Antileishmania Immunological Tests for Asymptomatic Subjects Living in a Visceral Leishmaniasis-Endemic Area in Brazil

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Abstract. The objective of this study was to evaluate the behavior of different tests used for the diagnosis of visceral leishmaniasis (VL) in asymptomatic subjects living in an endemic area. No gold standard is available for the diagnosis of asymptomatic infection with *Leishmania*. In continuation of a previous study, 1,017 subjects living in a VL-endemic area were clinically reevaluated. Of these, 576 had at least one positive serological test in a first assessment. About 3 years after the first evaluation, none of the subjects had progressed to clinical VL. Among this group, 246 subjects were selected, and five serological tests (enzyme-linked immunosorbent assay [ELISAp], ELISArK39, ELISArK26, indirect immunofluorescence test [IIFT] using *L. amazonensis* promastigote antigen, and an immunochromatographic test using rK39 antigen [TRALd]) and the Montenegro skin test (MST) were repeated. There was a significant increase in the number of subjects who tested positive in the MST, IIFT, ELISAp, and ELISArK39 in the second evaluation. For all tests, there were subjects who tested positive in the first evaluation and negative in the second evaluation. A positive result in the serological tests and MST in subjects from the endemic area studied did not indicate a risk of progression to VL and may only be temporary.

INTRODUCTION

In Brazil, the incidence of visceral leishmaniasis (VL) has increased over the years despite the control measures established by the Ministry of Health. These measures are mainly based on the identification and early treatment of human cases of the disease, serological surveys for canine leishmaniasis followed by euthanasia of infected dogs, and vector control (chemical and environmental management).¹

The role of asymptomatic humans infected with *Leishmania chagasi* in the transmission chain of American VL has not been well-established, a fact that might explain why the control of the disease is not effective. Classically, humans are not considered to be a reservoir of *L. chagasi* in the New World,^{2,3} but this possibility could be properly evaluated only if a feasible and unequivocal tool for the diagnosis of asymptomatic cases were available. In addition, the identification of new infected individuals in endemic areas would also serve as an indicator of the effectiveness of control measures designed to reduce the number of individuals exposed to *Leishmania*. However, the diagnosis of asymptomatic infection with *L. chagasi* is still a matter of controversy and requires larger studies.⁴

The standardization and validation of diagnostic methods for VL usually include patients with the classical clinical presentation of the disease and controls. The gold-standard techniques used for the detection of the parasite are not justified in asymptomatic cases of the disease, because they may pose a risk or excessive discomfort to the individual without providing evident benefit. Thus, indirect methods for the evaluation of exposure to the parasite have been widely used as tools for the diagnosis of subclinical infection.

As early as in 1964, Manson-Bahr and Southgate⁵ called attention to a group of subjects with a positive skin test and no

clinical history of leishmaniasis that did not develop the disease. Since then, the Montenegro skin test (MST) has become a diagnostic tool for previous infection with *L. chagasi* in VL-endemic areas and can be used to characterize asymptomatic infection according to various investigators.^{6–8}

Serological tests also frequently identify individuals who live in endemic areas and have a positive VL test but show no clinical signs or symptoms of the disease. These individuals are classified as asymptomatic carriers of *L. chagasi* by different investigators. Several tests have been used to estimate the frequency of subclinical infection in these areas, with enzyme-linked immunosorbent assay (ELISA) using promastigote antigen being the most cited method.^{9–11} Burns and others,¹² standardizing recombinant antigen rK39, suggested that ELISA using rK39 may serve as a tool for the diagnosis of asymptomatic cases.

In most studies regarding the diagnosis of asymptomatic cases of VL, the authors selected one serological test and/or skin test, and positive subjects were considered to be carriers of infection. However, comparison of five distinct serological tests performed at the same time showed low agreement between the results obtained.¹³ This panorama indicates the difficulty in selecting the best test for the detection of subclinical infection. Within this context, the present study evaluated the clinical and evolutionary behavior of a positive result in different VL serological tests and the MST in subjects from an endemic area.

SUBJECTS AND METHODS

First, 1,241 subjects living in the endemic area of Porteirinha underwent clinical and laboratory evaluation between 1998 and 1999 using five serological tests (ELISA using promastigote antigen [ELISAp], ELISA using recombinant K39 [ELISArK39], ELISA using K26 [ELISArK26] antigens, an indirect immunofluorescence test using *L. amazonensis* promastigote antigen [IIFT], and an immunochromatographic test using rK39 antigen [TRALd])¹³ and the MST.¹⁴ None of these subjects had a history of a diagnosis of VL or had

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received specific treatment. In continuation of this cross-sectional study, the same population was reassessed between January and August 2002, about 3–4 years after the first evaluation.

All 1,241 subjects of the initial study were invited by letter to attend the Health Service for clinical reevaluation. However, we encountered difficulties in the delivery of the letter, because there were errors in the address provided or the subject had moved to another town. In addition, not all subjects who received the letter appeared at the service. Therefore, a total of 1,017 subjects (82% of the initial sample) were clinically reassessed by anamnesis and physical examination performed by an infectologist.

Among these subjects, 246 were selected, and a new blood sample was collected. All initial serological tests and the skin test were repeated based on the following information: group I ($N = 122$) consisted of subjects who, in the initial study, tested positive for leishmaniasis in the IIFT and/or TRALd and/or MST and negative for Chagas disease by indirect hemagglutination and IIFT, and group II ($N = 124$) consisted of subjects who tested negative in all five serological tests for leishmaniasis and in the MST and who presented negative serology for Chagas disease. Group II was matched for gender and age to group I. Group I should include all subjects of the initial study who met the inclusion criteria established. However, some of the subjects of this group refused to participate, and difficulties in delivering the invitation letter were encountered. Therefore, 122 of 195 subjects invited were available for reassessment and were matched for gender and age to group II. There were no significant differences in the previous results between the group invited and the group studied eventually.

The serum samples were collected between June and October 2002 and were processed in November and December 2002. The TRALd, ELISArK39, and ELISArK26 tests were performed at the Laboratory of Immunology, Federal University of Triângulo Mineiro (UFTM). Another aliquot stored at -20°C was kept on dry ice and sent to the Laboratory of Leishmaniasis and Vaccines, Institute of Biological Sciences, Federal University of Minas Gerais (ICB/UFGM), Belo Horizonte, for ELISA and IIFT using promastigote antigen. All laboratory researchers were unaware of the origin of the samples. All serum samples were analyzed by ELISAp, ELISArK26, ELISArK39, IIFT, and TRALd using the procedures of the initial study as described below.

TRALd (rK39). For the immunochromatographic test, a kit (InBios International, Seattle, WA) consisting of paper strips coated with rK39 antigen was used according to manufacturer's instructions. The strips were stored at room temperature ($28\text{--}30^{\circ}\text{C}$) or were refrigerated ($2\text{--}8^{\circ}\text{C}$) and protected from humidity. The buffer solution was stored at $2\text{--}8^{\circ}\text{C}$. Readings were obtained 10 minutes later according to manufacturer's recommendations, and the results were reported as positive or negative.

IIFT. The IIFT was performed as described by Camargo¹⁵ using as antigen *L. amazonensis* (MHOM/BR/60/BH6) promastigotes in the exponential growth phase cultured in liver infusion tryptose (LIT) medium. Fluorescein isothiocyanate-labeled human immunoglobulin G (IgG) antiglobulin obtained from rabbit immune serum was used as conjugate (Biomanguinhos, Rio de Janeiro, Brazil). All samples testing positive at a dilution of 1:80 were considered to be reactive.

MST. Antigen of *L. amazonensis* promastigotes (IFLA/BR/67/PH8), produced at the Laboratory of Leishmaniasis and Vaccines, ICB/UFGM, was used at a standard concentration of 40 g/mL protein nitrogen diluted 1:10,000 in saline merthiolate.¹⁶ Antigen (0.1 mL) was applied intradermally to the anterior side of the left forearm. Readings were obtained 48–72 hours after injection, and the size of the papule was delimited with a ball-point pen.¹⁷ The reactions were considered to be positive when the mean of cross-sectional and longitudinal diameters was 5 mm or higher.

ELISA using recombinant antigens (ELISArK39 and ELISArK26). The assays were carried out according to Badaró and others¹⁸ using recombinant K39 and K26 antigens of *L. chagasi* produced by InBios International. The serum samples were diluted 1:50 and 1:100 and developed with peroxidase-conjugated protein A (Sigma Co., St. Louis, MO).

ELISA using promastigote antigen (ELISAp). The assay was carried out as described by Hommel and others¹⁹ and Voller and others²⁰ using as antigen *L. amazonensis* (MHOM/BR/60/BH6) promastigotes in the stationary growth phase cultured in LIT medium and lysed by sonication. Serum samples were tested at an initial dilution of 1:80.

A standard permitting comparison between the first and second evaluation was established to define the ELISA cut-off values. Because the tests using sera from subjects of the non-endemic area (Argentina) were not repeated, this group did not exist as a negative control for calculation of the cut-off. We, therefore, chose as a reference subjects from an endemic area who could serve as controls. The TRALd, IIFT, and MST, whose cutoff values are well-defined in the literature, were used as a parameter (TRALd: presence of a line of the labeled antigen, IIFT: $\geq 1:80$, and MST: ≥ 5 mm), and positive and negative results in the first and second evaluation could, therefore, be determined directly. In addition, the IIFT and TRALd presented the best sensitivity ($> 80\%$) in the initial study. Thus, for ELISA, the cutoff in the two evaluations was established based on the results of the three previous tests (IIFT, TRALd, and MST) as follows: subjects with a positive IIFT and/or TRALd and/or MST in the first and second evaluation were defined as positive, and subjects with a negative result in the three tests of the two evaluations were defined as negative. Next, the receiver operating characteristic (ROC) curve provided by the MedCalc 10.1 program (MedCalc Software, Mariakerke, Belgium) was used to determine the cutoff values of each ELISA assay based on the calculation of sensitivity, specificity, and likelihood ratios.

A positive result in each diagnostic test was compared between the first and second evaluation by the McNemar test. Optical densities were compared between the two groups by the Mann-Whitney test. Agreement between the diagnostic tests was determined by the κ coefficient. Statistical analysis was performed using the Statistica 8.0 software (Statsoft, Inc., Tulsa, OK). A P value < 0.05 was considered to be significant.

Informed consent was obtained from all participants, and the project was approved by the Board of UFTM (Resolution 196/96 of the National Research Council, Brazilian Ministry of Health).²¹

RESULTS

Clinical reassessment of the 1,017 subjects showed that none of them presented a clinical history or physical signs

TABLE 1
Cutoff values established for the ELISA assays based on the results of the ROC curve

Diagnostic test	Cutoff value	TP	FP	FN	TN	Sensitivity (%)	Specificity (%)	LR+	LR-	Area under the ROC curve (95% CI)	P value
First evaluation											
ELISAp	0.070	83	27	39	97	68.0	78.2	3.1	0.4	0.779 (0.722–0.829)	< 0.001
ELISArk39	0.308	72	33	50	91	59.0	73.4	2.2	0.6	0.667 (0.604–0.726)	< 0.001
ELISArk26	0.084	100	91	22	33	81.9	26.6	1.1	0.7	0.511 (0.446–0.575)	0.773
Second evaluation											
ELISAp	0.150	110	32	46	58	70.5	64.4	2.0	0.5	0.697 (0.636–0.754)	< 0.001
ELISArk39	0.323	102	46	54	44	65.4	48.9	1.3	0.7	0.580 (0.516–0.642)	0.031
ELISArk26	0.343	92	41	64	49	58.9	54.4	1.3	0.8	0.547 (0.483–0.610)	0.213

TP = true positive; FP = false positive; FN = false negative; TN = true negative; LR+ = positive likelihood ratio; LR- = negative likelihood ratio; 95% CI = 95% confidence interval.

compatible with VL (fever, paleness, visceromegaly, adynamia, or weight loss) during the interval between the first and second evaluation or at the time of the second evaluation. It should be mentioned that 19 cases of human VL occurred in Porteirinha, the study area, during the period investigated. All signs and symptoms reported by the 246 subjects were compared with the results of the laboratory tests, and no association was observed between positive tests and the presence of any symptom.

The ELISA cutoff values were established by the ROC curve, considering the optical density at which the tests presented, at the same time, the best sensitivity, specificity, and likelihood ratios (Table 1). This definition was used to evaluate the evolution of the serological tests over time in the 246 subjects reevaluated (Table 2). For the MST, IIFT ELISAp, and ELISArk39, a significant increase in the number of seropositive subjects was observed in the second evaluation. In contrast, a significant reduction in the number of positive subjects was observed for the TRALd and ELISArk26 when comparing the two assessments. For all tests, there were subjects who tested negative in a previously positive test.

The optical densities obtained with the three ELISA assays were significantly higher in the second evaluation than in the first one (Figure 1). This was even the case for ELISArk26, for which no increase in the number of seropositive subjects was observed.

Regarding the agreement between the results of the tests in the second evaluation, the best κ coefficients (0.42) were observed for the comparison of IIFT/ELISAp (Table 3). The TRALd results presented low agreement with ELISArk39 ($\kappa = 0.00$).

DISCUSSION

In the initial study, a positive result in at least one of the serological tests studied was observed in 576 (46.4%) subjects and in the skin test in 146 (11.7%) subjects.^{13,14} Clinical reassessment 3–4 years after the first evaluation revealed that none of the subjects who tested positive developed classical VL. This result would be expected for subjects with a positive MST. However, for the serological tests, we observed that the disease was not diagnosed during the incubation period on any occasion, as shown by other investigators in Brazil for ELISAp.^{9,10} In India, follow-up of asymptomatic individuals (i.e., household contact with patients with a recent diagnosis of the disease) who tested positive by ELISArk39 showed that 69% of them developed kala-azar within a period of 12 months.²² Our results agree with other investigators who clinically followed up asymptomatic individuals in Brazil who tested seropositive by ELISArk39, and we found that none of them developed the disease.^{4,23,24}

One aspect to be considered is the observation that, in the first study, the tests presented a specificity ranging from 96% to 100% using subjects from a non-endemic area as a reference. As a consequence, there was a false-positive rate of up to 4%. Similarly, part of the seropositive subjects followed-up in the endemic area may have had a false-positive test result.

Another limitation is that the 246 samples were retested 3–4 years after the first evaluation. Changes in the environmental conditions may have occurred during this period, which would interfere with the results. However, to test all samples at the same time, we would have to modify the storage

TABLE 2
Comparison of the results of the diagnostic tests between the first and second evaluation of subjects from a VL-endemic area

Diagnostic test	First evaluation	Second evaluation		
	Total positive	Remained positive	Became positive	Total positive
Positive	122/246 (49.6%)	105/122 (86.1%)	51/124 (41.1%)	156/246 (63.4%)
MST	71/246 (28.9%)	62/71 (87.3%)	52/175 (29.7%)	144/246 (46.3%)
TRALd	31/246 (12.6%)	3/31 (9.7%)	2/215 (0.9%)	5/246 (2.0%)
IIFT	31/246 (12.6%)	24/31 (77.4%)	63/215 (29.3%)	87/246 (35.4%)
ELISAp	110/246 (44.7%)	84/110 (76.4%)	58/136 (42.7%)	142/246 (57.7%)
ELISArk39	105/246 (42.7%)	75/105 (71.4%)	73/141 (51.8%)	148/246 (60.2%)
ELISArk26	191/246 (77.6%)	107/191 (56.0%)	26/55 (47.3%)	133/246 (54.1%)
Negative	124/246 (50.4%)	73/124 (58.9%)	17/122 (13.9%)	90/246 (36.6%)
MST	175/246 (71.1%)	123/175 (70.3%)	9/71 (12.7%)	132/246 (53.7%)
TRALd	215/246 (87.4%)	213/215 (99.1%)	28/31 (90.3%)	241/246 (98.0%)
IIFT	215/246 (87.4%)	152/215 (70.7%)	7/31 (22.6%)	159/246 (64.6%)
ELISAp	136/246 (55.3%)	78/136 (57.4%)	26/110 (23.6%)	104/246 (42.3%)
ELISArk39	141/246 (57.3%)	68/141 (48.2%)	30/105 (28.6%)	98/246 (39.8%)
ELISArk26	55/246 (22.4%)	29/55 (52.7%)	84/191 (44.0%)	113/246 (45.9%)

McNemar test ($P < 0.001$ for all comparisons between first and second evaluation).

FIGURE 1. Comparison of optical densities obtained with the ELISAp, ELISArK39, and ELISArK26 tests between the first and second evaluation (— = test cutoff; — = group mean).

conditions of the samples, with the initial samples being tested after 3–4 years of freezing, resulting in another type of bias.

The TRALd results need to be analyzed separately. In contrast to the other tests studied, the number of positive reactions was significantly smaller in the present investigation. This finding suggests differences in the interpretation of the test results in terms of their definition as positive or negative. The result of the TRALd depends on the visual evaluation of the presence or absence of a line labeled with rK39 on the immunochromatographic strip. In classical cases of the disease, this line is clearly visible and forms rapidly, with an unquestionable positive result in most cases. However, in asymptomatic individuals from endemic areas, this line is tenuous, and its interpretation may generate controversies. A lower quantity of antibodies in these subjects with asymptomatic infection may explain the lighter impression on the immunochromatographic strip. This observation was also made by Moreno and others,²⁵ who established, for the diagnosis of asymptomatic infection by TRALd, that the test result should be interpreted by three observers. The authors found wide disagreement between assessments and low reproducibility of the results ($\kappa = 0.14$).²⁵ This bias might explain the low agreement between the TRALd and ELISArK39 results, because both tests use the same antigen. In the present study, we observed the best

κ agreement between the results of tests that use similar antigens.¹³ Taken together, this rapid immunochromatographic test shows poor suitability for the diagnosis of asymptomatic *L. chagasi* infection.

The higher positivity rate obtained for the MST, IIFT, ELISAp, and ELISArK39 in the second evaluation might be associated with the permanence of the subject in the endemic area; the subject consequently continues to be exposed to the vector and thus, presents a chance of being infected within the interval of 3–4 years between assessments. An interesting finding was the increase of optical densities in the ELISA tests even in those subjects who continued to be positive. Repeated reinfections possibly exert a booster effect on the production of antileishmania antibodies, a fact that might explain the evident increase of optical density in the second evaluation.

With respect to a more detailed discussion of the MST results, the positivity rate obtained for the test increased from the first to the second evaluation, and as expected, most patients who tested positive in the first evaluation continued to be positive in the second. This result might be explained by possible new infections during the interval between the first and second evaluation. In addition, serial tests are known to trigger positivity in the subsequent test merely because of a booster effect of leishmanin.^{14,26} In contrast, one interesting

TABLE 3
 κ coefficients obtained for the different diagnostic tests in the second evaluation of subjects from a VL-endemic area

Comparisons	Agreement			
	Positive in both	Negative in both	Total agreement	κ (95% CI)
MST				
TRALd	1 (0.9%)	128 (97.0%)	129 (52.4%)	−0.02 (−0.16–0.11)
IIFT	46 (40.4%)	91 (68.9%)	137 (55.7%)	0.09 (−0.03–0.22)
ELISAp	74 (64.9%)	64 (48.5%)	138 (56.1%)	0.13 (0.01–0.25)
ELISArK39	80 (70.2%)	64 (48.5%)	144 (58.5%)	0.18 (0.06–0.30)
ELISArK26	69 (60.5%)	68 (51.5%)	137 (55.7%)	0.12 (0.00–0.24)
TRALd				
IIFT	4 (80.0%)	158 (65.6%)	162 (65.9%)	0.05 (−0.11–0.21)
ELISAp	3 (60.0%)	102 (42.3%)	105 (42.7%)	0.00 (−0.11–0.11)
ELISArK39	3 (60.0%)	96 (39.8%)	99 (40.2%)	0.00 (−0.10–0.10)
ELISArK26	1 (20.0%)	109 (45.2%)	110 (44.7%)	−0.03 (−0.14–0.09)
IIFT				
ELISAp	77 (88.5%)	94 (59.1%)	171 (69.5%)	0.42 (0.31–0.53)
ELISArK39	57 (65.5%)	68 (42.8%)	125 (50.8%)	0.07 (−0.05–0.19)
ELISArK26	53 (60.9%)	79 (49.7%)	132 (53.7%)	0.09 (−0.03–0.22)

finding was that nine previously positive subjects tested negative in the second evaluation. The MST is a delayed hypersensitivity reaction that depends on the cellular immune response to *Leishmania* antigens. Investigation of the subjects with a negative test result showed no immunosuppressive condition that would explain this behavior of the MST in any of them. Another possibility to explain this finding is a poor antigenicity of the antigen used in the second MST, as reported by other investigators.²⁷ However, this explanation does not apply, because an increase in the frequency of positivity was observed in the present evaluation.

Some investigators believe that a positive MST may indicate immunity of the individual against the disease.^{5,6} In this respect, Bern and others²⁷ showed that subjects from an endemic area with a positive MST presented a significantly lower chance to progress to classical VL than those with a negative MST. However, other investigators propose that, if this resistance really exists, it should be temporary, a suggestion that would explain the findings of the present study.^{28–30} In treated cases of the disease that progress to clinical cure, the MST classically becomes positive as a consequence of the restored immune response, which, in principle, should protect the individual against new relapses.^{31–33} However, some investigators have shown that this rate of post-clinical cure positivity is not as high as expected (about 40%) and may not increase over time post-treatment; additionally, negativity is not associated with a higher chance of relapse.^{27,32,34} These findings might be caused by the lack of a standard antigen used for the skin tests in different studies.

Difficulties exist in establishing a gold standard for the diagnosis of asymptomatic infection with *L. chagasi*.^{4,13,25} The comparison of tests may provide some additional information that contributes to the selection of the serological tests that best discriminates between infected and uninfected subjects. In this respect, ELISA assays, which are easily performed compared with IIFT and MST, would be an interesting option for this diagnosis. However, in the present study, none of tests presented a concordant positive result with the other tests or was superior for the identification of positive subjects. For confirmation of the results, it would be interesting to test the samples two times. This was not done because of the high cost of the large number of samples and number of tests evaluated. The ideal situation would be if only one test able to diagnose subclinical infection could be selected. It does not seem justifiable to routinely perform various tests only for detection of infection.

The low agreement between the results of the serological tests may indicate that each test detects different stages of infection in the group of asymptomatic subjects from the endemic area. On the basis of this assumption, all seropositive subjects would be carriers of asymptomatic infection, but the fact that none of the subjects progressed to clinical disease weakens this hypothesis. However, a low reproducibility of the serological tests used for the diagnosis of asymptomatic infection in the endemic area seems to be more likely. Therefore, the serological tests used here do not seem to be adequate tools for the diagnosis of asymptomatic infection.

Finally, the present study permits us to conclude that a positive result in the serological tests and in the MST in subjects from the endemic area studied did not indicate a risk of progression to VL and may only be temporary. Further studies are necessary to identify the best tool for the diagnosis

of asymptomatic infection with *L. chagasi* in endemic areas and based on a more precise diagnosis of infected individuals, to define their true role in the maintenance of VL in urban areas.

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