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# PERFORMANCE INDEXES OF A DOT-ENZYME-LINKED IMMUNOSORBENT ASSAY (dot-ELISA) AND AN ENZYME-LINKED IMMUNOSORBENT ASSAY (IgG-ELISA) FOR FIELD SURVEYS OF NEW WORLD LEISHMANIASIS.

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## SUMMARY

Diagnostic performance indexes of sensitivity, specificity, positive predictive value and efficiency were determined for dot-ELISA and IgG-ELISA tests in 340 leishmaniasis sera. Sensitivity of the dot-ELISA was significantly lower than IgG-ELISA's; the two tests had indexes of specificity and positive predictive value of the same magnitude. Seventy-eight sera gave a negative dot-ELISA test result and a positive IgG-ELISA test result. When sera were classified according to different criteria as how to interpret this diversity, the kappa statistic did not corroborate the classification indicating that the two tests display a substantial strength of agreement. The results presented indicate that performance indexes accrued in a survey where variables are well known may be extrapolated to other population studies if the disease presents itself as highly prevalent (due to a selection bias or not) and may be expected to discriminate a disease status among test positives.

**KEY WORDS:** New World leishmaniasis; Serology; Immuno enzyme tests; Dot-ELISA; IgG-ELISA.

## INTRODUCTION

One of the difficulties of New World leishmaniasis serology to disclose a disease-non disease status deals with the low level of circulating antibodies in cutaneous forms. A negative test result is often found in active cases making clinical diagnosis and parasitological tests, such as the finding of parasites in ulcers (lesion imprint)[10] or a Montenegro skin test[9] more dependable than serology. The difficulty increases regarding seroepidemiology surveys where a test possessing a high positive predictive value would facilitate the task as parasitology diagnostic tests are time-consuming and demand higher degree of expertise when compared to most serology tests.

When prevalence of a given disease is different from the presumed prevalence found in standardization stage, performance indexes such as sensitivity, specificity positive or negative predictive value and efficiency will vary accordingly; this is why before a serum survey starts it is necessary to run a pilot study to assess how the performance indexes found in strictly controlled conditions will be modified in the field and if the new indexes will still be able to show statistical significance regarding the discrimination between a disease non-disease status.

The dot enzyme-linked immunosorbent assay (dot-ELISA) for mucocutaneous leishmaniasis was standardized previosly[6] and its performance compared to that of the IgG-ELISA; the specificity, efficiency and positive predictive value of the dot-ELISA were higher than IgG-ELISA's ranging between 94.5% to 96.9%. The present report demonstrates the reasoning presented above as the same indexes were calculated for sera drawn from more recent cases displaying a presumed prevalence different from that of the standardization stage. We thought it should be investigated to which extent

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such changes would alter the previously found performance indexes and how this finding would influence serodiagnosis of New World leishmaniasis in field surveys.

## MATERIALS AND METHODS

Sera: three hundred and forty sera were submitted to an IgG-ELISA and to a dot-ELISA according to techniques already described[5, 6]. Three hundred and fourteen sera were collected by SUCAM (Superintendência de Campanhas de Saúde Pública, Ministério da Saúde do Brasil) from individuals with a diagnosis of cutaneous leishmaniasis of recent acquisition (1-4 months) by lesion imprint[10] and skin test reactivity[9]. Thirteen sera were classified as having mucosal involvement based on physical diagnosis. Twenty-six sera from the same geographical area on physical examination, skin test reactivity and skin biopsy were considered as negative controls for the purpose of assessment of performance indexes.

Blood was collected and parasitology tests conducted by SUCAM in Northern and Northeastern states of Brazil and sera were shipped frozen to the Seroepidemiology Laboratory of the Instituto de Medicina Tropical de São Paulo where they were diluted volume to volume in neutral glycerin and kept frozen at -20° C until tested.

**Leishmania major**-like promastigotes (MHOM/BR/71/49) grown in LIT culture medium for 7 days was used as antigen[2]. The dot-ELISA antigen was prepared according to GUIMARÃES et al., 1986[6] and the IgG-ELISA antigen prepared according to GUIMARÃES et al., 1981[5]. Briefly, promastigotes were suspended in 0.15 M NaOH and sonicated with 3 pulses of 20 seconds each; after extraction and neutralization with 0.3 N HCl the dot-ELISA antigen was centrifuged at 4,000 g x 10 min. and the antigen for IgG-ELISA was centrifuged at 30,000 g x 10 min.

Sera were diluted twofold and tested; in all tests one positive control serum and three pools of negative control sera were included. Negative control sera belonged to the laboratory's serum bank; they were found previously to have a negative serology for mucocutaneous leishmaniasis and Chagas'disease.

IgG-ELISA's reactivity endpoint was taken as the highest dilution yielding an absorbance value greater than the absorbance value of the average plus two standard deviations of the negative serum pools at a 1/20 dilution. For the dot-ELISA test sera were screened at a 1/40 dilution only; reactivity was assessed visually by comparison of the color development between test samples and negative control sera.

For statistical associations and for assessment of serology parameters IgG-ELISA results at a 1/40 reactivity level were considered. Standard algorithms were used for the computation of diagnostic performance indexes[4] and the 95% confidence limits were done using Diagval, a customized template for Lotus 123 (developed by E.L. Franco and R. Simons, unpublished software). The kappa statistic[3] was used to investigate the strength of the agreement between the dot-ELISA and IgG-ELISA and the z index (the radio of kappa/standard error of kappa) was used to ascertain the statistical significance of the kappa statistic.

## RESULTS

Serology parameters of sensitivity, specificity, positive predictive value and efficiency, and their corresponding 95% confidence limits by the dot-ELISA and IgG-ELISA are shown in table 1. No statistically significant differences were found between the 13 invasive leishmaniasis sera and the 301 cutaneous sera. The sensitivity and the efficiency of the dot-ELISA were lower than IgG-ELISA's; the specificity index and the positive predictive value were the same for both tests.

Two hundred and sixty-two sera agreed on a positive or negative result to both assays; seventy-eight sera gave a positive IgG-ELISA result and a negative dot-ELISA result. To investigate if a discrepancy in assessment of a serum status would influence serodiagnosis two criteria were used: the first criterion considered 9 such sera as "negative IgG-ELISA results" as its absorbance values were within 0.005 absorbance units of the ones used to establish the test's intra assay cutoff; the second criterion was to consider as negative all sera with a titer of 40 in the IgG-ELISA test (31 sera). The relative sensitivity and the relative specificity of dot-ELISA and IgG-ELISA for each of the situations are shown in table 2. The relative sensitivity of the dot-ELISA test increased from 61.6% to 72.7% and the relative sensitivity of the IgG-

ELISA did not change; the relative specificity of the dot-ELISA did not change with the different criteria while the relative specificity of the IgG-ELISA rose from 62.8 to 77.6 (table 2).

Table 1
Sensitivity, specificity, positive predictive value, efficiency and 95% confidence limits for dot-ELISA and IgG-ELISA tests in mucocutaneous leishmaniasis sera.

| Performance index | Serology test | |
|---|---|---|
| | Dot-ELISA | IgG-ELISA |
| Sensitivity | 41.1%[a] | 56.4% |
| C.L.[b] | 35.8%; 46.6% | 50.4%; 61.7% |
| Specificity | 95.8% | 82.9% |
| C.L. | 79.8%; 99.3% | 69.0%; 95.7% |
| Positive predictive value | 99.2% | 98.3% |
| C.L. | 95.8%; 99.9% | 95.2%; 99.4% |
| Efficiency | 45.0% | 58.6% |

[a] The interval contains the value which would be obtained by a random test not associated with disease attribute.
[b] confidence limits.

Table 2
Relative sensitivity, relative specificity and 95% confidence limits for dot-ELISA and IgG-ELISA tests in leishmaniasis sera.

| Test | Index (%) (95% confidence limits) | |
|---|---|---|
| | Relative Sensitivity | Relative Specificity |
| 78 sera (dot-ELISA negative, IgG-ELISA positive) | | |
| Dot-ELISA | 61.6[a] (54.7; 67.8) | 97.0[c] (92.7; 98.8) |
| IgG-ELISA | 96.9[b] (92.3; 98.8) | 62.8[d] (56.1; 69.1) |
| 69 sera considered as dot-ELISA negative, IgG-ELISA positive | | |
| Dot-ELISA | 64.4 (57.5; 70.8) | 97.3 (93.2; 98.9) |
| IgG-ELISA | 96.9 (92.3; 98.8) | 67.4 (60.1; 73.4) |
| 47 sera considered as dot-ELISA negative, IgG-ELISA positive | | |
| Dot-ELISA | 72.7 (65.6; 78.8) | 97.6 (94.0; 99.1) |
| IgG-ELISA | 96.9 (92.3; 98.8) | 77.6 (71.5; 82.7) |

[a] percentage of positive dot-ELISA tests/positive IgG-ELISA tests.
[b] percentage of positive IgG-ELISA tests/positive dot-ELISA tests.
[c] percentage of negative dot-ELISA tests/negative IgG-ELISA tests.
[d] percentage of negative IgG-ELISA tests/negative dot-ELISA tests.

Kappa statistic and z index were calculated for each of the instances; the value of kappa was 0.53 when the original 78 sera were considered as dot-ELISA negative/IgG-ELISA positive; when 9 sera were considered as IgG-ELISA negative the value of kappa was 0.58 and kappa was 0.70 when only 47 sera were considered as dot-ELISA negative/ IgG-ELISA positive; all kappa values correspond to a substancial degree of agreement between tests as defined by Feinstein. 1985[1]. The z value[3]. for each kappa statistic was 10.9, 11.6 and 13.3 respectively, indicating in all instances that there was a $p < 0.0001$ that results could be due to chance alone.

## DISCUSSION

The performance indexes accrued for sera collected in Northern and Northeastern regions in Brazil were similar to performance indexes found previously for the two tests[6]: the sensitivity of the dot-ELISA was lower than IgG-ELISA's whereas specificity and the positive predictive value of the dot-ELISA were higher than IgG-ELISA's (table 1). In this report, contrary to what was found previously[6], dot-ELISA's sensitivity index was not different from what would be found by a random test not associated with disease attribute, as for instance a coin-flip. As the specificity index for either test was higher than previously found[6], a very high value was achieved with the positive prediction for either test: 1 out of 100 dor-ELISA positive results would correspond to a false diagnosis of the disease (false positive) and 2/100 IgG-ELISA results would be false positive (table 1). This leads to the conclusion that the dot-ELISA is more apt than IgG-ELISA in disclosing a true diagnosis of the disease. In field surveys, or in any other situation where by means of a test result one tries to discriminate a diagnosis, it is this parameter, the positive predictive value and not sensitivity or specificity that allows for the discrimination between true positives and false positives to a test.

A similar change in performance indexes of sensitivity, specificity and positive predictive value

was seen for IgG-ELISA when different sets of leishmaniasis sera were used; when sera from long-standing patients were analysed (the same set of sera used for the standardization stage of dot-ELISA[3]) the sensitivity index was 93.3% (95% confidence limit 78.7-98.1) and the specificity index was 84.4% (95% confidence limit 73.5-91.3)[7]; when the present set of sera was used to assess the same indexes, IgG-ELISA sensitivity fell to 66.3% (95% confidence limit 60.8-71.4) and specificity was 77.5% (95% confidence limit 64.1-87.0)[10]. The positive predictive value of IgG-ELISA however, increased from 73.7% (95% confidence limit 58.0-85.0) in the standardization stage[7] to 94.6% (95% confidence limit 94.6-96.9) in field assay[8].

The comparison of each test positive or negative status led to the finding of 78 sera that were "dot-ELISA negative/IgG-ELISA positive". This led us to investigate if the discrepancies between test results were due to technical reasons (dot-ELISA titer was determined visually whereas IgG-ELISA's was determined by a spectrophotometer) and, if this phenomenon would influence the tests'diagnostic status as discrepancies in test results could give rise to different interpretations as to which category sera belonged: the "dot-ELISA negative/IgG-ELISA positive" or the "dot-ELISA negative/IgG-ELISA negative". Althought the relative sensitivity of the dot-ELISA and the relative specificity of both tests were influenced by the different criteria of assessment of a serum's positivity or negativity, as would be expected (table 2), the value of the kappa statistic for each of the situations varied between 0.53 and 0.70 indicating a substancial strength of agreement between tests[1]. The kappa statistic assesses the agreement between observers, methods or procedures discounting the proportion of agreements which is expected by chance alone; instead of the total proportion of observations on which there is an agreement being compared as a ratio to its maximum value (100%), the fraction of observations for which agreement can be attributed to the reproducibility of the observations rather than to mere chance, is compared as a ratio with its maximum posible value (1-P)[3]. The index z (kappa/standard error of kappa) indicates the statistical significance of the kappa statistic which can be assessed from the normal curve. Regardless of how many sera were considered in each category the two tests disclosed the same phenomenon, i.e., some sera reactive in the lower positive range of the IgG-ELISA assay gave negative dot-ELISA results and this decrease in reactivity did not interfere with dot-ELISA's ability to predict a disease non-disease status. The phenomenon seems dependent on the antigen used in each test, i.e., the dot-ELISA employs a partially soluble suspension as antigen whereas IgG-ELISA uses a soluble extract; the difference between extracts lies in the centrifugal force to which they are submitted which in turn interferes with the size of particles in suspension. In the standardization stage there were attempts to perform the dot-ELISA's using differently prepared antigens: a soluble antigen as IgG-ELISA's or of a fully particulate antigen as the one used by PAPPAS et al.[11], showed non-specific staining of antigen and conjugate controls. The removal of large debris from the parasite suspension resulted in an antigen without non-specific staining displaying maximum titer towards positive control sera on a checkerboard titration.

## RESUMO

**Índices de desempenho do dot-ELISA e do IgG-ELISA, em inquéritos soroepidemiológicos da leishmaniose do Novo Mundo.**

Índices de desempenho diagnóstico de sensibilidade, especificidade, valor preditivo positivo e eficiência foram determinados nas reações de dot-ELISA e IgG-ELISA em 340 soros de leishmaniose mucocutânea. A sensibilidade do dot-ELISA foi significativamente mais baixa que a da IgG-ELISA e os dois testes tiveram índices de especificidade e de valor de predição positivo da mesma magnitude. Setenta e oito soros tinham resultado negativo à reação de dot-ELISA e resultado positivo ao IgG-ELISA. Quando os soros foram examinados de acordo com diferentes critérios de análise, quanto a esta discrepância a estatística kappa não corroborou esta hipótese indicando que os dois testes concordavam substancialmente. Os resultados apresentados indicam que os índices de desempenho diagnóstico obtidos em trabalhos de campo onde haja boa caracterização das variáveis podem ser extrapolados a outros estudos populacionais desde que a doença se mostre de alta prevalência (devido a um viés de seleção ou não) e nestas condições se pode esperar que os índices façam o diagnóstico de doença entre os resultados positivos ao teste.

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