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Accuracy of an ELISA and indirect immunofluorescence for the laboratory diagnosis of American tegumentary leishmaniasis

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Summary   We compared the accuracy of ELISA and indirect immunofluorescence (IIF) using *Leishmania braziliensis* and *L. major*-like antigens and antigens from the Bio-Manguinhos kit for serological diagnosis of American tegumentary leishmaniasis (ATL). Cut-off values were defined by the area under the receiver-operating characteristic curve. For ELISA, statistical analyses revealed better accuracy [95.7% sensitivity, 100% specificity, 100% positive predictive value (PPV), 97.5% negative predictive value (NPV)] and reliability [intraclass correlation coefficient (ICC): 0.940] for *L. braziliensis* antigen compared with *L. major*-like antigen (78.7% sensitivity, 82.8% specificity, 73.3% PPV, 86.6% NPV, ICC: 0.833). ELISA optical density values obtained for both antigens were higher in mucosal forms of ATL. For IIF, sensitivity and specificity were 81.5 and 86.2%, respectively, for the *L. braziliensis* antigen, compared with 95.4 and 77.7% for the *L. major*-like antigen and 75.4 and 89.2% for the Bio-Manguinhos kit. No difference in the specificity of the IIF test was observed between antigens, whereas sensitivity differed between the *L. braziliensis* and *L. major*-like antigens and the Bio-Manguinhos kit. Parallel ELISA and IIF testing increased sensitivity, irrespective of the antigen employed, and serial testing increased overall specificity.

1. Introduction

Leishmaniasis is one of the main public health problems in Brazil and, according to WHO, is among the six most important parasitic diseases in Latin America. Leishmaniasis is considered a zoonosis, as animals are the main confirmed source of infection, with transmission occurring through a wild or domestic cycle.[1] The Brazilian Ministry of Health's guidelines recommend using indirect immunofluorescence (IIF) for the diagnosis of human and canine visceral leishmaniasis[2] but not for American tegumentary leishmaniasis (ATL). However, IIF is commonly employed as a routine diagnostic method for ATL in southeastern Brazil. The IIF test uses as its antigen a parasite that might differ from the etiological agent circulating in this region, mainly restricted to *Leishmania* (*Viannia*) *braziliensis*. Although not a fatal disease, ATL has relevant clinical implications, is 10 times more frequent in Brazil than visceral leishmaniasis, and also involves a broad spectrum of differential diagnoses. The large number of methods used for serological tests demonstrates the difficulty in providing a sufficiently accurate ancillary method for clinical diagnosis. Simplicity in execution and low cost are advantages of using serological tests as diagnostic and screening methods in areas where biopsy or histopathology are not feasible.

The objective of this study was to compare the performance of ELISA and IIF in the diagnosis of cutaneous or mucosal ATL caused by *L.* (*V.*) *braziliensis* among patients seen at Centro de Referência em Leishmanioses, Instituto de Pesquisa Clínica Evandro Chagas (CrLeish/IPEC), Fundação Oswaldo Cruz (Fiocruz), using *L. major*-like or *L.* (*V.*) *braziliensis* as antigens for the detection of anti-*Leishmania* IgG.

2. Materials and methods

A cross-sectional diagnostic study was designed for masked comparison of diagnostic techniques (ELISA and IIF) for ATL. The target population included the first serum sample of all patients with suspected ATL seen at CRLeish/IPEC between 2003 and 2005. The control group consisted of serum samples from subjects with negative Montenegro skin tests (MST).

Assays were carried out on 251 serum samples obtained from 94 patients with parasitological confirmation of ATL (74 with the cutaneous form, 14 with the mucocutaneous form and 6 with the mucosal form), 65 controls comprising patients with diseases included in the differential diagnoses of ATL (15 with pyodermitis, 10 with vascular ulcers, 3 with cutaneous tuberculosis, 2 with dermatophytosis, and 34 with sporotrichosis, an increasingly common diagnosis in Rio de Janeiro over the last few years), and 92 asymptomatic subjects with negative MSTs. Blood samples for the tests were collected from all cases and controls included in the study before the patients underwent MST. All selected patients were from the State of Rio de Janeiro and lived in areas considered to be endemic for leishmaniasis caused by *L.* (*V.*) *braziliensis*. All study subjects gave signed informed consent.

2.1. Antigens

Antigens used were obtained from the promastigote forms of *L. braziliensis* (MHOM/BR/75/M2903) and *L. major*-like (MHOM/BR/76/JOF) maintained in brain—heart infusion medium supplemented with 10% fetal bovine serum, 200 U/ml penicillin, 200 μg/ml streptomycin and 1% human urine. An initial inoculum of 1×10^6 promastigotes was collected in the stationary phase and washed three times in PBS. The sediment resulting from the last wash was resuspended in buffer containing protease inhibitors and submitted to ultrasound at 50/60 Hz (Transonic 310, Elma, Durhan, NC, USA) until intact parasites were no longer visible in the suspension by light microscopy. For ELISA, partially soluble antigen (PSA) was obtained by centrifugation at 12 000 *g* for 10 min and its protein content was determined by the Lowry method[3] using Folin phenol reagent. For IIF, intact promastigote forms (IPF) were stored in PBS containing 2% formalin.

2.2. ELISA

ELISA was carried out according to the method described by Voller et al.[4] Briefly, plates (PoliSorp, Nunc, Rochester, NY, USA) were sensitized with PSA at concentrations of 2.5 and 5.0 μg/ml for *L. braziliensis* and of 5.0 and 10.0 μg/ml for *L. major*-like, defined according to the cut-off of maximum sensitivity. Serum samples were diluted 1:40 in the reactions with *L. braziliensis* PSA and 1:20 in the reactions with *L. major*-like PSA. Anti-human IgG peroxidase conjugate (Sigma Chemical Co., St. Louis, MO, USA) was diluted 1:5000 for the *L. braziliensis* PSA and 1:1000 for the *L. major*-like PSA. The reactions were developed by the addition of developing solution containing hydrogen peroxide and ortho-phenyldiamine (Sigma). Absorbance was read at 492 nm [ELISA optical density (OD) readings] in an automated plate reader (Tecan, Spectran Classic, Mannedorf/Zurich, Switzerland). The accuracy study was performed using one single 'PSA reagent lot'. The reliability study included two readings using this same lot.

2.3. Indirect immunofluorescence

IIF was carried out for *L. braziliensis* and *L. major*-like IPF and antigens from the Bio-Manguinhos kit according to the

method described in Bio-Manguinhos kit insert.[5,6] The slides were examined under an Olympus BX 40 fluorescence microscope working in the epiluminescence mode at different times by three observers (observers A and B trained, and observer C under training). The accuracy study and inter-rater reliability were performed using one single reagent lot. To perform a reading with a different lot in our IIF reliability study, a second reagent lot was made up for *L. braziliensis* and *L. major*. Observer A made three readings at different times and, for the last reading, the second lot was used.

2.4. Statistical analysis

Parameters to describe the accuracy of a diagnostic test usually include sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV). Sensitivity is the probability that a test result will be positive when the disease is present. Specificity indicates the proportion of people without disease who have a negative test. VPP is the proportion of people with a positive test who actually have the disease and expresses the degree to which a positive test result represents disease. NPV is the comparable statistic for a negative test: the probability that the disease is not present when the test is negative.

Sensitivity, specificity, PPV and NPV were calculated with the WinPepi 4.0 computer Program for Epidemiological Analysis with their respective 95% CI[7] and compared using the χ^2 test, with $P < 0.05$ (all PEPI programs can be downloaded free from http://www.brixtonhealth.com). Statistical analysis was performed using SPSS WIN 11.0 (SPSS Inc., Chicago, IL, USA). The sensitivity and specificity of combined ELISA and IIF testing in series and in parallel using the various antigens were also calculated in order to determine whether a combination of these methods has a higher value in the serological diagnosis of ATL.

Cut-off values were established for ELISA OD measurements using a receiver-operating characteristic (ROC) curve, and the performance of the tests was compared based on the area under the ROC curve (AUC) using MedCalc 8.0 software, Mariakerke, Belgium (a license can be ordered online from http://www.medcalc.be). ROC curve analysis is used to evaluate the ability of a test to discriminate disease cases from normal cases or to compare the diagnostic performance of two or more diagnostic tests. In an ROC curve, the true positive rate (sensitivity) is plotted against the false positive rate (100 − specificity) for different cut-off points. Each point on the ROC plot represents a sensitivity/specificity pair corresponding to a particular decision threshold. A test with perfect discrimination between the two groups (no overlap in the two distributions) has an ROC plot that passes through the upper left corner (100% sensitivity, 100% specificity) and the area under the ROC curve equals 1. Therefore, the closer the ROC plot is to the upper left corner, the higher the overall accuracy of the test.[8]

The reliability of the continuous test (ELISA) was analyzed using the intraclass correlation coefficient (ICC) with a 95% CI, and that of the dichotomous test (IIF) was determined using the κ index.[9] Inter-rater reliability was used to evaluate the agreement between two classifications and is quantified by the κ index, which can be interpreted as follows: <0.20 poor; 0.21−0.40 fair; 0.41−0.60 moderate; 0.61−0.80 good; 0.81−1.00 very good. ICC (two-way model with fixed raters) produces measures of consistency of values within cases. The maximum value of an ICC is 1; the lower limit is an indeterminate negative value. It has been suggested that ICC values above 0.75 should be regarded as evidence of excellent, and values above 0.4 as evidence of good reliability.

The correlation between duration of the disease in days and ELISA OD readings was evaluated by Pearson's correlation coefficient. Correlation coefficients (ρ) express the degree to which two variables change correspondingly and may vary from 1 (highest possible degree of correlation) to −1 (lowest possible degree, or inverse correlation). Spearman's correlation coefficient was used to study correlations between OD and the ATL clinical form. Mean OD values were compared according to the clinical form by Student's *t* test.

3. Results

The performance of the tests according protein concentrations was compared based on the AUC and reached 0.99 for the *L. braziliensis* antigen at concentrations of 2.5 and 5.0 μg/ml, and 0.85 and 0.89 for the *L. major*-like antigen at concentrations of 5 and 10 μg/ml, respectively (Figure 1).

3.1. ELISA

ELISA carried out using the homologous *L. braziliensis* antigen showed significantly higher accuracy parameters than ELISA using the *L. major*-like antigen (Table 1).

The ELISA cut-off values defined based on the ROC curve were 0.230 for the *L. braziliensis* antigen (Figure 2A) and 0.095 for the *L. major*-like antigen (Figure 2B), suggesting that ELISA OD values were higher in the cutaneous and mucosal forms of ATL when using the *L. braziliensis* antigen compared with the *L. major*-like antigen.

Analysis of reliability (intra-rater) revealed a significantly lower ICC (95% CI) for the *L. major*-like antigen (0.833,

Table 1 Sensitivity, specificity and positive and negative predictive values (PPV and NPV) with their respective 95% CI of ELISA performed using the *Leishmania major*-like and *L. braziliensis* antigens

	L. major-like	*L. braziliensis*	*P*-value
Sensitivity (%)	78.7 (69.1−86.5)	95.7 (89.5−98.8)	<0.001
Specificity (%)	82.8 (76.0−88.3)	100.0 (97.7−100)	<0.001
PPV (%)	73.3 (63.5−81.6)	100.0 (95.9−100.0)	<0.001
NPV (%)	86.6 (80.2−91.7)	97.5 (93.8−99.3)	<0.001
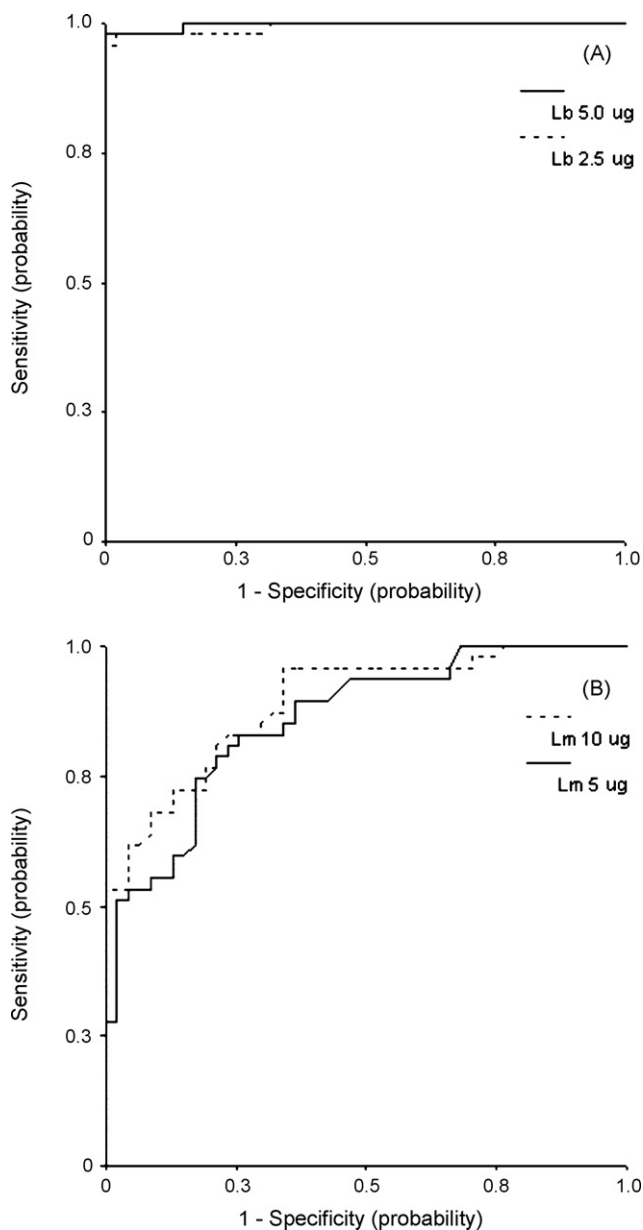
Figure 1   (A) The area under the receiver-operating characteristic (ROC) curve obtained for *Leishmania braziliensis* antigen at concentrations of 2.5 and 5.0 μg/ml (A) almost equals 1 (the ROC curve reaches the upper left corner of the plot). (B) By contrast, for *L. major*-like antigen at concentrations of 5.0 and 10.0 μg/ml, a more moderate performance is noted. In both cases, this occurs independently of concentrations used.

0.791; 0.867) compared with the *L. braziliensis* antigen (0.940, 0.924; 0.953) (*P* = 0.001).

ELISA using the *L. braziliensis* antigen showed a positive and moderate correlation (Spearman's correlation coefficient, ρ = 0.77, *P* = 0.001) between the mucosal/mucocutaneous clinical form and OD values, which were higher than those obtained for the cutaneous form and also higher than those observed with the *L. major*-like antigen (ρ = 0.064). The differences in mean OD values according to clinical form and antigen used did not reach statistical significance (Table 2).
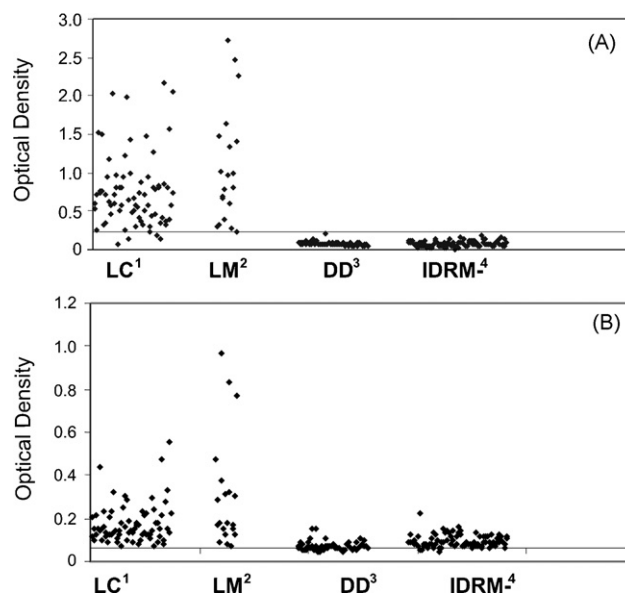
Figure 2   Distribution of ELISA optical density values obtained for (A) *Leishmania braziliensis* antigen (cut-off: 0.230) and (B) *L. major*-like antigen (cut-off: 0.095) in patients with American tegumentary leishmaniasis (cutaneous and mucosal/mucocutaneous forms), patients with other differential diagnoses and controls with a negative Montenegro skin test (MST). These are interactive dot diagrams, where the data of case and control groups are displayed as dots on vertical lines. A horizontal line indicates the cut-off point with the best separation (minimal false negative and false positive results) between two groups. [1]Cutaneous leishmaniasis; [2]mucosal and mucocutaneous leishmaniasis; [3]differential diagnosis; [4]controls with a negative MST.

For the *L. braziliensis* antigen ELISA test, positivity was 75.6 and 100.0% in patients with the cutaneous and mucosal forms of the disease, respectively, values close to those obtained with the *L. major*-like antigen for the cutaneous form (79.7%) but lower than those observed for patients with mucosal forms (75.0%).

A positive, although very weak (ρ = 0.34, *P* = 0.001), correlation was observed between the duration of ATL and ELISA OD measurements for both antigens tested.

3.2. Indirect immunofluorescence

Agreement between observers A and B was better for the *L. braziliensis* antigen (κ = 0.91) than for the *L. major*-like antigen (0.89) and Bio-Manguinhos kit (0.82). Agreement between observers A and C was 0.84, 0.84 and 0.54 for the *L. braziliensis*, *L. major*-like and Bio-Manguinhos kit antigens, respectively.

When kits from the same lot were used, inter-observer agreement was better for the *L. braziliensis* antigen (κ = 0.82) compared with the *L. major*-like antigen (0.69) and Bio-Manguinhos kit (0.69). However, when a different IPF lot was used for one reading, κ values of 0.68, 0.53 and 0.69, respectively, were obtained.

A significant difference in sensitivity (*P* = 0.006) and specificity (*P* = 0.03) was noted between the three antigens

Table 2 Mean (SD), median and range of ELISA optical density measurements performed using the *Leishmania braziliensis* and *L. major*-like antigens according to the clinical form of American tegumentary leishmaniasis

	L. braziliensis			*L. major*-like		
	CL[a]	ML[b]	*P*-value	CL[a]	ML[b]	*P*-value
Mean (SD)	0.743 (0.462)	1.106 (0.738)	0.161	0.160 (0.094)	0.309 (0.306)	0.158
Median	0.657	0.967		0.140	0.180	
Range	0.078−2.162	0.0967−2.720		0.042−0.582	0.071−1.204	

[a] Seventy-four patients with cutaneous leishmaniasis (CL).
[b] Twenty patients with mucosal leishmaniasis (ML) (including the mucocutaneous form).

Table 3 Sensitivity, specificity and positive and negative predictive values (PPV and NPV) with their 95% CI of indirect immunofluorescence using the *Leishmania braziliensis*, *L. major*-like and Bio-Manguinhos kit antigens

	L. braziliensis	*L. major*-like	Bio-Manguinhos kit	*P*-value
Sensitivity (%)	81.5 (70.0−90.1)	95.4 (87.1−99.0)	75.4 (63.1−85.2)	0.006
Specificity (%)	86.2 (79.0−91.6)	77.7 (69.6−84.5)	89.2 (82.6−94.0)	0.03
PPV (%)	73.4 (62.3−82.7)	63.5 (53.1−73.1)	79.6 (66.5−89.4)	0.137
NPV (%)	94.7 (88.9−98.0)	96.9 (91.2−99.4)	84.8 (77.8−90.4)	0.001

Table 4 Comparison between the indirect immunofluorescence (IIF) and ELISA techniques using the *Leishmania major*-like, *L. braziliensis* and Bio-Manguinhos kit antigens

	L. major-like			*L. braziliensis*			Bio-Manguinhos kit	
	IIF	ELISA	*P*-value	IIF	ELISA	*P*-value	IIF	*P*-value[a]
Sensitivity (%)	95.4	78.7	0.004	81.5	95.7	0.003	75.4	0.001
Specificity (%)	77.7	82.8	0.28	86.2	100.0	0.001	89.2	0.001
PPV (%)[b]	63.5	73.3	0.14	73.4	100.0	0.001	79.6	0.001
NPV (%)[c]	96.9	86.6	0.007	94.7	97.5	0.38	84.8	0.001

[a] Compared with *L. braziliensis* IIF.
[b] Positive predictive value.
[c] Negative predictive value.

used in the IIF tests. No significant difference in PPV was observed between the antigens used, whereas a significantly lower NPV was obtained with the Bio-Manguinhos kit (Table 3). Pairwise comparison showed a significantly lower sensitivity for the Bio-Manguinhos kit compared with the other two antigens (*P* = 0.001).

Positivity among patients with the cutaneous and mucosal forms was 94.1 and 76.9% for the *L. braziliensis* antigen, respectively, 96.0 and 92.3% for the *L. major*-like antigen, and 70.5 and 53.8% for the Bio-Manguinhos kit.

3.3. Comparison between ELISA and IIF

Agreement between the ELISA and IIF results was higher for the *L. braziliensis* antigen (84.3%) compared with the *L. major*-like antigen (67.1%).

Comparison between IIF and ELISA using the *L. major*-like antigen showed no significant differences in specificity (*P* = 0.28) or PPV (*P* = 0.14), whereas the two tests differed significantly in terms of sensitivity (*P* = 0.004) and NPV (*P* = 0.007). Comparison of the two techniques using the *L. braziliensis* antigen showed no significant difference in NPV (*P* = 0.38). Finally, comparison of IIF tests

using the *L. braziliensis* antigen and Bio-Manguinhos kit revealed a significant difference in all parameters analyzed (Table 4).

Parallel ELISA and IIF testing increased sensitivity, a desirable feature in screening and epidemiological studies, whereas serial testing increased specificity, which is necessary for confirmation of the diagnosis. Serial combination testing of ELISA using the *L. braziliensis* antigen and IIF using the *L. major*-like antigen yielded the best results, with a sensitivity of 94.0% and specificity of 100.0%.

4. Discussion

A negative MST is a valid parameter for the definition of the control group in ATL-endemic areas, where the prevalence of inapparent infection may be significant. MST can be persistently positive[10] in 5 to 70% of the population who never presented clinical disease.[11−13]

The differential diagnosis group in this study consisted of patients with clinical presentations similar to ATL and who were, therefore, assigned to the same care protocol, but for whom ATL was not confirmed by demonstration of the parasite. In our series, the most prevalent differential diagnosis

was sporotrichosis, a disease that clinically resembles ATL and may show epidemiological overlap with the latter.[14]

In agreement with our observation of higher sensitivity when the *L. braziliensis* antigen is used, several authors have reported better results with the use of homologous rather than heterologous antigens for immunological diagnoses.[15–17] Furthermore, *L. braziliensis* induces a greater antibody response compared with other *Leishmania* species responsible for ATL, especially *L. guyanensis*.[18] However, our results differ from those reported by Guimarães et al.[19] who showed that, although mean ELISA antibody titers were high in patients with mucocutaneous leishmaniasis from Vale da Ribeira (São Paulo) when using a homologous antigen (*L. braziliensis*), mean values for this antigen did not exceed those obtained for an heterologous antigen (*L. donovani*). Pedras et al.[20] also reported no difference in sensitivity (94.7%) between homologous and heterologous antigens for the detection of anti-*Leishmania* IgG antibodies.

Guimarães et al.[21], using a heterologous antigen (*L. major*-like) in ELISA assays, reported lower sensitivity (66.9%) and specificity (77.5%) for sera of patients from the north and northeast of Brazil. Nevertheless, these authors recommended the use of this heterologous antigen for serological tests, because of its high PPV (94.6%) despite a very low NPV (28.6%). Their findings differ from our results, which show lower PPV (73.2%) for the *L. major*-like antigen. However, a PPV of 100% and an NPV of 97.5% were obtained for the *L. braziliensis* antigen in our study, a finding that supports the use of homologous antigen for ELISA diagnosis of ATL.

Pedras et al.[20] reported higher antibody titers and more intense cellular immune response for mucosal forms of ATL. Valli et al.[22], using *L. braziliensis* and *L. mexicana* antigens, also reported high mean OD values in patients with the mucosal form (18/40). Although there was a significant and positive correlation between mucosal forms and higher ELISA OD using *L. braziliensis* in our sample, and our subjects showed slightly higher mean OD values, sample size (20/94) was probably insufficient to detect statistically significant differences between mean OD values of each group according to clinical forms.

Some studies have demonstrated a positive correlation of variable intensity between disease duration and ELISA OD values,[23,24] whereas others have not.[16] Mucosal forms of the disease might be a confounding factor, as they are associated both with higher OD values and a longer clinical course compared with cutaneous presentations.

Technician training and use of a different reagent lot influenced IIF reliability. Greater agreement between more experienced observers and for readings made at different moments by the same trained observer were noted for *L. braziliensis*. When the level of training was lower, the lower limit of the 95% CI included κ values that were moderate or good and varied according to the observer (0.54–0.84). This evidences the need for enhanced training when IIF is used, which is not always feasible in ATL-endemic regions.

A difference in performance was noted between the IIF test and ELISA. Accuracy parameters for IIF tests employing any of the three antigens reached point estimates that were always lower than those observed for ELISA using

the *L. braziliensis* antigen. In contrast to ELISA, none of the antigens employed alone in the IIF test provided both high sensitivity and specificity (Table 4). We also noted that simple agreement between ELISA and IIF was higher for the *L. braziliensis* antigen (84.3%) than for the *L. major*-like antigen (67.1%). However, when *L. braziliensis* antigen was used in IIF tests, sensitivity (81.5%) and specificity (86.2%) reached intermediate accuracy values between those observed for the *L. major*-like antigen and the Bio-Manguinhos kit (Table 3). As the reliability of the analysis obtained with the *L. braziliensis* antigen was better than that observed for the other antigens, use of this antigen in IIF is recommended.

Marzochi et al.[10] reported a higher sensitivity (90.4%) for IIF using an antigen then supposed to be *L. braziliensis* than that observed in our study (81.5%); however, it was later discovered that the antigen was an *L. major*-like antigen, and, thus, their reported sensitivity was in fact more similar to that described for the same antigen (95.4%) in our study. Contradictory results regarding sensitivity of *L. major*-like antigen have been reported by Guimarães et al.[21] (27.7%) and Oliveira-Neto et al.[13] (74.3%). In a subsequent study, Guimarães et al.[25] compared IIF using *L. braziliensis* and *L. major*-like antigens and reported results similar to ours, with a larger number of cross-reactions for the *L. major*-like antigen and a slightly higher PPV and NPV for the *L. braziliensis* antigen. Pedras et al.[20] reported a sensitivity of 100% for IIF using the *L. braziliensis* antigen in patients with the mucosal form of ATL, a finding probably related to the higher production of antibodies by these patients.

Analysis of multiple tests showed that the use of assays in parallel increases overall sensitivity, with values exceeding those noted for each test alone and reaching 100% in all combinations. The best results were obtained for ELISA performed with the *L. braziliensis* antigen, regardless of whether the assay was carried out simultaneously with an IIF test employing *L. major*-like or *L. braziliensis* antigen. Similar findings were noted for serial tests, which increase specificity, with the best results always being obtained for ELISA using the *L. braziliensis* antigen. Lira et al.[26] compared ELISA and IIF tests, both produced by Bio-Manguinhos, in the diagnosis of canine American visceral leishmaniasis by serial and parallel combination testing and suggested that parallel tests are more sensitive and more appropriate for epidemiological studies. Similarly, we observed an increase in sensitivity when parallel tests were used, whereas serial tests resulted in increased specificity.

For screening in epidemiological studies, parallel testing using the ELISA—*L. braziliensis* and IIF—Bio-Manguinhos kit sequence, which maximizes sensitivity, seems to be appropriate, despite the practical inconvenience of requiring the execution of two tests. By contrast, for diagnostic confirmation of suspected ATL, serial testing using the ELISA—*L. braziliensis* and IIF—*L. major*-like sequence, which maximizes specificity, is indicated. Thus, depending on the context of use, these different combinations might be used for clinical decision-making in *L. braziliensis*-endemic areas.

ELISA carried out with the *L. braziliensis* antigen showed higher accuracy and reliability in the serological diagnosis of ATL than the *L. major*-like ELISA and IIF using the *L. braziliensis* antigen. The results of this study support the recommendation that if only one test is to be used

as a diagnostic tool for suspected cases of the disease in *L. braziliensis*-endemic areas, it should be ELISA employing *L. braziliensis* antigen.

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