrifugation with Ficoll-Paque (Pharmacia, Uppsala, Sweden). DNA was extracted by phenol/chloroform/isoamyl alcohol and ethanol precipitated, resuspended in 30µl low TE buffer and stored at 4°C.

PCR amplification: a pair of primers were used to amplify a fragment of 120 bp of the conserved region of *Leishmania* kDNA minicircle [5 (G/C) (G/C) (C/G) CC (A/C) CTAT (A/T) TTACACCAACCCC - 3' and 5'-GGGGAGGGGCGTTCTGCGAA - 3']^{15, 32}. For all reactions 100ng of each primer, 2mM dNTP mixture, 1.5mM MgCl₂, 2.5 U *Taq* DNA polymerase (Amplitaq gold-Perkin Elmer) were used in a total reaction volume of 25ml, including 1ml of sample of DNA. The reactions were cycled in a thermal cycler (PTC-100, MJ Research, Inc). The following conditions were used: an initial denaturation step at 95°C for 4 min, followed by 30 cycles at 94° for 30 sec, 50° for 30 sec for annealing, 72° for 30 sec for extension. In the final cycle the extension step was extended to 10 minutes. Positive and negative controls were used in all reactions performed. A 100-bp DNA ladder (Gibco BRL®) was used as a marker. Samples were regarded as positive when revealing bands of 120bp. Negatives samples were tested for the human β-globin gene to confirm that DNA was not degraded and that inhibition in PCR exams had not occurred. Procedures to avoid carryover contamination from previously amplified DNA were used routinely¹⁹.

PCR product analyses and DNA hybridization: the reaction products were visualized in 5% polyacrylamide gel electrophoresis stained with silver²⁹. The amplified DNA was denatured and applied to nylon membranes (Biodyne A, Gibco

BRL®) using a bio-dot apparatus (Hybri-dot manifold-BRL®). Probes composed of cloned minicircles from *L. chagasi* were radiolabeled with ³²P - [α]dCTP using the Random Primer DNA Labeling System (Gibco BRL)³². Hybridization conditions were as previously described¹.

Positive and negative controls: true negative and positive controls; confirmed by clinical, parasitological, serological and molecular tests; were included in all diagnostic tests performed.

Reproducibility: ten percent of blood samples collected on filter paper and DNA samples were randomly selected and marked for retesting; each duplicate received a different number from the original sample.

Nutritional status: malnutrition was determined by analyzing the children's weight and height for age according to the population pattern of the Reference National Center Health Statistics, using EPI-NUT/EPI-INFO (version 6.04d). Participants over ten years were evaluated using the ratio of weight by square height.

Statistical analysis: the agreement between qualitative tests was estimated by the kappa statistic²⁸; quantitative results were compared by Pearson's correlation (r), using log titer +1 (IFAT) and absorbances (ELISA).

RESULTS

Cross-sectional study. The description of the population studied was realized elsewhere²².

Table 1 shows the estimated prevalence rates by each serological test performed in eluates. Only eleven (6.4%) samples were simultaneously positive in two techniques. If we consider as probably infected those samples which showed a positive result in at least one test performed, the estimated prevalence rate was 10.7% (171/1604).

Table 1 - Reactivity of the samples collected in filter paper in a serological survey (n=1,604) to identify the seroprevalence of infection by *Leishmania* in General Carneiro-Sabará/MG, 1998.

| Serological Tests | Positive samples | |
|-------------------------------|------------------|--------------------------------|
| | number | prevalence ^a IC 95% |
| IFAT | 38 | 2.4 (1.7-3.3) |
| ELISA ^b | 54 | 3.4 (2.6-4.4) |
| Strip test | 90 | 5.6 (4.5-6.9) |
| Positive in at least one test | 171 ^c | 10.7 (9.2-12.2) |

^aPrevalence = positive test/1604. ^bCrude antigen. ^c11 samples were positive in two tests simultaneously: IFAT and ELISA= 8 ; Strip test and ELISA or IFAT= 3

The evaluation of test reproducibility (duplicates) showed good agreement for IFAT (kappa 0.74, 95% CI 0.40-1.0), regular for ELISA (kappa 0.53, 95% CI 0.18-0.89) and poor agreement for the strip test (kappa 0.14, 95% CI -0.11-0.40). Pearsons' correlation for ELISA absorbency and IFAT titers for duplicates were r=0.63 and r=0.76, respectively.

Follow up phase. Two hundred and fifty-six participants were invited to return for reevaluation 12 months after the

cross-sectional study. One hundred and eight positive participants included all the participants that were positive in ELISA or IFAT (n=84) and 50% (n= 44) of those positive in strip tests (randomly chosen among those positive in this test; a decision based on the poor reproducibility of the test). A subset of 128 negative participants was randomly selected among those negative in all tests performed.

Thirty participants were lost during the follow up (26 among the positive group and 4 among the negative) due to refusals, death, relocation or insufficient biological material. The final sample evaluated comprised 226 participants: 102 positive and 124 negative. Comparison of the demographic characteristics, such as age, gender and period of residence in General Carneiro, revealed no differences ($p > 0.05$) between the positive subjected included and those not included in this phase. The negative participants showed a similar result ($p > 0.05$) among the negative population when the same variables were compared. The characteristics of the 226 participants are shown in Table 2. This group constitutes a cohort and the data showed refer to this first evaluation.

Table 3 shows the results of serological tests of the sera of the 226 participants examined. Positive in sera include both positive and negative in the cross-sectional study. It is worth noting that 130 (57.5%) were positive in at least one test and 49 (37.7%) were simultaneously positive in two or three techniques; 96 (42.5%) were considered negative in all the tests performed.

Table 2 - Distribution of some demographical and social characteristics of the 226 participants from General Carneiro, Sabará/MG evaluated by serological and molecular techniques to diagnose asymptomatic visceral leishmaniasis

| Variables | Number | Percentage |
|---|-----------------|------------|
| Gender | | |
| female | 119 | 52.7 |
| male | 107 | 47.3 |
| Race | | |
| white | 55 | 24.3 |
| black | 43 | 19.0 |
| mullato | 128 | 56.6 |
| Age groups | | |
| >10 years old | 160 | 70.8 |
| 1 -10 years old | 66 | 29.2 |
| Education ¹ | | |
| college and high school | 21 | 12.0 |
| elementary school | 135 | 77.1 |
| no schooling | 19 | 10.9 |
| Income (minimum wage) | | |
| 0-3 | 102 | 45.1 |
| >3-15 | 124 | 54.9 |
| Natural of metropolitan region ² | | |
| yes | 146 | 68.5 |
| no | 67 | 29.6 |
| Period of residence | | |
| 1-5 years | 55 | 24.3 |
| > 5 years | 171 | 75.7 |
| mean \pm SD | 16.0 \pm 11.3 | |
| median (IQR) | 13.5 (7/24) | |

IQR1/Q3= 25% and 75% inter quartil range

¹only for more than 7 years old; n=175. ²data for refusals, not known and not done are not shown

Table 3 - Reactivity of the 226 sera for Leishmania infection in follow-up phase, General Carneiro-Sabará/MG, 1999.

| Serological Tests | Positive samples | |
|-------------------------------|------------------|-----------------------------|
| | n ^a | proportion ^a (%) |
| IFAT | 74 | 32.7 |
| ELISA | 60 | 26.5 |
| rk39-ELISA | 57 | 25.2 |
| positive in at least one test | 130 ^b | 57.5 |

^a Proportion= positive test/226. ^b 49 samples among of 130 samples were positive in two or three tests simultaneously

Molecular test results. Among the 226 samples examined, 133 were positive in hybridization with a specific probe for the *L. donovani* complex.

Figure 1 shows a representative electrophoresis gel of PCR products and dot-blot hybridization with specific probes. The PCR and hybridization negative samples were analyzed by PCR with specific primers for the human β -globin gene; the tests confirmed the presence of human DNA in all samples. In addition, the evaluation of test reproducibility, 23 blind duplicates, revealed that PCR showed good agreement (kappa 0.89, 95% CI 0.67-1.0).

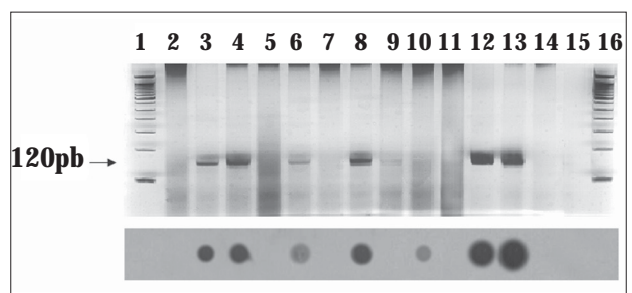


Figure 1 - Representative polyacrylamide gel showing an amplification product of 120bp from kDNA minicircles of Leishmania in blood samples from asymptomatic individuals and dot blot hybridization of the same PCR products performed with *L. donovani* complex specific kDNA probe. Lanes 3, 4, 6, 8 show samples that were positive in the PCR and hybridization assays; sample in lane 10 was positive for hybridization although the amplified products are not visible in the electrophoresis gel. Lane 9 shows a weakly PCR positive sample but negative for hybridization. Lanes 1 and 16: molecular size markers (100bp ladder). Positive controls: lane 12 - amplification product from the blood of a patient with kala-zar and lane 13 - DNA of cultured *L. chagasi*. Negative controls: lane 14 - non-infected individual and lane 15 - no DNA in the PCR reaction mixture.

Comparison among hybridization and serological methods. The comparison of the results obtained in hybridization and serologic tests is shown in Table 4. Among the 133 positive samples in hybridization, 74 (55.6%) were positive in at least one serological test and among the 93 negative in hybridization, 56 (60.2%) were positive in at least one serological test. Therefore, if the hybridization is considered a reference diagnostic test for asymptomatic VL, the sensitivity estimate of serological techniques ranged from 24.8 to 30.1% and specificity ranged from 63.4 to 76.3%.

Cross-reaction with Chagas' disease. Eight participants were identified as showing positive tests (Indirect Hemagglutination and IFAT) indicating possible *T. cruzi* infection; among these four were also reported as having Chagas' disease and six were positive in the hybridization or rk39-ELISA, suggesting a *T. cruzi/L. chagasi* coinfection.

Table 4 - Estimates of sensitivity and specificity of the diagnostic tests in identifying *L. chagasi* infection using the DNA hybridization as the reference test. General Carneiro -Sabará/MG, 1999.

| Serology | Positive in serology and hybridization | Sensitivity (IC 95%) ¹ | Negative in serology and hybridization | Specificity (IC 95%) ² |
|-------------------|--|-----------------------------------|--|-----------------------------------|
| IFAT | 40 | 30.1 (22.4-38.6) | 59 | 63.4 (52.8-73.2) |
| ELISA | 33 | 24.8 (17.7-33.0) | 66 | 71.0 (60.6-79.9) |
| rk39-ELISA | 35 | 26.3 (19.1-34.7) | 71 | 76.3 (66.4-84.5) |
| Serological tests | 74 | 55.6 (46.8- 64.3) ³ | 37 ⁴ | 39.8 (29.8-50.5) |

¹ positives in each serological test and hybridization /133 positive participants in hybridization. ² negatives in each serological test and hybridization /93 negative participants in hybridization. ³ positives in at least one serological test and hybridization/133 positive participants in hybridization. ⁴ negatives in all serological tests and in hybridization/93 negative participants in hybridization

Clinical examination. Signs and symptoms related to visceral leishmaniasis were neither observed nor reported during clinical examination. Only six participants (one child) showed low hematocrit (< 30%), but with no significant differences in all other clinical criteria used to define infection; (fever, hepatomegaly and/or splenomegaly and weight loss).

Malnutrition. Eight malnourished children were identified, though none of them presented progression to clinical disease. Two were positive in hybridization and four of them were positive only in serological tests.

Criteria for *L. chagasi* infection. Due to the disagreements found in the tests to identify asymptomatic infection using different diagnostic methods and the fact that which subjects were in fact infected remains undefined, different criteria were proposed to define *L. chagasi* infection. Criterion 1, greater specificity: include only participants that were positive in the crude antigen test and the specific antigen test and were confirmed by hybridization for *L. chagasi*; 17 (7.5%) participants fulfilled this criterion. Criterion 2, parasite presence: include all hybridization positive participants; 133 (58.8%) individuals were identified. Criterion 3, positive serology and hybridization: include all participants with a positive hybridization and presenting, at least, one positive serological test (crude or specific antigen); 74 (32.7%) participants were considered as infected by *L. chagasi*.

DISCUSSION

Herein, the presence of asymptomatic infection by *L. chagasi* in an urban area of Southeast Brazil was shown. The prevalence rates of *L. chagasi* infection estimated in a cross-sectional study, by serological methods, ranged from 2.4% (IFAT) to 5.6% (strip test), depending on the serological test used. When considering those samples that gave positive results in at least one of the tests performed, the estimated prevalence rate of infection was 10.7% (171/1604).

The lack of concordance among the serological tests was a limitation of the present investigation. The discordance among the serological tests can not be explained by errors in conducting the tests, because the evaluation of test reproducibility revealed a good agreement for IFAT, and regular for ELISA; only the strip test revealed poor concordance in the duplicates. The

correlations of absorbances (ELISA) or titers (IFAT) in duplicates also were good. Therefore, it can be concluded that serological methods are not appropriate for diagnosing asymptomatic infection in areas of low disease prevalence and occurrence of cutaneous leishmaniasis.

The DNA hybridization technique identified a greater number of positive participants when compared to the serological tests performed. Assuming DNA hybridization as the reference test for identifying individuals with active *L. chagasi* infection, the participants who showed positive serology and negative hybridization results can be considered as false-positives or self-cured of their infection. The positive serology in self-cured subjects could be a consequence of a gradual and slow decrease in titers, as demonstrated in other infections⁸ The serological tests were able to identify the presence of infection in 30.1, 24.8 and 26.3% of those with positive hybridization (IFAT, ELISA and rk39-ELISA, respectively). Therefore, when the hybridization is considered the reference test, serology (positive for at least one serological test) was able to identify 55.6% (74/133) of the individuals who were truly infected. Furthermore, serology was able to identify 39.8% (37/93) of the individuals who were truly negative; specificity of 63.4, 71.0 and 76.3%, for IFAT, ELISA and rk39-ELISA, respectively.

The greatest challenge of this investigation was to understand the disagreement among the diagnostic methods used to identify asymptomatic *L. chagasi* infection. The different antigens used to perform the tests may explain, in part, the low concordance and correlation among serological tests. The antigen rk39 presents high sensitivity and specificity for the *L. donovani* complex and has been associated with active disease². In the present study, among those positive in hybridization, rk39 showed high specificity (71/93= 76.3%), but low sensitivity for asymptomatic individuals (35/133=26.3%). It is also known that ELISA with crude antigen and IFAT present some limitations, such as cross-reacting with other parasitic infections (eg *T. cruzi* and other leishmaniasis) or the detection of past infection^{2, 26}. Therefore, the use of only one serologic test may not reflect the true prevalence of an infection in a population at risk, leading to misclassification bias in epidemiological studies.

The different algorithms proposed to identify infection present specific advantages and limitations. Criterion 1 is more rigorous in defining the infection; however, some true positives could have been excluded because the definition requires positive results in molecular and serological tests with crude and specific antigens. Sera from infected patients may show distinctive recognition of crude and specific antigens and patients with asymptomatic or self-healing infections may present low or undetectable levels of anti-k39 antibodies². Criterion 2 suggests the presence of circulating parasites in the blood, including individuals that were positive in hybridization, independent of the serological results. In criterion 3 some positives were probably excluded because the algorithm is dependent on a serological test and, as mentioned elsewhere, asymptomatic individuals present low levels of antibodies and minute amounts of circulating parasites when compared to symptomatic patients^{2, 26}. Thus, the performance of the serological tests

should be interpreted differently in epidemiological studies devised to identify asymptomatic infection in a population and in studies conducted to diagnose clinical cases or to confirm suspected cases. Moreover, negative hybridization may be due to the amount of blood used, which could have been insufficient to permit the extraction of parasite DNA²³.

The lack of a gold standard technique to confirm an active *L. chagasi* infection in asymptomatic individuals is an important drawback in population studies. The use of invasive procedures to detect the parasite (spleen, bone marrow, lymph node aspirates) it is not justified for ethical reasons when the patient does not show any symptoms of the disease, which was the case in the present study. Blood culture is not the method of choice to prove the presence of the parasite due to the low sensitivity when assayed in asymptomatic carriers²⁷. Although the molecular methods are not easily available for routine testing, DNA hybridization should be preferred in the identification of asymptomatic infection. The hybridization is more specific than serological tests in identifying active infection and can be used to discriminate false positives and false negatives. However, although it is high sensitivity, hybridization can not identify 100% of the true positive, since parasite presence may be intermittent in peripheral blood²⁰.

Other important finding of this study is the absence of clinical disease. The occurrence of asymptomatic and subclinical illness with nonspecific symptoms has been reported in investigations conducted in Northeastern Brazil. However, in these areas clinical cases have been found and the ratio of infected/diseased persons ranged from 6.5:1 to 28:1^{3,6,16}. In the area studied, a possible explanation for the absence of the disease would be the low level of malnutrition in the population studied, an important prognostic factor for disease progression^{4,9}. In this investigation, only eight malnourished children were identified and none developed clinical signs or symptoms of VL. Similar results were obtained in cohort studies carried out with children in northeastern Brazil, where asymptomatic and subclinical cases also did not progress to the acute form of VL^{6,17,18}. If *L. chagasi* infection does not progress to visceral leishmaniasis within the first or second year after infection, it is unlikely to do so in otherwise healthy individuals¹⁸. It is becoming clear that asymptomatic infections should be considered an important aspect of VL epidemiology. Published results suggest that phlebotomine sand flies do not acquire the infection from asymptomatic individuals¹⁰. Nevertheless, questions regarding the competence of asymptomatic individuals to infect the vector, especially when found in high numbers, remain unsolved¹¹.

The present VL control strategies in Brazil are based on the occurrence of symptomatic cases; it is important from a public health perspective, to understand the role of asymptomatic cases in maintaining the infection in urban areas and to identify risk factors leading to disease progression. Unfortunately, the disagreements in the test results observed in the present investigation suggest that the available diagnostic tests are not adequate for identifying asymptomatic infection. Based on the present data, the PCR products submitted to hybridization with

specific probes seem to discriminate the real infections and will improve confidence in diagnosing asymptomatic subjects in areas of low prevalence of clinical disease.

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