FIELD EVALUATION OF ENZYME-LINKED IMMUNOSORBENT ASSAY FOR THE SERODIAGNOSIS OF TUBERCULOSIS

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Suggested running head: SERODIAGNOSIS OF TUBERCULOSIS

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SUMMARY An enzyme-linked immunosorbent assay (ELISA) was evaluated as a serodiagnostic test for active tuberculosis in La Paz, Bolivia. ELISA was compared with sputum smear in 277 persons presenting to the Instituto de Torax and was used for screening in 1458 military personnel. The test was performed under field conditions on 4 μl samples of capillary blood obtained by finger prick. The ELISA was found to have a sensitivity of 69% and a specificity of 88%. Sputum smear had a sensitivity of 79% and a specificity of 100%. ELISA was found to have undiminished sensitivity and specificity in patients who were sputum-negative, and the two tests could be combined to achieve a sensitivity of 92% and specificity of 88%. Positive and negative predictive values were highest for populations with tuberculosis prevalences in the range of that seen among patients presenting to the Instituto de Torax in Bolivia, but ELISA also led to the diagnosis of tuberculosis in 5 of 1458 the soldiers tested in the screening program.

Key Words: tuberculosis, serology, ELISA, serodiagnosis, sputum smear
Introduction

The introduction of enzyme-linked immunosorbent assay (ELISA) techniques has resulted in a resurgence of interest in the development of serodiagnostic tests for active tuberculosis. Following the initial study by Nassau, Parsons, and Johnson (1), a number of investigators have reported promising results with this technique (2-14). We have been particularly interested in using ELISA in developing countries with high tuberculosis prevalences, and we have found ELISA to be sensitive and very specific in studies performed in Argentina (14) and China (Ma, Wang, and Daniel, to be published). In those studies we used Mycobacterium tuberculosis antigen 5 prepared by immunoabsorbent affinity chromatography (15).

The present study was undertaken to determine whether ELISA could be performed under typical developing country conditions using simplified techniques without specialized equipment or instrumentation. We used an antigen prepared from a monoclonal immunoabsorbent potentially unlimited in its availability. ELISA was compared directly with sputum smear, the current standard procedure for the diagnosis of tuberculosis in most developing countries.

Methods

Selection of Patients

All new patients admitted to the in-patient and ambulatory pulmonary services of the Instituto de Torax in La Paz, Bolivia during a 14 week period were enrolled in the study. Enrollment was disrupted at times by civil unrest, a general strike, and a strike of health workers. The Instituto de Torax is the largest public facility providing tuberculosis treatment care in La Paz. It serves patients not only from the city but
also from surrounding regions. The majority of patients are indigent. Tuberculosis case rates in Bolivia are among the highest in the world (16).

Each patient entered into the study was seen by a physician. A history was obtained and physical examination performed. Chest radiographs were ordered on most patients, but often were not taken because of lack of supplies. Sputum was obtained from every patient who was able to produce an appropriate sample. Capillary blood was obtained by finger prick from every patient. Patients were classified by diagnosis as pulmonary tuberculosis, probable pulmonary tuberculosis, and not tuberculosis. Patients were considered to have tuberculosis who presented with a compatible clinical history, a positive sputum culture, and a compatible chest radiograph, if available. Two of the 61 patients in this category also had extrapulmonary tuberculosis. Patients with pleurisy with effusion were categorized separately. The diagnosis of tuberculous pleurisy was accepted if the culture of pleural fluid was positive or if the pleural biopsy demonstrated granulomas. Patients were classified as probable tuberculosis who presented with a history strongly suggestive of tuberculosis and either a chest radiograph typical of tuberculosis without sputum available or a positive sputum smear without culture available. Three of the 29 patients in this category had extrapulmonary lesions consistent with tuberculosis. Patients were considered nontuberculous who presented with an atypical pulmonary history and a negative chest radiograph or culture or a nonpulmonary history and no available chest radiograph or sputum examination. Sixty-one patients were excluded because data were insufficient to permit classification.

Studies of Military Personnel

ELISA serology was performed in 1458 unselected active duty military personnel in 6 Bolivian army regiments. One year of military service is
obligatory in Bolivia for males at age 18, and entrance examination includes a brief medical history and physical examination by a physician but does not include a chest radiograph. Among large segments of the Bolivian population, military service is highly desired because it provides a form of employment when other gainful occupation is not available. Thus, military personnel represent a sample of asymptomatic young adult males.

ELISAs were carried out at individual military barracks. Chest radiographs were obtained on all individuals with positive ELISA tests. Those with abnormal chest radiographs were further investigated with sputum examinations, and the diagnosis of tuberculosis was made in individuals with a positive sputum smear. Those with normal chest radiographs were considered not to be tuberculosis. Facilities were not available for further examination of soldiers with negative ELISA tests.

**Sputum Examination**

A single sample of expectorated sputum was collected from each patient. The sputum was digested with sodium hydroxide, centrifuged, and the pellet recovered. The pellet was resuspended in distilled water, neutralized with hydrochloric acid, and recentrifuged. This pellet was smeared and stained by the Ziehl-Neelsen technique for microscopic examination and cultured on duplicate Lowenstein-Jensen slants.

**Enzyme-linked immunosorbent**

Enzyme-linked immunosorbent assay (ELISA) of IgG antibody was performed by the method of Benjamin and Daniel (9) modified to allow its performance with capillary blood samples. The antigen used to sensitize microtiter plates was prepared by immunoabsorbent affinity chromatography (15) from immunoabsorbents prepared with monoclonal antibody TB-C-1 (17). This antigen contained several mycobacterial proteins.
Blood was obtained by finger prick, with 4 μl drawn into a disposable microcapillary pipette and diluted directly into 300 μl of phosphate buffered saline containing Tween (9) to which heparin had been added in a concentration of 1 U/ml, yielding a final dilution of blood of 1:75. All samples were identified only by study number, and the individual performing the ELISA was unaware of the clinical diagnosis. Three 50 μl aliquots of diluted blood were placed in replicate wells of a microtiter plate and the ELISA reaction carried out as previously described (9). A color standard of substrate reacted with enzyme diluted to an optical density of .25 and a positive reference serum diluted 1:500 in 1% bovine serum albumin were included on each plate. When the color intensity of the reference serum wells was equal to that of the color standard, all of the wells were graded by visual inspection against a white paper background. Wells with color equal to or greater than the reference serum wells were considered positive, and a blood was considered positive if 2 of the 3 replicate wells were positive.

Results

Characteristics of Study Population

The classification by diagnosis of the 277 subjects enrolled in the study and age and sex data for each group of subjects are presented in table 1. The patients with tuberculosis were somewhat younger than the nontuberculous subjects, reflecting the fact that in Bolivia, as in many other very high prevalence areas, tuberculosis remains a disease of the young adult.

Prevalence of Tuberculosis

If the patients with definite and probable tuberculosis are considered against all of study subjects, then the prevalence of tuberculosis in the population of individuals presenting to the Instituto de Torax was 36%. If
the 61 unclassified patients and 4 patients with nondiagnostic pleural biopsies are excluded from the denominator, then the prevalence was 47%.

**ELISA and Sputum Smear Results**

The results of the ELISA and sputum smear examinations are presented in table 2 for all classified subjects. ELISA was positive in 42 of 61 patients with pulmonary tuberculosis, yielding a sensitivity of 69% ± 6 (standard error of the proportion). It was negative in 99 of 113 nontuberculosis patients, a specificity of 88% ± 3. The sputum smear had a sensitivity of 79% ± 5 and a specificity of 100%. No sputum could be obtained from 50 persons in the group of subjects who did not have tuberculosis. Excluding these patients did not significantly change the calculated specificity of either ELISA or sputum smear. The null hypothesis that the agreement between ELISA and sputum smear results was no better than chance was tested using the kappa statistic (18). This hypothesis was rejected (p 0.05). The value of kappa (0.42) indicated fair to good agreement beyond chance between the two sets of results. Thus, while the two assays identified patients who were related by having the same disease, sufficient independence of the two tests was found to suggest that there may be utility in employing both assays.

Among patients with pleurisy with effusion, ELISA was positive in 4 of 7 patients with tuberculosis and 1 of 4 patients in whom a specific diagnosis could not be made. These results do not differ significantly from those obtained in the larger study population of patients with pulmonary tuberculosis.

**Results in Military Personnel**

Among 1458 soldiers tested, 46 were found to have positive ELISA tests. After review of chest radiographs and subsequent clinical and laboratory investigation, 5 were found to have tuberculosis. Two additional cases of
tuberculosis were recognized among soldiers who had negative ELISA tests but who presented to army physicians with symptoms during the course of the study.

Discussion

Among the more important conclusions which can be drawn from this study is that it is possible to perform large numbers of ELISAs under conditions typical of those present in third world countries of high tuberculosis prevalence. For the studies performed in army barracks, all necessary supplies were carried in a single box to the site of study. After preliminary experiments in laboratory, ambient tap water was used to prepare phosphate buffered saline containing Tween for use in washing microtiter ELISA plates in the field. As performed in this study with 22 tests on each ELISA plate, the cost of reagents and supplies at purchase price in the United States is approximately $.10 per test. No major equipment is required.

The sensitivity of ELISA for the diagnosis of tuberculosis in bacteriologically positive patients which was achieved in this study was equal to or greater than that which we have previously reported for studies performed in our laboratory (9,14). In this study, ELISA was less specific than in our previous investigations. This decrease in specificity may be in part due to reading of ELISA tests by eye without the use of a plate reader. A major factor which probably resulted in a relatively low specificity was our choice of antigen. We employed antigen eluted from immunoabsorbents prepared with monoclonal antibody TB-C-1 (17). We chose this antigen because its origin from a mouse hybridoma monoclonal immunoabsorbent assured a continuing large supply. We have subsequently shown (17) that this antigen contains multiple constituents, some of which are shared with environmental mycobacteria.
In this study a single sputum smear was very sensitive for the diagnosis of tuberculosis, substantially more so than reported by Alluoch in a similar situation (19). This could be due to a high proportion of advanced cavitary disease in the patients presenting to the Instituto de Torax. It should be noted, however, that we used smears on processed and concentrated sputum samples whereas others have used direct sputum smears in which organisms are less easily demonstrated.

Since the user of a diagnostic test does not have a priori knowledge of the subject status, it is important to consider not only sensitivity but also predictive values and accuracy of the test (20,21). Predictive values and accuracy depend upon disease prevalence in the population being tested. In table 3, the test characteristics of ELISA and sputum smear obtained from our data in patients classified as pulmonary tuberculosis and as not tuberculosis are given for a tuberculosis prevalence of 40%, the approximate prevalence found in patients presenting to the Instituto de Torax during the enrollment period. Test characteristics for ELISA and sputum smear are each presented separately. Test characteristics of ELISA in subjects with negative sputum smears are given. Finally, the characteristics resulting from using first a sputum smear and then subsequently performing ELISA on those persons with a negative sputum smear or no sputum smear available are shown. In this study ELISA performed well, recognizing 69% of patients with pulmonary tuberculosis with a positive predictive value of 79% and a negative predictive value of 81%. It did not perform as well as the sputum smear.

In patients with a negative sputum smear, the favorable test characteristics of ELISA were also present. Thus it is reasonable to combine the two tests, screening first with sputum smear and examining by ELISA those with a negative sputum smear or those from whom sputum could
not be obtained for examination. When the data are analyzed in this fashion, the sensitivity reaches 92%. The positive predictive value is high (83%) and the negative predictive value very high (94%). The combination of a negative sputum smear and a negative ELISA offers substantial assurance that the patient does not have tuberculosis.

In figure 1 the positive and negative predictive values of ELISA are plotted against tuberculosis prevalence. These are highest when disease prevalence is 30 to 50%. As a diagnostic test, ELISA has little role in low prevalence populations. However, the negative predictive value is very high when disease prevalence is low. Thus, ELISA might have a value as a screening test to exclude tuberculosis in situations where the cost is justified and within the limits of a 69% sensitivity. Our data obtained in soldiers provide a model for this situation. The ELISA identified 46 individuals. Among these persons, 5 cases of tuberculosis were found by ELISA. Calculating from our specificity data, two other cases probably existed in the population screened (in fact, two other symptomatic cases were identified).

We conclude from this study that ELISA may provide a useful adjunct to sputum smear for the diagnosis of tuberculosis in areas where disease prevalence is high. As with other diagnostic tests, consideration of the test's characteristics is important in its application. ELISA is likely to be of greatest use in those situations where sputum examination is negative or cannot be performed, either because the patient cannot provide a sputum sample or because the disease is extrapulmonary as exemplified by the patients with tuberculosis pleurisy seen in this study.
Acknowledgements

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References


<table>
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<th>Classification</th>
<th>Number</th>
<th>Median Age</th>
<th>Sex</th>
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<tr>
<td>Pulmonary tuberculosis</td>
<td>61</td>
<td>25.9</td>
<td>36</td>
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<tr>
<td>Probable pulmonary tuberculosis</td>
<td>29</td>
<td>20.7</td>
<td>21</td>
</tr>
<tr>
<td>Not tuberculosis</td>
<td>113</td>
<td>33.7</td>
<td>62</td>
</tr>
<tr>
<td>Unclassified</td>
<td>61.</td>
<td>26.7</td>
<td>27</td>
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<tr>
<td>Pleurisy</td>
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<td></td>
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<tr>
<td>Tuberculosis</td>
<td>7</td>
<td>21</td>
<td>5</td>
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<tr>
<td>Nonspecific</td>
<td>4</td>
<td>44</td>
<td>3</td>
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### TABLE 2
ELISA AND SPUTUM SMEAR RESULTS

<table>
<thead>
<tr>
<th>ELISA and Smear Result</th>
<th>Pulmonary Tuberculosis (n=61)</th>
<th>Probable Pulmonary Tuberculosis (n=29)</th>
<th>Not Tuberculosis (n=113)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA pos., smear pos.</td>
<td>34</td>
<td>5</td>
<td>0</td>
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<tr>
<td>ELISA pos., smear neg.</td>
<td>8</td>
<td>2</td>
<td>8</td>
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<tr>
<td>ELISA neg., smear pos.</td>
<td>14</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>ELISA neg., smear neg.</td>
<td>5</td>
<td>5</td>
<td>55</td>
</tr>
<tr>
<td>ELISA pos., no smear</td>
<td>0</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>ELISA neg., no smear</td>
<td>0</td>
<td>3</td>
<td>44</td>
</tr>
</tbody>
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TABLE 3

CHARACTERISTICS OF ELISA, SPUTUM SMEAR, AND SPUTUM SMEAR FOLLOWED BY ELISA IF SMEAR NEGATIVE OR NOT AVAILABLE IN A POPULATION WITH A TUBERCULOSIS PREVALENCE OF 40%

<table>
<thead>
<tr>
<th>ELISA in Sputum</th>
<th>ELISA in Smear-Negative Subjects</th>
<th>Sequential Smear and ELISA</th>
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<tbody>
<tr>
<td>Sensitivity</td>
<td>.69</td>
<td>.79</td>
</tr>
<tr>
<td>Specificity</td>
<td>.88</td>
<td>1.00</td>
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<tr>
<td>Fraction of test results positive</td>
<td>.35</td>
<td>.32</td>
</tr>
<tr>
<td>Positive predictive value</td>
<td>.79</td>
<td>1.00</td>
</tr>
<tr>
<td>Negative predictive value</td>
<td>.81</td>
<td>.87</td>
</tr>
<tr>
<td>Accuracy of prediction</td>
<td>.80</td>
<td>.92</td>
</tr>
<tr>
<td>Error of prediction</td>
<td>.20</td>
<td>.09</td>
</tr>
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</table>
Legend for Figure

Fig. 1. Positive and negative predictive values of ELISA plotted against tuberculosis prevalence with 95% confidence limits shown. Both values are highest when the disease prevalence is between 30% and 50%.
PREDICTIVE VALUES

TUBERCULOSIS PREVALENCE

PREDICTIVE VALUE

NEGATIVE PREDICTIVE VALUE

POSITIVE PREDICTIVE VALUE