Diagnostic performance indices for immunofluorescent tests and enzyme immunoassays of leishmaniasis sera from northern and north-eastern Brazil

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*A total of 341 sera were screened for anti-*Leishmania *IgA, IgG, and IgM antibodies by immunofluorescent (IF) tests and enzyme immunoassay (ELISA). Altogether, 292 of the sera originated from patients with clinically as well as parasitologically diagnosed (positive lesion imprint or the Montenegro skin test) cutaneous leishmaniasis; 49 of the sera were from controls from the same base population.*

In terms of diagnostic performance, the ELISAs for IgG and IgM yielded indices of diagnostic utility, and the positive predictive value for the IgG-ELISA was 94.6%. A remarkably high specificity (100%) was obtained with the IgA-IF test, but its sensitivity was very low.

Introduction

The leishmaniases are endemic in Latin America from northern Mexico and southern Texas to northern Argentina; in Brazil they occur mainly in the northern and north-eastern regions of the country. Over the period 1979–85, 40 985 cases occurred in Brazil and in 1985 alone a total of 11 508 were reported (*1*, *2*). The *Leishmania mexicana* and *L. braziliensis* complexes are present in endemic areas in Brazil and usually cause a cutaneous leishmaniasis (CL) only, but if the disease is not treated, the oropharynx mucosa of patients infected with *L. b. braziliensis* can be invaded and destroyed. *Leishmania* spp. can be identified using monoclonal antibodies (*3*), isoenzyme methods (*4*), or DNA probes (*5*); however, such tests have yet to be standardized. For field work or routine diagnosis in in- or outpatient departments, mucocutaneous leishmaniasis (MCL) is diagnosed clinically followed by lesion imprint (*6*) or delayed-type skin tests (*7*). Labelled-antibody techniques, such as the immunofluorescent (IF) test (*8*) and enzyme immunoassays (ELISAs) employing either soluble antigens (*9*, *10*) or particulate antigens (Dot-ELISA) (*11*), have also been developed for the serodiagnosis of the leishmaniases.

Diagnostic performance indices for the IF and ELISA methods have only recently been estimated for a group of leishmaniasis patients who exhibited long-standing or severe symptoms and for controls without any previous contact with the etiological agent (*12*). Although very useful for test standardization, such a series does not reflect the epidemiological pattern of the disease as found in endemic areas.

In the present study, 341 sera obtained from 292 patients with CL and 49 controls from endemic areas in Brazil were screened for the presence of anti-*Leishmania* anti-IgG, anti-IgA, and anti-IgM antibodies using IF and ELISA methods. Subsequently, the diagnostic performance indices of sensitivity, specificity, positive predictive value, negative predictive value, and efficiency were calculated to determine whether such serological tests could be used to diagnose the leishmaniases.

Materials and methods

Sera

A total of 341 sera were collected at regional offices of the Superintendência de Campanhas de Saúde Pública, Brazilian Ministry of Health (SUCAM), in northern and north-eastern Brazil. The lesion imprint (*6*) and Montenegro skin tests (*7*) were used to make a diagnosis of CL. Thirteen sera from patients who

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had a clinical diagnosis of MCL were also screened, but the results obtained were used only to calculate the performance indices (Tables 1 and 2).

Sera were collected from untreated cases and were shipped frozen to the Seroepidemiology Laboratory, Instituto de Medicina Tropical de São Paulo, where they were assayed for anti-*Leishmania* IgG, IgM and IgA antibodies by IF and ELISA. Sera from a group of 49 controls, who were either in good health or had clinical and pathological diagnoses of skin diseases such as yaws, chromomycosis, eczema, varicose ulcers, or sporotrichosis, but negative parasitological tests for *Leishmania* spp., were also collected. Controls and cases originated from the same base population.

Antigens

IF antigens. The IF antigens were prepared as described by Guimarães et al. (*8*) from 7-day-old *Leishmania major*-like (MHOM/BR/71/49) promastigotes grown in liver infusion and tryptose (LIT) culture medium (*13*).

ELISA antigens. The ELISA antigens were prepared as described by Guimarães et al. (*10*) from 7-day-old, LIT-cultured *L. major*-like (MHOM/BR/71/49) promastigotes.

IF tests

IF tests for the detection of the IgG and IgM antibodies were performed using the technique described by Guimarães et al. (*8*), while the IgA antibodies were detected using the IF tests described by Shaw & Lainson (*14*); conjugates were heavy chain specific. The IgM antibodies were detected after absorption of sera by heat-aggregated immunoglobulin (*15*).

The end-point titre was taken to be the final dilution to give a continuous, bright green membrane and flagellum fluorescence. A positive and a negative control serum were included in all tests.

ELISA methods

ELISAs were carried out using the procedure described by Guimarães et al. (*10*) with an anti-human IgG (γ-chain specific) or an anti-human IgM (μ-chain specific) conjugated to horseradish peroxidase (type VI),[a] according to the method described by Nakane & Kawaoi (*16*). The chromogen was 200 μl of a 5.2-mmol/l solution of 5-aminosalicylic acid and 1.5 mmol/l hydrogen peroxide.[a] In all assays the following controls were included: a positive serum, three pools of negative serum containing 10 sera each (previously found to be negative for anti-*Leishmania* and anti-*Trypanosoma* antibodies by IF and ELISA tests), conjugate, and antigen. The end-point titration was taken to be the absorbance just greater than the mean + 2 standard deviations of negative serum pools at a 1:20 dilution.

IgM antibodies were detected after absorption of sera by heat-aggregated immunoglobulin (*15*).

Statistical analysis

For a given test, the serological titre for each sample was used to construct 2×2 contingency tables to further classify sera according to disease attribute (CL or not; leishmaniases or not). The frequencies of true positives, true negatives, false positives, and false negatives with respect to disease attribute could then be determined. Diagnostic indices such as sensitivity, specificity, positive and negative predictive value, and efficiency were calculated using previously described formulae (*17*), while 95% confidence intervals were estimated by Wilson's method (*18*) using Diagval (E.L. Franco & R. Simons, unpublished software, 1985), a customized template for Lotus 123.

Results

Skin and lesion imprint tests

A total of 194 patients (47.9% of respondents) were evaluated using the Montenegro skin test and 276 (55.7% of respondents) by the lesion imprint test.

Serological tests

The proportion of sera with titres that were above the prescribed cut-off level for each test ($\geq$ 1:40 in the IgG-ELISA, 1:10 in the IgA-IF, and $\geq$ 1:20 for the IF tests and the IgM-ELISA) was used to compare the anti-*Leishmania* response for each antigen.

Immunofluorescent tests. *IgG-IF test*—27.7% of CL sera had titres $\geq$ 1:20. *IgM-IF test*—20.5% of sera had titres $\geq$ 1:20. *IgA-IF test*—3.1% of CL sera (9 patients) exhibited a positive response. One of these patients had experienced symptoms for 2 months, while five had had symptoms for 3–6 months, indicating that the invasive stage of the disease may occur earlier than that estimated by Shaw & Lainson (*14*). Five of the sera were collected in the northern Amapá State and the remainder in north-eastern states of the country; two patients were 7 years old. Parasite findings for four patients were positive, while those for one patient were negative; also, five patients had a

[a] Sigma Chemical Co., St. Louis, MO, USA.

positive Montenegro skin test. There was a positive association between the results of the IF-IgA test and serum origin ($P = 0.0015$).

Enzyme immunoassays. The distribution of titres for IgG antibodies was as follows: 32.5% of sera had titres $<1:40$, while 67.5% had titres $>1:40$. There was a positive association between the IgG-ELISA titre and serum origin ($P = 0.0366$) as well as the age ($P = 0.0006$) and sex ($P = 0.0174$) of patients.

IgM-ELISA—the distribution of IgM titres was as follows: 44.5% were $<1:20$, while 55.5% were $>1:20$. There was a positive association between the IgM-ELISA titres and the serum origin ($P = 0.0001$) and age ($P = 0.0126$) of patients, since 72.0% of children's sera (18 out of 25 individuals aged 1–17 years) and 55.6% of adult's sera (148 out of 266 individuals aged 18–49 years) gave positive IgM-ELISA results.

Diagnostic performance indices

By basing diagnosis of leishmaniasis on a positive lesion imprint test or a positive Montenegro skin test and the preselected cut-off titre for each test, it was possible to evaluate the relative clinical and epidemiological utility of the five anti-*Leishmania* serological tests investigated. Tables 1 and 2 show the results of such an analysis in the form of diagnostic performance indices and their respective 95% confidence intervals for all five tests for the disease groups CL and CL plus MCL. As indicated by the total number of correct diagnoses, both positive and negative (efficiency index), the best disease indicator was the IgG-ELISA followed by the IgM-ELISA for both groups. These were the only tests with sensitivity indices and predictive values (positive and negative) as well as specificity indices that had values significantly different from those expected by chance alone.

Discussion

Although the study was carried out using antigens prepared from *L.major*-like parasites, *L.b.braziliensis* and *L.b.guyanensis* are the prevalent organisms in the survey area. At present, parasites of the *L.braziliensis* complex cannot yet be cultured to yield the large mass of promastigotes required to perform the serological tests described.

As shown in Table 1, the efficiency indices of the ELISAs, which are a measure of a test's ability to detect diseased or non-diseased status, were twice those of IF tests, regardless of whether CL sera were used to calculate the index or whether sera from the 13 invasive MCL cases were also included. Also, the ELISAs had sensitivity and specificity indices and positive and negative predictive values for both CL and MCL sera that were significantly different from those that would have arisen by chance alone (Tables 1 and 2).

The IF tests had significant specificity indices (87.8% for the IgM-IF and 100.0% for the IgA-IF); however, the IgG-IF specificity indices were lower than those of the IgG-ELISAs for CL or MCL sera. The significance of the specificity index is of no assistance in making a diagnosis by exclusion of false positive results, since the positive predictive value of

Table 1: **Performance indices for immunofluorescent (IF) tests and enzyme immunoassays (ELISAs) for antigens in sera from patients with leishmaniasis in northern and north-eastern Brazil**

	Performance index (%)		
	Sensitivity	Specificity	Efficiency
Cutaneous leishmaniasis versus controls			
IgG-IF	27.7 (22.9,3.31)[a,b]	71.4 (57.6,82.1)	34.0
IgM-IF	20.5 (16.3,25.5)[b]	87.8 (75.8,94.3)	30.2
IgA-IF	3.1 (1.6,5.7)[b]	100.0 (0.927,1.00)	17.1
IgG-ELISA	66.9 (61.3,72.1)	77.5 (64.1,87.0)	68.4
IgM-ELISA	56.6 (50.8,62.3)	72.9 (59.0,83.4)	59.0
Leishmaniases[c] versus controls			
IgG-IF	28.2 (23.4,33.5)[b]	71.4 (57.6,82.1)	31.2
IgM-IF	21.0 (16.8,25.9)[b]	87.8 (75.8,94.3)	30.2
IgA-IF	4.3 (2.5,7.9)[b]	100.0 (92.7,100.0)	17.6
IgG-ELISA	66.3 (60.8,71.4)	77.5 (64.1,87.0)	67.9
IgM-ELISA	56.2 (50.5,61.8)	72.9 (59.0,83.4)	58.5

[a] Figures in parentheses are 95% confidence intervals.
[b] The interval includes the value that would be obtained by chance alone.
[c] Both cutaneous and mucocutaneous leishmaniasis.

Table 2: **Performance indices for immunofluorescent (IF) tests and enzyme immunoassays (ELISAs) for antigens in sera from patients with leishmaniasis in northern and north-eastern Brazil**

	Performance index (%)	
	Positive predictive value	Negative predictive value
Cutaneous leishmaniasis versus controls		
IgG-IF	85.3 (76.8,91.0)[a,b]	14.2 (10.4,19.1)[b]
IgM-IF	90.9 (81.5,95.6)[b]	15.6 (11.8,20.4)[b]
IgA-IF	100.0 (70.1,100.0)[b]	14.8 (11.4,19.0)[b]
IgG-ELISA	94.6 (94.6,96.9)	28.6 (21.6,36.8)
IgM-ELISA	92.6 (87.7,95.6)	22.0 (16.3,29.1)
Leishmaniases[c] versus controls		
IgG-IF	86.0 (77.9,91.5)[b]	13.8 (10.1,18.6)[b]
IgM-IF	91.4 (82.5,96.0)[b]	15.1 (11.4,19.8)[b]
IgA-IF	100.0 (77.2,100.0)[b]	14.4 (11.1,18.5)[b]
IgG-ELISA	94.8 (90.9,97.0)	27.3 (20.6,35.3)
IgM-ELISA	92.8 (88.0,95.7)	21.2 (15.7,28.1)

[a] Figures in parentheses are 95% confidence intervals.
[b] The interval contains the value that would be obtained by chance alone.
[c] Both cutaneous and mucocutaneous leishmaniasis.

the three IF tests lacked significance (Tables 1 and 2).

The performance of the IF and ELISA tests was also investigated for a series of sera from long-standing cases of leishmaniasis (*12*), and, as expected, the indices differed from those reported above. Of note was the performance of the IgM-ELISA. For the sera from the long-standing cases the performance indices for this test were not significantly different from those that would have arisen by chance alone; however, in the present study, probably because the cases were of shorter standing, the IgM-ELISA indices were significantly different from those that would have arisen by chance.

In general, whenever the performance indices were significantly different from those that would have arisen by chance alone (both for the long-standing cases and those in the present study), the values of the indices accrued in this study were the lowest; in contrast, the positive predictive value for the IgG-ELISA increased from 73.7% for the long-standing cases to 94.6% for those in the present study, thereby permitting the detection of true positives from among a greater number of suspected cases. When the sera used in the present study were screened by the Dot-ELISA test (Guimarães et al., unpublished results, 1989) the specificity index increased to 91.4% and the positive predictive value to 98.8%. Our results for the ELISAs show therefore that serological tests for cutaneous leishmaniasis may be used for diagnosis, even with an *L. major*-like heterologous antigen.

The diagnostic ability of the IgA-IF test is still not clear; for example, although its sensitivity index was not significantly different from that which would have arisen by chance, nine cases that were diagnosed as CL only had positive IgA-IF tests. Since none of the controls had cross-reactive results, these patients represented true invasive cases of the disease (MCL), which were still in the cutaneous stage, and, unless given adequate therapy and followed up, invasion of the oropharynx mucosa could result.

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Résumé

Indices de qualité du diagnostic pour les épreuves d'immunofluorescence et les épreuves immuno-enzymatiques de sérodiagnostic de la leishmaniose dans le nord et le nord-est du Brésil

On a examiné 341 échantillons de sérum (292 de sujets atteints de leishmaniose cutanée et 49 de sujets témoins) en provenance du nord et du nord-est du Brésil, par immunofluorescence (IF) et épreuves immuno-enzymatiques (ELISA) de recherche des anticorps IgG, IgA et IgM anti-*Leishmania*. Le diagnostic a été confirmé sur empreinte de la lésion et par cuti-réaction de Montenegro.

Les épreuves sérologiques présentaient différents degrés d'association avec les marqueurs épidémiologiques. Par exemple, pour l'IgG-ELISA on observait une association positive entre les résultats et l'origine géographique du sérum, de même qu'avec le sexe et l'âge des malades; en revanche, pour l'IgM-ELISA on n'observait une association positive qu'avec l'origine géographique du sérum et avec l'âge des malades.

Parmi les méthodes étudiées, les meilleurs indicateurs de la maladie étaient les résultats de l'IgG-ELISA et de l'IgM-ELISA. La valeur prédictive positive de cette dernière épreuve était de 94,6%, ce qui permet de déceler les sujets positifs vrais parmi un plus grand nombre de cas suspects qu'il n'était auparavant possible de le faire; les autres

indices de qualité pour les épreuves ELISA différaient significativement de ceux qui auraient été obtenus par pur hasard. L'IgG-IF avait le plus faible intérêt clinique, car ses indices de sensibilité et de spécificité n'étaient pas significativement supérieurs à des indices obtenus par pur hasard.

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