Evaluation of serological diagnostic indices for mucocutaneous leishmaniasis: immunofluorescence tests and enzyme-linked immunoassays for IgG, IgM and IgA antibodies

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The sensitivity, specificity, positive predictive value, negative predictive value, and efficiency of immunofluorescence (IF) and enzyme-linked immunoassays (ELISA) for IgG, IgM and IgA antibodies were assessed on sera from mucocutaneous leishmaniasis patients and controls. The sensitivity of the IgG-ELISA test was 93.3% with 95% confidence interval higher than what could be due to a random test not associated with the disease. The specificity of all tests, except the IgM-ELISA, gave indices that could not have been due to chance. The IgG-ELISA and IgG-IF had the highest positive predictive value and the kappa statistic showed that the strength of agreement between the disease and the test was strongest for IgG-ELISA. The IgG-ELISA had a negative predictive value with 95% confidence limits that were not due to chance alone. Efficiency was highest for IgG-ELISA and IgG-IF. These results were obtained using sera from patients with severe or long-standing disease and from controls in whom the disease was ruled out by a negative Montenegro skin test. In field surveys where the differences between cases and controls are less easy to define the diagnostic indices of these tests may vary with the disease prevalence.

Introduction

Mucocutaneous leishmaniasis is diagnosed on clinical grounds supported by tests which include (*a*) identification of the organism in a lesion imprint or skin biopsy (*1*); (*b*) identification of the species and subspecies of the etiological agent by different means such as the use of monoclonal antibodies (*2*), isoenzyme profiles (*3*) or DNA probes (*4*); and (*c*) determination of the host's response to the agent by means of the Montenegro skin test (*5*).

Serological tests for the diagnosis of the disease by immunofluorescence and enzyme-linked immunoassays have yet to be criticaly evaluated as far as their performance results are concerned, so their contribution to the diagnosis of the disease has been played down by other issues such as the differentiation stage of the parasite (*6*) and the homologous or heterologous relationship of the antigen in the serogical test to the etiological agent (*6*). However, it has been shown that the geometric mean titre of leishmaniasis sera was higher when promastigotes were used as antigen than amastigotes (*7*), and that the results when using a homologous antigen such as *Leishmania braziliensis braziliensis* promastigotes in immunofluorescence tests may vary with the number of days the organisms are grown in the culture medium (*8*).

As far as diagnostic indices like sensitivity, specificity, positive predictive value, negative predictive value and efficiency are concerned, antibody-labelled tests such as the IF and the ELISA required evaluation relating the clinical findings of mucocutaneous leishmaniasis with the test results. In order to evaluate the performance of these tests and indices we studied the sera from inpatients in the Department of Dermatology of the University of São Paulo Medical School. Patients and controls were subjected to all the diagnostic procedures currently used in this department, the blood samples were submitted to the serological tests, and the results were then statistically analysed.

In the present study all the leishmaniasis patients were severe or long-standing cases of the disease. In a separate study the same diagnostic

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indices will be calculated for less severe and/or more recent cases of the disease to see whether there are any changes in the indices and to discover what might have caused them.

Materials and methods

Sera

Sera were screened for the presence of anti-*Leishmania* antibodies of the IgG, IgM and IgA class by immunofluorescence tests (IF) and enzyme-linked immunoassays (ELISA). Blood samples were drawn from 30 patients with mucocutaneous leishmaniasis, 34 with deep mycoses, and 30 normal controls in the Dermatology Department, University of São Paulo Medical School. In the case of the mucocutaneous leishmaniasis patients the blood was taken before treatment and weekly thereafter; six patients who were submitted to a second therapeutic series were bled weekly; the deep mycoses patients and normal controls were bled once. All the sera were sent to the Seroepidemiology Laboratory, Instituto de Medicina Tropical de São Paulo, where they were diluted in an equal volume of neutral glycerin and kept frozen until tested. Data on all the study patients included their age, sex, occupation, geographical origin, and response to the Montenegro skin test; for the leishmaniasis patients we included the results of lesion imprint and lesion biopsy and, for the deep mycoses patients, the results of biopsy for fungus identification.

Antigen

Leishmania major-like (MHOM/BR/71/49) promastigotes were used as the source of antigen for the IF and ELISA tests; organisms were cultured in LIT (liver infusion tryptose) medium (*9*) for 7 days at 25°C; preparation of the ELISA antigen, an alkaline promastigote extract, has been described elsewhere (*10*). The IF antigen consisted of formalin-fixed promastigotes (*11*).

ELISA assays

The IgG-ELISA and IgM-ELISA assays were carried out using, respectively, an anti-human IgG (gamma-chain specific) serum[a] and an anti-human IgM (mu-chain specific) serum[a] conjugated to horseradish peroxidase according to techniques described previously (*10*). The sera were screened for the presence of IgM antibodies after absorption with heat-aggregated gammaglobulin (*12*). In the ELISA assays the following controls were used: 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 titration endpoint was taken as the absorbance greater than the average +2 standard deviations of negative serum pools at a 1:20 dilution.

IF tests

The IF tests were carried out using anti-human IgG, anti-human IgM, and anti-human IgA FITC conjugates which had been made specific for each heavy chain according to techniques described previously (*11, 13*). Titration of the IgM antibodies was performed after absorption with heat-aggregated gammaglobulin (*12*).

All the IF tests were examined at ×400 magnification by fluorescence microscopy (Carl Zeiss). The last dilution to give a continuous bright green membrane and flagellum fluorescence was considered as the endpoint for that particular specimen. In all tests, both negative and positive control sera were included.

Statistical analysis

The serological titres found for each serum sample tested were used to construct 2 × 2 contingency tables where the sera were further classified, according to the disease's presence or absence (leishmaniasis or not), as positive or negative. This procedure allowed for the assessment of the frequencies of true positive, true negative, false positive and false negative results with respect to the diseases's presence. Diagnostic indices such as sensitivity, specificity, positive and negative predictive value, and efficiency were calculated using previously described formulae (*14*) and the estimation of their 95% confidence limits was calculated according to the method of Wilson (*15*). The kappa statistic for the strength of the agreement between disease and serological test and the *z* statistic were determined as shown by Fleiss (*16*).

Results

The study was carried out on serum samples from 94 individuals, 80 males and 14 females, aged between 11 and 83 years (average 47 years, median 36 years); 28 males and 2 females had a clinical and parasitological diagnosis of mucocutaneous leishmaniasis. All the mucocutaneous leishmaniasis patients had a positive Montenegro skin test, the others being negative. One of the deep mycosis patients and one of the controls had an IgG-ELISA titre of 640 and no other evidence of leishmanial infection by clinical, epidemiological, parasitological or serological parameters. Of the ulcer imprints, 13.3% were positive for *Leishmania* sp. among the mucocutaneous patients,

Table 1: **Diagnostic indices of immunofluorescence and enzyme immunoassays for the serological diagnosis of mucocutaneous leishmaniasis in patients from the Hospital das Clínicas/FMUSP**

Test	Index (%)				
			Predictive value		
	Sensitivity	Specificity	Positive	Negative	Efficiency
IgG-IF	56.7 (0.392; 0.726)[a,b]	89.1 (0.791; 0.946)	70.8 (0.508; 0.851)	81.4 (0.708; 0.888)	78.7
IgM-IF	16.7 (0.073; 0.336)[b]	93.7 (0.850; 0.975)	55.6 (0.267; 0.811)[c]	70.6 (0.602; 0.709)[b]	69.1
IgA-IF	13.3 (0.053; 0.297)[b]	90.6 (0.810; 0.956)	40.0 (0.168; 0.687)[c]	69.0 (0.585; 0.779)[b]	65.7
IgG-ELISA	93.3 (0.787; 0.981)	84.4 (0.735; 0.913)	73.7 (0.580; 0.850)	96.4 (0.879; 0.990)	87.2
IgM-ELISA	46.7 (0.302; 0.637)[b]	56.2 (0.441; 0.677)[c]	33.3 (0.210; 0.484)[b]	69.2 (0.557; 0.801)[b]	53.2

[a] Figures in parentheses are the 95% confidence limits.
[b] These intervals include the value which would be obtained by a random test not associated with disease attribute.

and the skin biopsy was positive in 70.0% of these cases. There was no report of *Leishmania* organisms in the biopsies from the deep mycosis group.

The indices for sensitivity, specificity, positive and negative predictive value and their respective 95% confidence intervals, and efficiency (as defined in ref. *15*) are shown in Table 1. In the cases of mucocutaneous leishmaniasis the indices were determined for the first bleeding only. IgG-ELISA was the only test that showed a high sensitivity (93.3%), the other tests displaying index values that could have been due to chance alone; specificity for all tests, except for IgM-ELISA, ranged from fair to high (84.4% to 93.7%). The IgG tests (IgG-ELISA and IgG-IF) had positive predictive value indices that were not due to chance alone, but only the IgG-ELISA had a significant negative predictive value (Table 1).

The geometric mean titres of the different serological tests are shown in Table 2. For the IgM antibodies there was no significant difference in the geometric mean titres in the IF or ELISA tests between control and diseased sera, while for the IgG antibodies and for IgG-ELISA there was, respectively, a 6 times and 38 times difference in the geometric mean titres between control and diseased sera.

Leishmaniasis patients were bled once every week for the duration of treatment. Before therapy 17 out of 30 had a positive IgG-IF with titres in the range of 20 to 160; after one month 10 out of 30 remained positive when therapy was discontinued for all but 6 leishmaniasis patients. Blood samples were withdrawn for 6 more weeks at the end of which only one of the patients had a positive IgG-IF test. In the IgG-ELISA tests, 28 out of 30 patients had a positive result with titres in the range of 40 to $\geqslant 2560$, nine samples remaining positive after 4 weeks; four of these patients remained positive until the eighth week and at the ninth week only one was positive.

Discussion

Parasitological tests were performed on the patients to corroborate the clinical diagnosis of mucocutaneous leishmaniasis; the imprint had a sensitivity of 13.3%, biopsy had a sensitivity of 70.0%, and the disease had an average evolution of 36 months. The low sensitivity of the imprint was probably due to the duration of the disease since fewer parasites were

Table 2: **Geometric mean titres for the immunofluorescence tests and enzyme immunoassays in mucocutaneous leishmaniasis serology (Hospital das Clínicas/FMUSP)**

Test	Control sera		Leishmaniasis sera
	Normals	Mycoses	
IgG-IF	1.3	1.4	8.6
IgM-IF	1.5	1.3	1.5
IgG-ELISA	3.1	7.6	288.0
IgM-ELISA	5.7	10.1	5.4

recovered from skin lesions the longer the duration (*1*).

Of the five serological tests carried out on blood samples with the object of finding out if the results would be good enough to recommend them as a diagnostic test for mucocutaneous leishmaniasis, only IgG-ELISA was able to disclose a disease or non-disease status which was not due to chance alone (Table 1). In the IgG-ELISA the 95% confidence limits for sensitivity, specificity, and positive and negative predictive values were higher than the 95% confidence limit of a totally random test such as the coin-flip. Such a random test would have a 50% sensitivity, 50% specificity, and 95% confidence limits of 33.1% and 66.8% (for sensitivity), 38.1% and 61.9% (for specificity), 20.4% and 46.2% (for the positive predictive value), and 53.8% and 79.6% (for the negative predictive value), all values lower than the ones shown for IgG-ELISA in Table 1. In the case of the IgG-ELISA the kappa statistic corroborated this finding indicating a $P<0.0001$, that the agreement between the tests and disease status could be due to chance alone (Table 3); also, a kappa statistic value of 0.73, according to Feinstein (*18*), indicates a substantial strength of agreement between the disease and the test result.

Table 3: **The kappa statistic and the standard error of kappa for mucocutaneous leishmaniasis serology tests (Hospital das Clínicas/FMUSP)**

Test	Kappa statistic	Standard error	*z* value[a]
Immunofluorescence:			
IgG-IF	0.48	0.07	6.8[b]
IgM-IF	<0.0	n.d.[c]	n.d.
IgA-IF	0.07	0.07	1.0
Enzyme immunoassays:			
IgG-ELISA	0.73	0.18	4.1[b]
IgM-ELISA	0.16	0.10	1.6

[a] *z* value = kappa value/standard error of kappa.
[b] $P < 0.0001$.
[c] n.d. = not done.

The specificity of the IgG-IF, IgM-IF and IgA-IF and the positive predictive value of the IgG-IF test met the criteria mentioned above, i.e., that their respective 95% confidence limits were higher than what could be due to chance alone.

Table 1 shows that the sensitivity and the negative predictive value of the IgG-IF test were no different from what would be expected of a random agreement between test and disease; however, Table 3 shows the probability that this could happen ($P<0.0001$). This apparent discrepancy was due to the fact that the IgG-IF specificity index of 89.1% was high enough to overcome its low sensitivity index of 56.7% resulting in a kappa index of 0.48, corresponding to a moderate strength of agreement between disease and test (*17*). The difference in performance between tests disclosing IgG class antibodies, i.e., the IgG-IF and the IgG-ELISA, is still stressed when its efficiency index, meaning the test's ability to disclose true negatives and true positives with regard to disease, were compared: although both tests had the same extremely low probability of chance agreement ($P<0.0001$), the efficiency index of IgG-IF was 78.7 while that for IgG-ELISA was 87.2. The geometric mean titres of the diseased sera and controls in the IgG-IF and the IgG-ELISA tests also reflected this observation since the difference in geometric mean titres between control and diseased sera on the IgG-ELISA showed a 38-fold increase whereas this difference was 6-fold for IgG-IF.

It could be argued that the low sensitivity of the IgG-IF test, as seen here, was due to the species of the antigen and/or the differentiation stage used in performing the tests. This seems not to be the case because when sera from the present study were used to standardize an IF antigen of *L.b. braziliensis* promastigotes, serum titres were lower than when *L.major*-like promastigotes were the antigen, depending on the number of subcultures of *L.b.braziliensis* (*8*). Also, the use of promastigotes as antigen for serological tests in mucocutaneous leishmaniasis was shown by Pappas et al. (*7*) to give a statistically significant higher geometric mean titre than when amastigotes, either grown *in vitro* or purified from hamster lesions, were used as the antigen.

L.b.braziliensis and *L.b.guyanensis* are the predominantly circulating species in Brazil but *L.major*-like promastigotes were chosen to perform the serological tests for technical reasons: its yield in liquid media made possible the production of enough cells to prepare the IgG-ELISA antigen (200 mg of freeze-dried promastigotes in each batch). Also, *L.major*-like promastigotes react very well with control sera without giving rise to non-specific staining in the IF tests or non-specific binding to inert supports in the ELISA tests.

The IgA-IF test did not show a statistically significant sensitivity and this may indicate that the IgA immune response is short-lived and was no longer detected in sera after 36 months of evolution. As reported by Shaw & Lainson (*13*), a positive IgA test result was indicative of mucosal invasion by the parasite; in this study the invasive nature of the infection was even more stressed by the perforation of the nasal septum in some cases but this did not result in positivity of the IgA-IF test.

With these results, only the IgG-ELISA test showed indices of sensitivity, positive predictive value, negative predictive value, and efficiency that have diagnostic value and may be used as a subsidiary tool in the diagnosis of mucocutaneous leishmaniasis. In the case of the specificity index, this was higher in the IgG-IF test (89.1%) than with IgG-ELISA (84.4%); this result, however, was not extended to the positive predictive value of the IgG-IF test.

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Résumé

Evaluation des indices de diagnostic sérologique de la leishmaniose mucocutanée: immunofluorescence et épreuves immuno-enzymatiques de recherche des IgG, IgM et IgA.

On a soumis les sérums de patients atteints de leishmaniose mucocutanée et de mycoses profondes et ceux de témoins sains à des épreuves de recherche des anticorps antileishmaniens des classes IgG, IgM et IgA par immunofluorescence et épreuves immuno-enzymatiques (ELISA). Tous les sujets de la série étudiée ont été soumis à une cutiréaction de Montenegro et chez tous les malades on a pris une empreinte de lésion et/ou une biopsie cutanée, suivant les protocoles de diagnostic en usage au Département de Biologie de l'Ecole de Médicine de l'Université de São Paulo. On a fait une prise de sang chez les cas et les témoins avant le début du traitement, et les sujets atteints de leishmaniose ont été soumis à une prise de sang hebdomadaire pendant toute la durée de celui-ci.

La durée moyenne de la maladie chez ces sujets était de 36 mois. La prise d'empreinte de la lésion avait une sensibilité de 13,3% et la biopsie cutanée a révélé la présence d'amastigotes dans 70,0% des cas.

On a évalué pour les épreuves de recherche des anticorps antileishmaniens les indices de diagnostic suivants: sensibilité, spécificité, valeur prédictive positive, valeur prédictive négative, efficacité, intervalle de confiance à 95%.

L'IgG-ELISA était la seule épreuve à donner des indices statistiquement significatifs de sensibilité, de spécificité, de valeur prédictive positive et négative et d'efficacité, puisque ses deux limites de confiance à 95% se situaient au-dessus de l'intervalle de confiance à 95% d'une épreuve aléatoire sans rapport avec la maladie, par exemple le tirage à pile ou face; en revanche, l'indice de sensibilité de l'IgG-IF et sa valeur prédictive négative ne différaient pas de ceux que l'on pouvait attendre d'une épreuve aléatoire.

L'IgM-IF avait un indice de spécificité élevé et des limites de confiance à 95% statistiquement significatives, tandis que les indices de l'IgM-ELISA ne différaient pas de ceux d'un test purement aléatoire. L'interprétation de la valeur diagnostique des épreuves de recherche des IgM peut avoir été faussée du fait de la prise de prélèvements chez des cas de leishmaniose déjà anciens.

Normalement, une épreuve IgA-IF positive est indicative de l'invasion des muqueuses par le parasite; mais, bien que de nombreux participants à l'étude aient déjà une perforation de la cloison nasale, leurs résultats en IgA-IF étaient négatifs. Il est nécessaire d'approfondir cette question afin d'élucider la signification de la réponse en IgA et sa longévité.

Dans la présente étude, les indices ont été obtenus par examen de sérums prélevés chez 30 cas de leishmaniose grave ou ancienne et 64 témoins ne présentant aucun signe d'infestation actuelle ou passée.

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