

COMPARISON ON THE PERFORMANCE OF *Leishmania major*-LIKE AND *Leishmania braziliensis braziliensis* AS ANTIGEN FOR NEW WORLD LEISHMANIASIS IGG-IMMUNOFLUORESCENCE TEST.

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SUMMARY

The performance of an antigen of *L. major*-like promastigotes for the serological diagnosis of mucocutaneous leishmaniasis in the IgG-immunofluorescent test was compared to that of an antigen of *L. braziliensis braziliensis*. Each antigen was used to test two hundred and twenty-four sera of etiologies such as mucocutaneous leishmaniasis, deep mycoses, toxoplasmosis, malaria, Chagas' disease, visceral leishmaniasis, anti-nuclear factor, schistosomiasis, rheumatoid factor and normal controls. Agreement between responses to each antigen was high: 77.2% of leishmaniasis sera agreed on a positive or a negative result to both antigens and 91.1% of control sera.

Cross reactivity was restricted to Chagas' disease sera, visceral leishmaniasis, anti-nuclear factor and paracoccidioidomycosis. The quantitative response of leishmaniasis and Chagas' disease sera to both antigens was evaluated by a linear regression; although the y-intercept and the slope were different for each antigen, neither was better than the other in the disclosure of anti-*Leishmania* antibodies. In the case of Chagas' disease sera the *L. major*-like antigen was better than *L. braziliensis*' to disclose cross-reacting antibodies.

KEY WORDS: *L. major*-like; *braziliensis braziliensis*; IgG; immunofluorescence test.

INTRODUCTION

The use of *Leishmania braziliensis braziliensis* (Lbb) as antigen in serology surveys of New World leishmaniasis poses many problems related to the discrepancy between the large amounts of organisms needed for the preparation of the antigen and the state-of-the arte of Lbb promastigote in vitro culture. Until recently, Lbb was grown in biphasic media such as NNN¹⁵ as this medium is very sensitive and when fetal calf serum is not added, relatively inexpensive. Culture of parasites in biphasic media has as major disadvantages the fact as they are based on blood products that cannot be standardized and the quality of the antigen may vary from one batch to another; also, the presence of red blood cells may

interfere with serology results¹¹. In 1978, Childs et al.⁴ proposed Schneider's *Drosophila* medium to be used for the culture of New World leishmania. Its advantages are that promastigotes can be grown free of red blood cell contamination and the medium is commercially available; its disadvantages are its high cost and the need for fetal calf serum as supplement, a highly expensive component which's activity varies between lots and has no suitable substitute¹¹.

To overcome such difficulties researchers have resorted to the use of other organisms as antigen for IF tests; these can be parasites that belong to the same complex as Lbb such as *L.b.*

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*panamensis*¹⁶, parasites of another complex as *L. mexicana amazonensis*⁵, or *L. major*-like (*Lm*)¹⁰. *Lm* grows very well in liquid media and cells display maximum titer towards positive standard sera in 7 days when grown in LIT culture medium⁶.

The use of *Lm* as antigen for serology tests may be even greater than reported in the literature as many researchers unknowingly used *L. major*-like organisms in lieu of *L. braziliensis* before the work by Momen et al., 1983 was published¹³. In it, strains generically referred to as *L. braziliensis* were later identified by several analytical methods as being phenotypically similar to a reference *L. major* strain rather than to *Lbb* reference strains.

Although *Lm* is a more convenient organism to use for serology work than *Lbb* a question remained whether it could be as suitable an antigen as *Lbb* to put in evidence anti-*Lbb* antibodies. Previous work had indicated that small differences only were found in serum titers of a small sample of positive standard sera in the IgG-immunofluorescent test (IgG-IF) with *Lbb* or *Lm*^{ns} antigen³. The present work investigates this hypothesis by means of a larger sample of positive sera, represented by mucocutaneous leishmaniasis sera of patients in the invasive stage or not, and negative sera represented by normal controls as well as by sera of diseases due to other trypanosomatids and sera from unrelated pathologies.

MATERIALS AND METHODS

Sera

Two sets of sera were used. Set 1 made up of one hundred and thirty five specimens comprised three subsets: leishmaniasis sera, normal control and deep mycoses. Seventy-eight were drawn from patients with a clinical, immunological and parasitological diagnosis of leishmaniasis (invasive stage or not) by means of a Montenegro skin test¹⁴ and skin imprint or skin biopsy⁵; twenty-five sera were drawn from normal controls and thirty-two sera were drawn from deep mycoses patients in whom diagnosis was established by appropriate diagnostic tests. Normal controls and deep mycoses patients were given a Montenegro skin test. Set 2 was made up of eighty-nine sera of etiologies such as malaria ($n=19$), toxoplasmosis ($n=21$), Chagas' disease ($n=20$), anti-nuclear factor ($n=18$), rheumatoid factor ($n=5$), visceral leishmaniasis ($n=2$) and

shistosomiasis ($n=4$); sera were kept frozen, diluted v/v in neutral glycerin at the Seroepidemiology laboratory of the Instituto de Medicina Tropical de São Paulo. All sera had diagnostic serology results and when appropriate, parasitological tests such as parasite identification or xenodiagnosis.

Antigens

Lm promastigotes (*M/HOM/BR/71/49*) grown in LIT culture medium⁶ for seven days and *Lbb* promastigotes (*M/HOM/BR/75/M2903*) grown for five days in Schneider's *Drosophila* medium supplemented with 20% fetal calf serum¹¹ were used to prepare antigens for the IgG-IF tests according to techniques already described^{8,3}.

IF test

Tests were done according to techniques already described⁸. Sera were diluted twofold (starting at a 1/10 dilution); end point titration was considered as the last dilution to display a bright green, continuous membrane and flagellum fluorescence. An anti human IgG (gamma chain specific) fluorescein isothiocyanate conjugate (BioLab-Merieux, Brazil) was optimally diluted to ensure maximum reactivity by means of block titration of serum and conjugate. A Carl Zeiss fluorescent microscope (Oberkochen, Germany) at 400 x with an oil immersion objective, dark field condenser and interference filter KP-500 was used.

Statistical analysis

After titration of each serum with *Lm* and *Lbb* antigen samples were grouped according to their positivity or negativity in the test as defined by a previously determined cutoff. Results were used to construct contingency tables and the kappa statistic was used to investigate the probability of the association of each serum to each antigen⁷; hypotheses were tested at a 5% critical level. Performance indices of sensitivity, specificity and positive predictive value were calculated by means of Diagval, a customized template for Lotus 123 (developed by E.L. Franco and R. Simons, unpublished software).

End point titration of each sample with *Lm* or *Lbb* antigen was transformed into $\log(x+1)$ and a linear regression was calculated using a

Statgraphics™ software (Statistics Graphic Corp., 1987). A Student's t test for each hypothesis was calculated by the software.

RESULTS

Leishmaniasis sera showed a probability of agreement between the antigens equal to 0.78. Eleven sera (14.1%) had a positive test with Lm antigen and a negative test with Lbb antigen and 6 sera gave the opposite result (7.7%). The kappa statistic was 0.56 with a probability $p < 0.0001$ of random agreement between test results (Table 1).

Negative control sera showed an agreement probability of 0.91; seven sera (4.8%) had a positive Lm test and a negative Lbb test and 6 sera (6.2%) showed the opposite. The kappa statistic for negative control sera was 0.64 with a $p < 0.0001$ of random agreement between tests. Statistical analysis of each category of negative control sera by the kappa statistic (Table 1) showed that there was a $p < 0.0001$ of random agreement for the category of normal control sera or malaria sera and a $p = 0.0423$ for deep mycoses sera; there was random agreement for anti-nuclear factor sera ($p = 0.1310$) as well as for Chagas' disease sera ($p = 0.0784$) (Table 1). Visceral leishmaniasis sera and shistosomiasis sera were not tested for the kappa statistic. The agreement probability for all sera was 0.87 with a kappa statistic of 0.67.

A linear regression was calculated plotting titers for IF tests done with the Lm antigen as the independent variable. When all 224 sera were analysed the resulting equation was $y = 0.16 + 0.80x$, the y-intercept corresponding to a Lbb titer 1.17 greater than Lm's, correlation coefficient (r) 0.76 (95% confidence limits $0.70 < r < 0.81$), $p = 2.54 \times 10^{-43}$ of chance agreement; the resulting equation for leishmaniasis sera ($n = 78$) was $y = 0.18 + 0.84x$, y-intercept corresponding to a Lbb titer 1.20 greater than Lm's, $r = 0.67$ (95% confidence limits $0.52 < r < 0.78$), $p = 2.14 \times 10^{-11}$ (Table 2).

A linear regression for Chagas' disease sera was calculated plotting Lbb antigen serum titers as the x variable: the resulting equation was $y = 1.56 + 0.11x$, y intercept equal to titer 4.77 greater than Lm's, $r = 0.43$ (95% confidence limits $-0.01 < r < 0.74$), $p = 0.06$ (Table 2).

Sensitivity of the IgG-IF test when Lm was

used as antigen was 44.9% (95% confidence limit $38.6 < S < 60.2\%$) and for Lbb antigen was 48.7% (95% confidence limit $35.8\% < S < 57.8\%$); specificity was 85.6% (95% confidence limit $78.8 < Sp < 90.3\%$) for Lbb and 84.9% for Lm antigen (95% confidence limit $78.8 < Sp < 90.3$).

The positive predictive value (PPV) for the Lm antigen was 65.0% (95% confidence limit $52.4\% < PPV < 75.8\%$) and for Lbb antigen was 62.5% (95% confidence limit $49.4\% < PPV < 74.0\%$). PPV was also calculated using simulated prevalence levels: at a 1% prevalence level PPV for Lm antigen was 2.6%, and for Lbb antigen was 3.4%; at a 10% prevalence level PPV for Lm antigen was 24.9% and for Lbb was 27.5%; at a 25% prevalence level PPV was 49.8% and 53.0%, (Table 3).

DISCUSSION

Diagnostic performance indices of sensitivity, specificity and positive predictive value for the IgG-IF test with Lm as antigen were within the 95% confidence limits of indices accrued previously when an evaluation study was carried out¹⁰. When the sensitivity, specificity and positive predictive value of the IF-IgG test using Lbb as antigen were compared to the ones accrued using Lm as antigen it was seen that the sensitivity of the test using one antigen was comprised within the 95% confidence limit of the other.

The probability of agreement of serum categories and antigens was high: 78% for leishmaniasis sera and 91.1% for negative control sera (Table 1). The lack in test specificity was restricted to Chagas' disease sera, visceral leishmaniasis, deep mycoses and anti-nuclear factor. The first two categories cross-react with mucocutaneous leishmaniasis sera¹; in order to put in evidence specific antibodies to any of them it is necessary to adsorb sera by the heterologous antigens (*L. major*-like and *L. donovani chagasi* in the case of Chagas' disease and *T. cruzi*, *L. donovani chagasi* and/or *L.b. braziliensis* in the case of leishmaniasis)²; in the case of sera with anti-nuclear activity no technique establishing such distinction is available. The deep mycoses group had two paracoccidioidomycosis sera with conflicting results: one serum was positive with the Lm antigen and negative with Lbb, the reverse was true for the other serum; patients of this disease may have

a positive Montenegro skin test in the absence of other epidemiological data for New World leishmaniasis¹² and paracoccidioidomycosis sera cross-react with the Lm antigen in IF test and enzyme immunoassays (M.C.S. Guimarães, unpublished results).

Lm or Lbb antigens did not show non-specific reactivity with antibodies to agents of other infectious diseases prevalent in endemic areas of New World leishmaniasis. As seen from Table 1, the probability of random agreement between any antigen and malaria sera was $p < 0.0001$. The agreement was made up of 15 negative pairs and 4 positive ones; since malaria sera do not react non-specifically with Lm antigen(s)⁹ the latter represent true positives for leishmaniasis as well as malaria; as malaria sera were part of set 2, it is not known whether patients had ulcers or scars suggestive of the disease.

There was an agreement of 1.00 between test

results to any antigen and schistosomiasis or toxoplasmosis sera (Table 1); the kappa statistic was not calculated for the two categories because test results were represented by negative responses only to antigens, meaning a division by zero in the kappa statistic and consequently, a undefined value of it.

The quantitative response of leishmaniasis and Chagas' disease sera to antigens was investigated by means of linear regressions as shown in Table 2. The y-intercept and the slope were different for each antigen and each set of sera (Table 2) but the correlation coefficient of leishmaniasis sera ($r = 0.67$) indicated that the scatter of experimental points around the least-square line (random error) was small; the y-intercept of 0.18 (t value 7.85, t critical 1.99, $df = 77$, $p = 0.05$) indicated that the magnitude of the constante error for leishmaniasis was null. The value of the t statistic for leishmaniasis sera (Table 2), indicates that neither antigen performs better than the other in the disclo-

Table 1
Ratio of agreement and kappa statistic of antigens *Leishmania major*-like and *L.b. braziliensis* in the IgG-immunofluorescence test.

Serum	N ^a	Agreement ^b	Kappa statistic	z ^c	p ^d
All sera	224	0.87	0.67	9.98	<0.0001
Leishmaniasis	78	0.78	0.56	5.03	<0.0001
Negative sera	146	0.91	0.64	7.80	<0.0001
Categories of negative sera:					
Malaria	19	1.00	1.00	4.36	<0.0001
Deep mycoses	32	0.94	0.59	2.03	0.0423
Normal controls	25	1.00	undefined ^e		
Toxoplasmosis	21	1.00	undefined		
Rheumatoid factor	5	1.00	undefined		
Visceral leishmaniasis	2	0.50	n.d. ^f		
Schistosomiasis	4	1.00	n.d.		
Chagas' disease	20	0.70	0.40	1.76	0.0784
Anti-nuclear factor	18	0.78	0.36	1.51	0.1310

a N = number of sera

b agreement = probability of agreement between two randomly selected observers on a randomly selected subject

c z = kappa statistic/standard error of kappa

d p = probability

e undefined = division by zero in the kappa statistic

f n.d. = not done

Table 2

Performance correlation of *L.b. braziliensis* as antigen on the IgG-immunofluorescence test by means of linear regression.

	y-intercept	slope	r ^a	t test	probability
All sera	0.16	0.80	0.76	17.39	2.54.10 ⁻⁴³
Leishmaniasis sera	0.59	0.53	0.67	7.85	2.14.10 ⁻¹¹
Chagas' disease sera	1.56	0.11	0.43	2.04	0.06

a r = correlation coefficient

sure of anti-*Leishmania* antibodies in the IgG-IF test; the same was seen when all sera were analysed statistically by means of the linear regression, as table 2 shows.

For Chagas' disease sera a value of 2.04 for the t statistic (critical t 2.08, n = 20, p = 0.05) indicates an association between Lm antigen and anti *T. cruzi* antibodies; however, this point needs to be analysed further by means of samples with a larger number of Chagas' disease sera; then, the difference between t values might change towards the indication that neither antigen performs better than the other when Chagas' disease sera are used.

We conclude that instead of *L.b. braziliensis*, *L. major*-like can be used as antigen for IgG-IF tests as this organism is easier to cultivate than Lbb and performs in a similar way on the IgG-IF test. However, as the PPV for either organism indicates a number of positive results in the IgG-IF test will be made of false-positives (from 51.6% to 26.0% according to the antigen and to 95% confidence limits); the simulation of the positive predictive values according to different prevalence levels

as shown in Table 3 indicates that in spite of the high specificity index displayed by the test (84.9%) only at prevalence levels of 75% or more a false-positivity rate of 10% is reached. Negative predictive value, is very high even at prevalence levels of 1% (99.3% for Lm antigen and 99.4% for Lbb antigen) (Table 3). A negative IgG-IF test using either antigen is indicative of no-disease but a positive IgG-IF test result indicates the need for further assessment of the true-positive status by another diagnostic procedure.

RESUMO

Comparação do desempenho de antígenos de *Leishmania major*-like e *Leishmania braziliensis braziliensis* no teste de imunofluorescência-IgG para leishmaniose mucocutânea.

O desempenho de um antígeno de promastigotas de *L. major*-like para o diagnóstico sorológico de leishmaniose mucocutânea pelo teste de imunofluorescência-IgG foi comparado com o desempenho de um antígeno de *L. braziliensis braziliensis*. Cada antígeno foi usado para testar

Table 3

Positive predictive value and negative predictive value for IgG-immunofluorescence test at simulated levels of prevalence using *L. major*-like (Lm) or *L.b. braziliensis* (Lbb) as antigen.

Prevalence (%)	Positive predictive v. (%)		Negative predictive v. (%)	
	Lm	Lbb	Lm	Lbb
1	2.6	3.4	99.3	99.4
2	5.7	6.6	98.7	98.8
5	13.3	14.93	96.6	96.9
10	24.9	27.5	93.3	93.8
15	34.4	37.4	89.7	90.4
20	42.7	45.8	86.1	88.9
25	49.8	53.0	82.2	83.4
50	74.9	77.2	60.6	62.6
75	89.9	91.0	33.9	35.7
100	100.0	100.0	0.0	0.0

224 soros de etiologias como leishmaniose mucocutânea, micoses profundas, toxoplasmose, malária, doença de Chagas, leishmaniose visceral, fator anti-núcleo, esquistossomose mansônica, fator reumatóide e controles normais. A concordância entre as respostas a cada antígeno foi grande: 77,2% dos soros de leishmaniose mostraram resultado positivo para ambos os antígenos ou negativo, assim como 91,1% dos soros controle negativos. Reações cruzadas ficaram restritas à doença de Chagas, leishmaniose visceral, fator anti-núcleo e paracoccidioidomicose. A resposta quantitativa dos soros de leishmaniose e doença de Chagas foi avaliada pelo método de regressão linear; embora a intercessão com o eixo y e o "slope" fossem diferentes para cada antígeno nenhum deles se mostrou melhor na evidência de anticorpos anti-*Leishmania*. O antígeno de *L. major*-like mostrou-se melhor que o de *L.b. braziliensis* na evidência de anticorpos em soros de doença de Chagas.

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