Evaluation of Dot Enzyme-Linked Immunosorbent Assay for Mucocutaneous Leishmaniasis and Comparison with Microplate Enzyme Immunoassay

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A dot enzyme-linked immunosorbent assay (dot ELISA) was evaluated and compared with a standard microplate ELISA (immunoglobulin G [IgG] ELISA) for the serological diagnosis of mucocutaneous leishmaniasis. The two assays were used to test 113 serum specimens from the following groups: normal individuals and patients with deep mycoses, toxoplasmosis, mucocutaneous leishmaniasis, visceral leishmaniasis, Chagas' disease, malaria, and schistosomiasis. Both tests exhibited cross-reactivity when testing specimens from cases of visceral leishmaniasis and Chagas' disease. The dot ELISA proved to be economical with respect to use of reagents and was easy to perform. Interpretation could easily be made by visual inspection of reaction endpoints in the nitrocellulose disks, obviating the need for spectrophotometric readings. There were no significant differences in sensitivity between the dot ELISA and the IgG ELISA at a cutoff level either of 20 or 40. However, its most remarkable feature was the high specificity compared with that of the IgG ELISA. Because of its ease of performance and high sensitivity and specificity, the dot ELISA should be an excellent test to be executed in the field during seroepidemiological surveys.

Leishmaniasis is a disease endemic in Brazil and is caused by at least three different protozoan parasites. One such agent, *Leishmania braziliensis braziliensis*, is able to invade the mucosae of the nose and soft palate, and if not adequately treated, this may lead to extensive destruction of cartilage and tissue scarring. Other leishmaniasis, such as *Leishmania braziliensis guyanensis* and *Leishmania mexicana amazonensis*, cause only a cutaneous disease. In 1983, Pappas et al. (8) reported that a dot enzyme-linked immunosorbent assay (dot ELISA) for the serological diagnosis of visceral leishmaniasis was a rapid and economical method. When the anti-immunoglobulin G (IgG) conjugate used was replaced by an anti-gamma-chain-specific conjugate, there was a decrease in the rate of false-positive results, which resulted in a more specific test (9). In this paper we report a similar dot ELISA for the serodiagnosis of mucocutaneous leishmaniasis following the procedure described by Pappas et al. (8). Modifications introduced in this assay resulted in a highly specific and sensitive test with high predictive values with respect to disease status, as ascertained by comparison with a standard ELISA which requires antigen-coated plates.

**MATERIALS AND METHODS**

**Antigen preparation.** Eight-day-old, LIT-cultured (6) promastigotes from *L. braziliensis* 49 were used as the source of antigen. The strain was characterized as *Leishmania major*-like by monoclonal antibody serotyping by G. Grimaldi, Fiocruz, Rio de Janeiro. Cultured parasites were washed three times with 0.01 M phosphate-buffered saline (pH 7.2) and freeze-dried. A total of 100 mg of parasites were suspended in 20 ml of 0.15 M NaOH and sonicated at 40 KHz with three pulses, 20 s each. Particles were extracted during a 6-h period at 4°C, and the solution was neutralized to pH 7.2 with 0.3 N HCl. The suspension was further extracted overnight and centrifuged at 4,000 × g for 10 min to remove large debris. The protein concentration was determined by the Waarburg-Christian method, as cited by Layne (7). Nitrocellulose membranes (pore size, 0.22 μm; Millipore Corp., Bedford, Mass.) were cut into 5-mm disks and handled with forceps. Antigen in 1-μl amounts (1.8 μg of protein) was dotted on the dull side of disks with a 10-μl Hamilton syringe. Antigen was fixed onto the disks by drying for 30 min at 37°C. In some experiments, decreasing concentrations of antigen were dotted onto the disks. Sensitized disks were stored at −20°C in a tightly closed screw-cap jar until use.

**Dot ELISA.** Disks were allowed to reach room temperature and were placed in flat-bottom microtitration plate wells (Alfesa Ltda., São Paulo, Brazil). A positive control serum, three pools of negative control sera, and antigen and conjugate controls were included in every experiment. Antigen disks were blocked overnight with 75 μl of a 5% skim milk solution (wt/vol) in triethanolamine-buffered saline (pH 7.5) (TBS). This solution had been previously heated in a water bath at 100°C for 15 min and then filtered to inactivate milk proteases (S. Hoshino-Shimizu, personal communication). In previous experiments, we tested different incubation times with the skim milk solution and also incubation with 5% bovine serum albumin in TBS and 1 and 2% normal rabbit serum in TBS. No differences in background reactivity were seen when the blocking solution was incubated for 1 or 2 h as compared with overnight incubation. Neither bovine serum albumin-TBS nor normal rabbit serum-TBS adequately blocked background nonspecific reactivity. The blocking solution was discarded, and 50 μl of serially diluted serum samples in 5% skim milk-TBS was added to each well and incubated at 37°C for 30 min. Dilutions were aspirated off disks, and these were washed by shaking (three times, 10
min each) with 100 µl of 0.05% Nonidet P-40 (Shell Química do Brasil, São Paulo, Brazil) in TBS (vol/vol). The washing solution was removed, and 50 µl of a dilution of a horseradish peroxidase-labeled anti-human IgG (gamma chain specific) conjugate (enzyme/protein weight ratio, 0.3) was added to each well and incubated for 30 min at 37°C. The optimum dilution of the conjugate was found to be 1/150 by block titration of twofold dilutions of the conjugate. The conditions of conjugate preparation and evaluation were those described by Guimarães et al. (4). The conjugate was removed, and washings were conducted as described above. The chromogen 4-chloro-1-naphthol (Sigma Chemical Co., St. Louis, Mo.) was dissolved in anhydrous methanol (stock solution, 3 mg/ml) and stored in a dark bottle at room temperature for up to 14 days. Immediately before use, 10 µl of TBS, 4 µl of 30% H₂O₂, and 2 ml of the chromogen stock solution were mixed together and added in 50-µl amounts to each well, and the plates were incubated for 30 min at 37°C. The chromogen solution was discarded, and washings were conducted as described above. The development of blue-purple dots on disks when compared with negative serum control pools and antigen and conjugate controls was considered to be evidence of positivity. Color development in the controls was negligible.

Sera. Two sets of sera were used: set 1, made up of 93 specimens divided into four subsets (28 normal controls, 32 deep mycoses, 5 toxoplasmosis, and 28 mucocutaneous leishmaniasis); and set 2, made up of five malaria, five visceral leishmaniasis, five Chagas' disease, and five schistosomiasis. Diagnosis in set 1 was established by means of clinical, epidemiological, and parasitological data. All but the five toxoplasmosis patients were given a Montenegro skin test. Etiology in the deep mycoses subset was assessed by means of other appropriate skin tests and culture or biopsy from the lesion to identify the agent. Set 2 was made up of sera kept at the Seroepidemiology Laboratory of the Sao Paulo Tropical Medicine Institute. All specimens had positive serology results establishing diagnosis and also positive parasitological tests such as parasite identification or xenodiagnosis. In addition to the dot ELISA, all sera were submitted to standard IgG immunofluorescence (IgG IF) and IgG ELISAs with promastigote antigens and anti-IgG conjugated antibodies were performed using techniques described previously (5, 6).

Statistical analysis. Diagnostic performance of the dot ELISA and the standard ELISA was comparatively evaluated as follows. Serological titers obtained for all sera could be construed dichotomously as either positive or negative by using two different cutoff dilutions for each test, 1/20 and 1/40. This allowed a construction of 2 × 2 contingency tables with frequencies of true-positive, true-negative, false-positive, and false-negative results with respect to the disease attribute of the specimens (cases of mucocutaneous leishmaniasis being considered the disease group). Standard diagnostic indexes such as sensitivity, specificity, predictive values of positive and negative results, and efficiency were calculated by using previously described formulas (3). Interval estimation around these indexes were computed by the method of Wilson, by using 95% binomial confidence bands (10). All specimens constituted a panel assembled randomly from different preexisting collections, forming a distorted picture of the actual prevalence of the target disease, mucocutaneous leishmaniasis. Because predictive value indexes are not only test related but also dependent on the prevalence of the disease in a given setting, these values were considered solely for within-assay comparisons with results from the standard ELISA.

FIG. 1. Titer frequency distribution of dot ELISA results in 93 sera from set 1. (a) Overall distribution. (b) Distribution according to disease or nondisease attribute (mucocutaneous leishmaniasis or other parasitic diseases).

RESULTS

The dot ELISA titer frequency distribution for sera in set 1 is shown in Fig. 1. The overall distribution of titer frequencies behaved as a bimodal curve with its lowest point at titer 40 (Fig. 1a). This is best seen in Fig. 1b, where sera were discriminated according to their disease or nondisease attribute. A rise in the control serum frequency distribution at titer 160 was due to one serum in the deep mycoses subset from a Montenegro-negative patient who also had positive serology for Chagas' disease but no clinical evidence of concomitant mucocutaneous leishmaniasis.

All mucocutaneous leishmaniasis patients and five (15.6%) deep mycoses patients had positive Montenegro skin tests. However, the latter five samples yielded negative results by the dot ELISA and by the IgG IF test. As judged by the IgG ELISA, one of these specimens had a titer of 80, whereas the four other had titers of ≥20. Among the 28 mucocutaneous leishmaniasis sera, only 2 were judged negative by the dot ELISA. Of the latter specimens, one was also negative by both the IgG ELISA and IgG IF, and the other had a titer of 640 by IgG ELISA and was negative by IgG IF. The remaining 26 sera had titers ranging between 80 and 2,560.

In the deep mycoses subset, two sera from Montenegro-negative patients had titers of 40 and 160. The first was also negative by IgG ELISA and IgG IF, and the other had an IgG ELISA titer of 640, negative IgG IF, and positive serology for Chagas' disease, as described above. All five toxoplasmosis sera yielded negative dot ELISA and IgG IF results. However, three specimens from this group had titers of ≥20 by the IgG ELISA.

All 28 patients from the normal control group had negative Montenegro skin tests. Three sera in this group exhibited dot ELISA titers of 20, and the remaining specimens were negative. One of these had an IgG ELISA and IgG IF titer of 20, another was negative by IgG ELISA and had a titer of 20 by IgG IF, and the third had an IgG ELISA titer of 20 and no reactivity by the IgG IF. Among the sera judged negative by the dot ELISA technique, one, from a keloid patient, was also negative by IgG IF. However, this specimen exhibited a titer of 640 by IgG ELISA and negative serology for Chagas' disease.

In set 2, four out of five malaria sera gave negative results...
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TABLE 1. Frequencies of test results obtained with the dot ELISA and the IgG ELISA at two different cutoff levels for the serological diagnosis of mucocutaneous leishmaniasis (results with sera from set 1)

<table>
<thead>
<tr>
<th>Test and cutoff titera</th>
<th>Frequency of result</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>False</td>
<td>True</td>
</tr>
<tr>
<td>Dot ELISA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>26</td>
<td>5</td>
<td>60</td>
</tr>
<tr>
<td>40</td>
<td>25</td>
<td>2</td>
<td>63</td>
</tr>
<tr>
<td>IgG ELISA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>26</td>
<td>28</td>
<td>37</td>
</tr>
<tr>
<td>40</td>
<td>26</td>
<td>12</td>
<td>53</td>
</tr>
</tbody>
</table>

a Set 1 sera are divided into the following subsets: 28 normal controls, 32 deep mycoses, 5 toxoplasmosis, and 28 mucocutaneous leishmaniasis ("true" disease cases were assumed to be the last subset, whereas the remaining 65 specimens were taken as negative controls).

b Specimens reacting at a titer greater than or equal to the cutoff level were considered positive.

and one had a titer of 40. Four schistosomiasis sera were also negative by the dot ELISA. The remaining schistosomiasis serum yielded a titer of 40 by the dot ELISA and had positive serology for Chagas’ disease, as determined by current serological tests used in our laboratory. All visceral leishmaniasis sera and all five Chagas’ disease sera had titers of at least 40 by the dot ELISA and by the IgG ELISA.

Frequencies of serological results for specimens in set 1, with dot ELISA and IgG ELISA at two different cutoff titers, are displayed in Table 1. The combined frequencies of false results, both positive and negative, were lower for both tests with the higher cutoff level of 40. These frequencies were used in the computation of diagnostic test indexes with their respective 95% confidence limits (Table 2). For both cutoff levels, the dot ELISA ranked as a superior test (except for sensitivity measured at a cutoff of 40). The index which most remarkably resolved the diagnostic performance of both tests was specificity. In fact, at each cutoff level, specificities and their 95% confidence intervals were completely exclusive. Efficiency, as a measure of all correct diagnoses, both positive and negative, was also an index that allowed resolution between performances of the two tests.

DISCUSSION

The dot ELISA for mucocutaneous leishmaniasis, as reported here, is a specific means of establishing serological diagnosis of the disease. It also has technical advantages such as the use of minute amounts of antigen, antibody, conjugate, and chromogen solution. In preliminary experiments, we did not observe any differences in efficiency of blocking nonspecific reactivity with incubations of 1 h, 2 h, or overnight. Therefore, we chose an overnight incubation period as more convenient for completion of the assay. If, however, the blocking period were shortened to 1 h, the total length of time required to perform a dot ELISA would be similar to that required for the execution of an IF test, i.e., ca. 3.5 h. Another advantage of this technique is that it obviates the need for spectrophotometric readings, which are required in classical plate or cuvette ELISA techniques. This is a desirable feature if assays are to be done in a field laboratory.

As disadvantages of the dot ELISA the following may be listed; the high cost of nitrocellulose membranes and the test requirement of a low dilution of conjugate. These are, nevertheless, of small importance since 150 disks or more can be punched out from one sheet and the small volume of conjugate used resulted in a test no more expensive than a standard ELISA or IF.

Cross-reactivity with both visceral leishmaniasis and Chagas’ disease became evident when specimens from set 2 were tested. All sera reacted at least at a titer of 40 (with both ELISA versions). Such cross-reactivity, nonetheless, to be expected inasmuch as most serological tests using crude parasite preparations tend to reflect the extensive antigenic sharing among those flagellates (1). It is likely, however, that purified antigen preparations, if used in the dot ELISA, might yield more specific results when testing specimens from patients with Chagas’ disease or visceral leishmaniasis. In fact, Araujo (2) did not observe cross-reactivity between a Trypanosoma cruzi antigen and mucocutaneous leishmaniasis sera. He attributed this finding to the centrifugation step used during the antigen preparation procedure which removed coarse particles from the antigen lysate.

In set 2 there was another serum which displayed a titer of 40. It belonged to a patient with Plasmodium falciparum malaria acquired in Mato Grosso state, where mucocutaneous leishmaniasis is also endemic. Since it was not possible to obtain data on this patient’s Montenegro skin test, the possibility of this being a true-positive serum cannot be ruled out. This patient, however, had a negative IgG IF test for mucocutaneous leishmaniasis, as well as negative serology for Chagas’ disease. It is noteworthy that seroepidemiological surveys for mucocutaneous leishmaniasis performed in malaria-endemic regions do not indicate any cross-reactivity with antimalarial antibodies (M. C. S. Guimaraes, B. J.

TABLE 2. Diagnostic performance indexes of the dot ELISA and IgG ELISA at two different cutoff levels for the serological diagnosis of mucocutaneous leishmaniasis

<table>
<thead>
<tr>
<th>Test and cutoff titer</th>
<th>Sensitivity (95% confidence limit)</th>
<th>Specificity (95% confidence limit)</th>
<th>Predictive value Positive</th>
<th>Predictive value Negative</th>
<th>Efficiency</th>
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<tbody>
<tr>
<td>Dot ELISA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>92.9 (77.4–98.0)</td>
<td>92.3 (83.2–96.7)</td>
<td>83.9 (67.4–92.9)</td>
<td>96.8 (89.0–99.1)</td>
<td>92.5</td>
</tr>
<tr>
<td>40</td>
<td>89.3 (72.8–96.3)</td>
<td>96.9 (89.5–99.2)</td>
<td>92.6 (76.6–97.9)</td>
<td>95.5 (87.5–98.4)</td>
<td>94.6</td>
</tr>
<tr>
<td>IgG ELISA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>92.9 (77.4–98.0)</td>
<td>56.9 (44.8–68.2)a</td>
<td>48.2 (35.4–61.2)</td>
<td>94.9 (83.1–98.6)</td>
<td>67.7</td>
</tr>
<tr>
<td>40</td>
<td>92.9 (77.4–98.0)</td>
<td>81.5 (70.5–89.1)</td>
<td>68.4 (52.5–80.9)</td>
<td>96.4 (87.7–99.0)</td>
<td>85.0</td>
</tr>
</tbody>
</table>

a Results with sera from set 1 computed from frequencies in Table 1.

b Interval includes the value which would be obtained by a random test not associated with the disease attribute.

A completely random test with independent outcomes (positive or negative results) with respect to the disease attribute of the specimens (a coin-flip-like test, for instance) would present equal sensitivity and specificity of 50%. However, predictive values are dependent on the relative proportion of samples which belong to the disease group. In the present series, the predictive values of positive and negative results from a totally random test would be 30.4% and 70.2%, respectively. The IgG ELISA used at a cutoff of 20 yielded very poor specificity (56.9%) which was not significantly different from that which would be exhibited by a hypothetical no-association test. Ninety-five percent confidence bands computed for this observed value included 50%. Although the sensitivity for the dot ELISA at a cutoff of 40 was numerically lower than that obtained for the IgG ELISA at the same cutoff, this difference could be attributed to chance alone. On the other hand, the specificity for the dot ELISA at a cutoff of 40 was much higher than that obtained for the IgG ELISA at either level. As a corollary, predictive values of positive tests with the dot ELISA were considerably higher than those with the IgG ELISA.

Because sensitivities were not different across tests and cutoffs, the predictive values of negative results also did not differ noticeably. Diagnostic efficiency, however, reflects the ratio of false-positive and false-negative results obtained in each test combination. By focusing on this index alone, the dot ELISA, when used at a cutoff of 40, seemed to be the best test combination.

Selection of an appropriate test to be used in seroepidemiological surveys should be based on objective criteria of cost effectiveness, ease of use in the field, and diagnostic performance. Use of test indexes represents a straightforward means of scoring the magnitude of such a performance. In addition, interpretation of the relative value of these indexes is made easier if they are expressed together with estimates of dispersion at predetermined confidence levels. With this set of criteria, the dot ELISA was an excellent test for the serological diagnosis of mucocutaneous leishmaniasis.

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LITERATURE CITED