Comparative Assessment of the Leprosy Antibody Absorption Test, *Mycobacterium leprae* Extract Enzyme-Linked Immunosorbent Assay, and Gelatin Particle Agglutination Test for Serodiagnosis of Lepromatous Leprosy

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Leprosy is still a major health problem in many tropical and subtropical areas, where it afflicts approximately 12 million people, even though the total number of officially reported cases for 1990 was 3.7 million (31). Currently, leprosy is diagnosed and classified according to clinical, histopathological, and bacteriological criteria (9). Nevertheless, in many developing countries where leprosy is endemic, resources for these purposes are often very limited. Therefore, it is necessary to have simple, cost-effective, and rapid methodologies which could be complementary to or even substitute for conventional diagnostic procedures. Serodiagnosis is the most affordable alternative, and consequently several different tests for leprosy with a wide array of effectiveness have been proposed and evaluated (17, 24, 31).

The aim of the present work was to assess the performance and effectiveness of three leprosy tests, using a heterogeneous mixture of antigens of *Mycobacterium leprae*, and the third using a specific antigenic derivative of the same agent. The first was the fluorescent leprosy antibody absorption (FLA-ABS) test, developed by Abe et al. (1), which is an indirect immunofluorescence test with whole *M. leprae* as the antigen and suspicous sera previously absorbed with intact *M. bovis* BCG and *M. vaccae* and a cardiolipin-lecithin solution. The second was an indirect enzyme-linked immunosorbent assay (ELISA) using plates coated with soluble antigen from sonicated *M. leprae* (28). Finally, we included the *M. leprae* particle agglutination (MLPA) test (22), a recently developed test involving passive agglutina-

tion of sensitized gelatin particles with chemically synthesized *M. leprae* natural trisaccharide-phenyl propionate-bovine serum albumin (NT-P-BSA).

Studied sera included samples obtained from 45 patients treated for lepromatous leprosy diagnosed and classified according to the standard clinical, histopathological, and bacteriological methods at the Centro Dermatológico Dr. Ladislao de la Pascua, Mexico City, Mexico. Also, 21 serum samples from newly discovered and untreated cases of pulmonary tuberculosis, all of which were identified after clinical manifestations and through culture-positive results at the Clinica de Enfermedades del Aparato Respiratorio, Secretaría de Salud, Mexico City, Mexico, were used. As healthy controls, two groups were selected. The first consisted of 99 serum samples from the area of Mexico with the highest rate of leprosy (the state of Sinaloa), where the leprosy rate is 1.28 cases per 1,000 inhabitants, and the second consisted of 99 serum specimens from Mexico City, where the prevalence of the disease is low (0.14 cases per 1,000 inhabitants). No known histories of direct contact with patients with leprosy or apparent manifestations of leprosy were reported either for tuberculosis cases or for healthy controls. All sera were kept frozen at −70°C until use.

For the FLA-ABS test, *M. leprae* grown in armadillos and supplied by E. E. Storr, Medical Research Institute, Florida Institute of Technology, Melbourne, Fla., was purified from the liver by the method of Prabakaran et al. (27). The acid-fast bacilli were counted by the method standardized by Shepard and McRae (30), and the suspension was diluted with phosphate-buffered saline (PBS), pH 7.2, to a concentra-

tion of 1 × 10⁸ to 1.5 × 10⁸ acid-fast bacilli per ml. The
were titrated in fourfold dilutions up to 1:10,000 g at 4°C for 30 min, and the resulting pellet was suspended in 2 ml of PBS. Microorganisms were disrupted by sonication on ice for 15 min at 250 W on a Sonifier cell disrupter (Model W-370, Heat System; Ultrasonics, Inc., North Tonawanda, N.Y.). The sonicated material was microscopically examined for the absence of intact bacilli and centrifuged at 20,000 \( \times \) g for 15 min at 4°C, and the sediment was discarded. The supernatant was filtered through a membrane filter (0.22-μm pore size), the protein concentration was adjusted to 5 mg/ml, and the supernatant was stored at −70°C until used.

ELISA plates (Nunc-Immunoplate Maxisorp F96 polystyrene microtiter plates; Nunc A/S, Kamstrup, Roskilde, Denmark) were sensitized with 100 μl (500 mg of protein) of \( M. \) leprae extract. After blocking with 1% BSA (grade IV; Sigma in PBS, the plates were incubated with test or control sera diluted to 1:1,000 in the same solution used for blocking. The wells were then washed, and the plate was incubated at room temperature with polyclonal goat anti-human immunoglobulin conjugated to horseradish peroxidase (Sigma) at a 1:1,000 dilution. After 1 h of incubation, freshly prepared 0.4-mg/ml o-phenylenediamine with hydrogen peroxide was used as the enzyme substrate. The reaction was stopped with 4 N sulfuric acid, and the optical density was read at 490 nm in a Dynatech MR580 MicroELISA autoreader. A cutoff value of 0.197 was calculated as the sum of the mean optical density obtained for healthy individuals from the area where leprosy is endemic (0.117) and two standard deviations (0.040).

The quantitative gelatin particle agglutination test (MLPA) with particles sensitized with the semisynthetic trisaccharide of phenolic glycolipid I (PGL-I) (NT-P-BSA) was performed with a Serodia*-Lepra diagnostic kit (Fujirebio Inc., Tokyo, Japan), which was kindly donated by the manufacturer. Twofold serum dilutions from 1:4 to 1:4,096 were made in the wells of the supplied U-bottom microtiter plate. Twenty-five microliters of sensitized particles were added to each serum dilution, and after 2 h of incubation at room temperature, agglutination patterns were read and interpreted according to the manufacturer's instructions. Positive results were recorded when agglutination was observed in the 1:32 final dilution and higher dilutions.

Specificity, sensitivity, and positive and negative predictive values were calculated for each test, by the method of Galen and Gambino (19). The percent agreement between two tests was determined by the following formula: 100 \( \times \) number of results in agreement/total number of samples tested. Comparison between each pair of procedures was tested by Cohen's kappa test (16), and a 95% confidence interval was calculated. The \( \kappa \) value was corrected for chance and ranged from −1 to 1, where a \( \kappa \) of 1 indicates perfect agreement, a \( \kappa \) of −1 indicates perfect disagreement, and a \( \kappa \) of 0 indicates agreement expected by chance alone.

Table 1 shows the results obtained with the FLA-ABS test, \( M. \) leprae extract ELISA, and MLPA test with sera from patients and controls. Some sera from individuals from

coverslips. They were read at a magnification of ×600 with a fluorescence microscope (Immunopan; American Optical, Buffalo, N.Y.), and no less than 50 fields were observed and counted for each smear. Results were reported as positive when most bacilli showed the strong characteristically green fluorescence of fluorescein isothiocyanate and as negative when it was not possible to detect fluorescence or when the nonfluorescent bacilli outnumbered the fluorescent ones.

The \( M. \) leprae extract ELISA was performed with \( M. \) leprae obtained from infected armadillos as described above. The bacillary suspension was centrifuged at 10,000 \( \times \) g at 4°C for 30 min, and the resulting pellet was suspended in 2 ml of PBS. Microorganisms were disrupted by sonication on ice for 15 min at 250 W on a Sonifier cell disrupter (Model W-370, Heat System; Ultrasonics, Inc., North Tonawanda, N.Y.). The sonicated material was microscopically examined for the absence of intact bacilli and centrifuged at 20,000 \( \times \) g for 15 min at 4°C, and the sediment was discarded. The supernatant was filtered through a membrane filter (0.22-μm pore size), the protein concentration was adjusted to 5 mg/ml, and the supernatant was stored at −70°C until used.

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the area where leprosy is not endemic and from patients with tuberculosis could not be studied by the MLPA test because of insufficient sample volumes. It is evident that the FLA-ABS test is the best method to detect almost all leprosy cases under treatment, as it yielded no false-positive results for patients with tuberculosis and a very low rate of false-positive results for healthy populations.

Table 2 depicts data for sensitivity, specificity, and predictive values for each of the studied tests. Because of the high circulation of M. leprae in areas of endemicity, we considered it necessary to analyze the results of each of the tests separately, thus comparing leprosy patients first with the healthy population from the area of low endemicity and second with those from the area of high endemicity. All methods demonstrated similarly high specificities, but only the FLA-ABS test displayed adequate sensitivity. Positive and negative predictive values were also more reliable with the FLA-ABS test, and the only other comparable method for yielding positive results was the MLPA test.

Results showing the concordance between each pair of tests are given in Fig. 1. No two tests agreed absolutely, and the differences observed could be attributed to reactivity with separate antigens or epitopes in each test, but further studies will be necessary to clarify this.

A low degree of specificity due to cross-reactive antibodies elicited by casual or deliberate contact with mycobacteria other than M. leprae is one of the most serious limitations in leprosy serodiagnosis. Hence, in order to seek the proportion of false-positive results due to these factors, the healthy controls used here were selected from two epidemiologically distinct areas, one of high endemicity and the other of low endemicity. For both groups, contact with environmental mycobacteria and the effect of BCG vaccination, which is still compulsory in Mexico, are the factors to be explored, while in the former group another factor is the immunological impact of the wide circulation of M. leprae. Our results showed that all three evaluated tests displayed good enough specificity; with the FLA-ABS test, the small number of positive reactions with both control groups agrees with previously reported data (1, 4, 6, 21, 23), but the absence of false-positive results with the MLPA test differs from observations in other laboratories in studies in which the only important difference from the present work is the ethnic background of the control population. Thus, Izumi et al. found 7.7% false-positive results in Japan (22), Chanteau et al. found 9.3% false-positive results in Polynesia (10), and Dhidayuthapani et al. found 17% false-positive results in India (13). It is highly probable that genetic traits, along with other factors yet to be defined, are responsible for such discrepancies. In this study, it was not possible to establish the origin of antibodies which gave positive reactions in healthy individuals, but the ages of positive controls displayed a random distribution, a fact which could rule out a major participation of antibodies generated after BCG vaccination and supports their probable presence due to frequent contact with environmental nonpathogenic mycobacteria.

The case of M. tuberculosis is special because it is still a major pathogen in developing countries and very often its geographical distribution overlaps with that of M. leprae. Here, a small but highly representative sample of patients with confirmed, active, untreated pulmonary tuberculosis was selected and studied. The best results were obtained with the FLA-ABS test with no false-positive reactions, supporting similar data reported by us (4) as well as other authors (1, 23). Undoubtedly, extensive absorption with whole-cell M. vaccae and M. bovis BCG is able to confer high specificity to the test. In contrast, the M. leprae extract ELISA resulted in the lowest mycobacterial specificity, a likely result of antigen sharing between M. leprae and other mycobacteria (34). With the MLPA test, despite the recognized exclusiveness of PGL-I for M. leprae (33), two of the tuberculosis patient samples were positive without a satisfactory explanation. Such a finding is not unusual, and in some but not all of the reported studies in which native PGL-I or its synthetic oligosaccharides were used, a few positive reactions in tuberculosis patients sera have occurred (12, 18, 26, 32). Regardless of whether these reactions were due to the recognition of analogous glycolipids in M. tuberculosis, this phenomenon does not invalidate the usefulness of PGL-I moiety-based tests in leprosy serodiagnosis.

### Table 1. Results of the FLA-ABS test, M. leprae extract ELISA, and MLPA test for patients with leprosy and individuals without leprosy

<table>
<thead>
<tr>
<th>Group</th>
<th>FLA-ABS test</th>
<th>M. leprae extract ELISA</th>
<th>MLPA test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. positive/total</td>
<td>% Positive</td>
<td>No. positive/total</td>
</tr>
<tr>
<td>Healthy individuals from an area where leprosy is not endemic</td>
<td>1/99</td>
<td>1</td>
<td>5/99</td>
</tr>
<tr>
<td>Healthy noncontacts from an area where leprosy is endemic</td>
<td>5/99</td>
<td>5</td>
<td>3/99</td>
</tr>
<tr>
<td>Untreated pulmonary tuberculosis</td>
<td>0/21</td>
<td>0</td>
<td>9/21</td>
</tr>
<tr>
<td>Patients with lepromatous leprosy</td>
<td>43/45</td>
<td>95.6</td>
<td>21/45</td>
</tr>
</tbody>
</table>

*At a 1:1,000 serum dilution.*

### Table 2. Sensitivity, specificity, and predictive values of the FLA-ABS test, M. leprae extract ELISA, and MLPA test

<table>
<thead>
<tr>
<th>Test</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLA-ABS test</td>
<td>0.95</td>
<td>0.95</td>
<td>0.89</td>
<td>0.98</td>
</tr>
<tr>
<td>M. leprae extract</td>
<td>0.47</td>
<td>0.97</td>
<td>0.88</td>
<td>0.80</td>
</tr>
<tr>
<td>ELISA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MLPA test</td>
<td>0.58</td>
<td>0.95</td>
<td>1.00</td>
<td>0.86</td>
</tr>
</tbody>
</table>

*In comparison with results of healthy individuals from an area where leprosy is highly endemic.*
and indicates only that special care be taken in the interpretation of individual positive results.

The low sensitivity of the *M. leprae* extract ELISA (47%) resulting from the high number of false-positive reactions precludes its use in serodiagnosis of leprosy. Interestingly, a similar sensitivity for patients with lepromatous leprosy with a BCG extract ELISA has been reported (15), suggesting that the reactivity of most antibodies in these patients is against antigens shared by both species.

The FLA-ABS test showed an outstanding sensitivity of 95% which, along with the aforementioned high specificity, makes it an excellent candidate to be used for a wide variety of leprosy studies, except for the assessment of treatment efficacy (2, 3, 7, 14, 20, 29). Nevertheless, in developing countries it is difficult to set up this test because it is expensive and requires well-trained personnel for its correct interpretation. These features, along with the high proportion of household contacts who have antibodies detectable with the assay (1, 2, 4, 6, 21, 23), limit its performance to well-developed laboratories to be carried out as a confirmatory test for individuals with a definite clinical status and epidemiological background.

A remarkable decrease of anti-PGL-1 antibodies after treatment has been observed with several methods (5, 8, 11, 12, 25), including the MLPA test (10, 13, 22). Thus, our value of 58% sensitivity is appropriate for a mixed population that includes lepromatous patients with regular and irregular treatments. Unfortunately, the sample size was not large enough to assess the statistical significance of the relationship between the MLPA test and the extent or success of treatment. However, the diminished reactivity in the MLPA test as a consequence of successful treatment does not affect the effectiveness of the test in epidemiological surveillance, detection of new cases, and monitoring of the therapeutic response. Advantages resulting from its low cost, the high number of samples that can be processed daily, and the ease of carrying out the test and interpreting results make the MLPA test the most suitable diagnostic tool for small laboratories with limited resources and for large-scale and field serological studies.

In conclusion, the MLPA and FLA-ABS tests are complementary tests for leprosy which must be set up for different purposes: the MLPA test for primary screening, epidemiological surveillance, and therapy monitoring in multibacillary cases and the FLA-ABS test for confirmatory and research studies.

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