

FINAL REPORT

ELISA Serodiagnosis of Tuberculosis in Bolivia

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INTRODUCTION

Tuberculosis is a disease of enormous importance in the world and in Bolivia. A rapid serologic test for the diagnosis of tuberculosis would be of great importance in countries such as Bolivia where fiscal resources are insufficient to deal with the tuberculosis problem using measures commonly employed in more affluent countries.

In a number of laboratory studies we have found that enzyme-linked immunosorbent assay (ELISA) can be applied to the serodiagnosis of tuberculosis. The present investigation was undertaken in an attempt to bring this technology to Bolivia and establish its utility in third world countries. We proposed to (1) train Bolivian personnel in ELISA techniques and establish a working assay suitable for field use in Bolivia, (2) compare the assay with sputum smear for the diagnosis of tuberculosis in a Bolivian clinic, and (3) employ the assay for screening of a general population in Bolivia. As a result of this study we hoped to obtain data which would allow assessment of the utility of ELISA serodiagnosis of tuberculosis in Bolivia and similar high prevalence areas with meager fiscal resources.

PERSONNEL

The following individuals participated in some or all aspects of the study;

Case Western Reserve University

Thomas M. Daniel, Professor of Medicine, Principal Investigator

Sara M. Debanne, Assistant Professor of Biometry

John A. Sawyer, Post-doctoral Research Fellow

Anne McLean Griffin, Post-doctoral Research Fellow

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Angela Sanson, Research Assistant
 Instituto Nacional de Laboratorios de Salud, (INLASA) and
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 Graciela L. de Murillo, Director, INLASA
 Enrique Pinto M., Chief of Pulmonary Medicine, Instituto
 Nacional de Torax
 Edmundo Cespedes A., Physician
 Patricia Espinosa M., Senior Technician

WORK ACCOMPLISHED

Training of Bolivian Scientists

At the inception of the project Patricia Espinosa travelled to Cleveland for a period of three months and Graciela Murillo for a period of one month. Ms. Espinosa worked side-by-side with the laboratory staff in the laboratory at CWRU performing ELISAs on a daily basis. Special emphasis was placed on the effects of varying experimental conditions and trouble shooting. Dr. Murillo joined with Drs. Daniel and Debanne in developing detailed protocols for the study in Bolivia.

During this initial training period, twelve hours of formal classroom instruction in experimental protocol design, data analysis, and statistics were organized and presented to Dr. Murillo and Ms. Espinosa by Drs. Daniel and Debanne. Later, during the course of visits to Bolivia, an additional twelve hours of instruction were presented to all of the Bolivian participants by Drs. Daniel and Debanne.

Establishment of ELISA in Bolivia

All supplies and equipment necessary for ELISA were purchased or prepared in Cleveland and transported to Bolivia. Mycobacterial antigen which was prepared for the project in Cleveland using immunoabsorbent affinity chromatography techniques developed by Dr. Daniel. In order that the antigen employed would be available throughout the study and indefinitely thereafter, monoclonal antibody produced by a recently developed murine hybridoma was used to prepare immunoabsorbents for antigen preparation. This resulted in an antigen which was qualitatively different from that which has been used in other ELISA studies. This may have had some influence on the results obtained.

The standard ELISA technique was modified to facilitate its use in the field under third world conditions. It was demonstrated that the buffer which is used in large volume for washing ELISA plates could be made with ordinary table salt and ambient tap water. In place of an electronic plate reader, color development in ELISA plates was judged by comparing color

intensity against a white paper background with a prepared standard color solution. The most important modification was that the test was carried out on 3 or 4 ul samples of capillary blood obtained by finger prick and diluted directly into tween containing buffered saline to which heparin 1 unit/ml had been added. This allowed immediate testing in the field and eliminated technical and cultural problems associated with obtaining larger blood samples and serum. By direct comparison in 40 tuberculosis patients and 50 control subjects, a 1:75 dilution of capillary blood was found to correspond in this test with the usual 1:80 dilution of serum.

There were a number of operation problems encountered during the study, but they were not related to the study design or to the assays employed. Rather they were external problems resulting from the economic and political instability of Bolivia during the time of study. Strikes, work stoppages, and episodes of violent civil unrest interrupted work at the Instituto de Torax and occasionally at INLASA. Never-the-less, all of the proposed work was accomplished.

ELISA Results

The results of the two major ELISA studies completed are presented in detail in a manuscript draft which is attached as an appendix to this report. This manuscript is in the process of preparation for publication in a medical research journal. Because journals of this type will not publish data which has been previously released, confidentiality is requested. The results of this study should not be released until publication has occurred.

ELISA Compared with Sputum Smear. Among 277 subjects enrolled and studied at the Instituto Nacional de Torax in La Paz, Bolivia, ELISA was found to have a sensitivity of 69% and a specificity of 88% for the diagnosis of tuberculosis. 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 a specificity of 88%. Positive and negative predictive values were highest when results were projected to hypothetical populations with prevalences in the range of those seen among patients presenting to the Instituto de Torax.

ELISA in Military Personnel. Screening of 1458 active duty soldiers was carried out and 46 positive ELISAs were obtained. The 46 soldiers with positive ELISAs were subjected to chest X-ray, and sputum examination of the X-ray was abnormal. Five cases of active tuberculosis were recognized in this manner.

DISCUSSION AND CONCLUSIONS

A simplified ELISA for the serodiagnosis of tuberculosis was developed and established in Bolivia. It was performed on more than 1800 subjects by Bolivian laboratory personnel. Although the laboratory facilities at INLASA are more elaborate than those typically seen in smaller cities in the third world, large numbers of tests were performed under conditions truly representative of third world field conditions. The per test cost was ultimately reduced to less than \$.10 at United States purchase price and ignoring the cost of antigen produced for this test in the laboratory at Case Western Reserve University.

The ELISA test was found to be both sensitive and specific. It was not as good as a smear of concentrated sputum, but added substantially to the diagnostic yield when combined with sputum smear. The specificity was less than that achieved in other studies of ELISA. This may be in part a product of reading test plates by eye without the use of spectrophotometric instruments. Another major factor, however, may be that the antigen chosen for this study was less highly purified than that used in other investigations.

This study demonstrated that ELISA can be implemented in a country such as Bolivia for the serodiagnosis of tuberculosis. Whether that will ever happen will depend on many operational considerations. Among these is the availability of antigen for the test. Currently, appropriate antigens are available only from research laboratories. However, interest in this approach to the diagnosis of tuberculosis is growing, and it seems likely that appropriate antigens will become available from commercial sources.

The ultimate use of ELISA serodiagnostic tests and all tests for the detection of tuberculosis will be determined as much by factors dependent upon the disease problem as perceived by society as by the efficacy of the test itself. The demands of patients, the perception of physicians, and the norms of health infrastructure bodies will all be important. In this context, predictive values are very likely to influence the acceptability of any test. Our data have been presented in this manner, and the high predictive values of ELISA for the serodiagnosis of tuberculosis suggest that it may have an important future role.

FIELD EVALUATION OF ENZYME-LINKED IMMUNOSORBENT
ASSAY FOR THE SERODIAGNOSIS OF TUBERCULOSIS¹⁻³

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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 μ l 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

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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 μ l drawn into a disposable microcapillary pipette and diluted directly into 300 μ l 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 μ l 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% \pm 6 (standard error of the proportion). It was negative in 99 of 113 nontuberculosis patients, a specificity of 88% \pm 3. The sputum smear had a sensitivity of 79% \pm 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.

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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.

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TABLE I

CLASSIFICATION AND CHARACTERISTICS OF STUDY SUBJECTS

Classification	Number	Median Age	Sex	
			Male	Female
Pulmonary tuberculosis	61	25.9	36	25
Probable pulmonary tuberculosis	29	20.7	21	8
Not tuberculosis	113	33.7	62	54
Unclassified	61	26.7	27	34
Pleurisy				
Tuberculosis	7	21	5	3
Nonspecific	4	44	3	1

TABLE 2
ELISA AND SPUTUM SMEAR RESULTS

ELISA and Smear Result	Pulmonary	Probable Pulmonary	Not
	Tuberculosis (n= 61)	Tuberculosis (n= 29)	Tuberculosis (n= 113)
ELISA pos., smear pos.	34	5	0
ELISA pos., smear neg.	8	2	8
ELISA neg., smear pos.	14	7	0
ELISA neg., smear neg.	5	5	55
ELISA pos., no smear	0	7	6
ELISA neg., no smear	0	3	44

TABLE 3

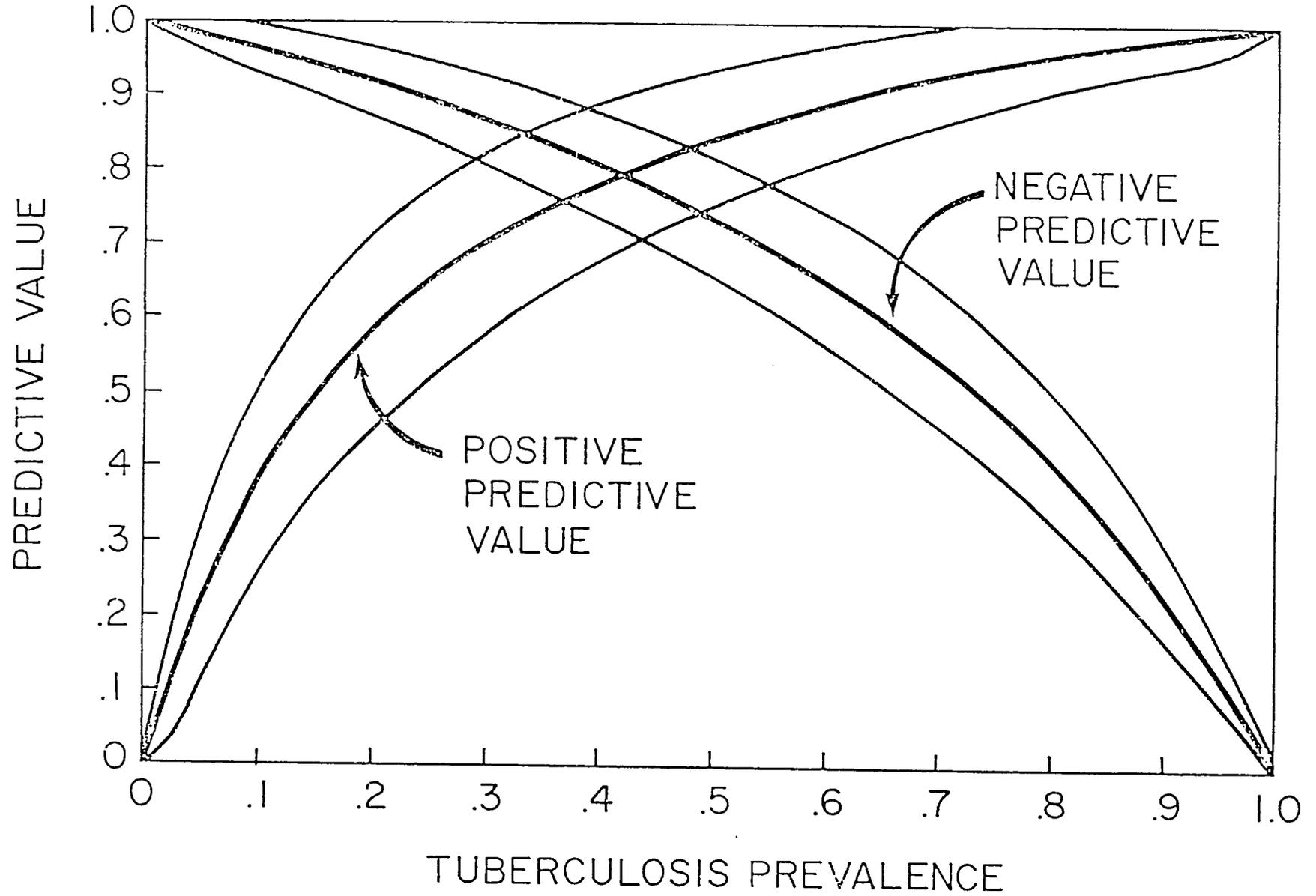
CHARACTERISTICS OF ELISA, SPUTUM SMEAR, AND SPUTUM
 SMEAR FOLLOWED BY ELISA IF SMEAR NEGATIVE OR
 NOT AVAILABLE IN A POPULATION WITH A TUBERCULOSIS
 PREVALENCE OF 40%

	ELISA	Sputum Smear	ELISA in Smear-Negative Subjects	Sequential Smear and ELISA
Sensitivity.	.69	.79	.62	.92
Specificity	.88	1.00	.87	.88
Fraction of test results positive	.35	.32	.32	.44
Positive predictive value	.79	1.00	.76	.83
Negative predictive value	.81	.87	.77	.94
Accuracy of prediction	.80	.92	.77	.89
Error of prediction	.20	.09	.23	.11

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



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