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ORIGINAL ARTICLES

Value of an ELISA for Mycobacterial Antigen Detection as a Routine Diagnostic Test of Pulmonary Tuberculosis

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SUMMARY **A double antibody sandwich ELISA was carried out with commercially available anti-BCG and peroxidase labeled anti-BCG, for the detection of mycobacterial antigens. By using purified protein derivative of tuberculin (PPD) as the antigen, the lowest detection limit of the assay was found to be 0.05 μg/ml. At the cut off level of absorbance index (AI) ⩾ 5, positive results of ELISA were obtained from 24/25 sputum specimens which were positive for staining of acid fast bacilli (AFB), 5/16 specimens positive for culture of *Mycobacterium tuberculosis* and 67/69 specimens positive for both tests. The assay was positive in only 11/164 specimens negative for both staining of AFB and culture of *M.tuberculosis*, 4 of which were known to have tuberculosis. Thus, with sputum specimens, the sensitivity, specificity, efficiency, positive predictive value and negative predictive value of the ELISA were 87.27, 93.29, 90.88, 89.72 and 91.62 percent respectively. Positive results were also obtained in 2/111 sputum specimens which were positive for other bacteria but the presence of AFB in these specimens could not be ruled out. With pleural fluid specimens, positive ELISA with AI >1 was found in 3/26 specimens of patients with tuberculous pleurisy and 0/11 of those with malignancy. Twenty-six sera and urine specimens of tuberculous patients and also all control specimens (138 sera and 86 urine specimens) assayed, gave negative ELISA results (AI < 1).**

Tuberculosis is a disease which was recognized more than a century ago. Since the time that Robert Koch identified the etiologic agent of this disease by staining and culture of *Mycobacterium tuberculosis,* the routine diagnosis has not differed substantially from what he used.[1] Although various immunodiagnostic techniques, based on the detection of mycobacterial antigens[2-12] or specific antibodies,[9,11,13-27] have been reported, they have not come into wide spread clinical use. This is due to two main problems. Firstly, some procedures cannot be confirmed for their validity,[3,10,11] some lack of sensitivity,[13,14,17,19-21,23,26] or specificity.[8,16] Secondly, the reagents used in some of them are not generally available[5,7,15,22,24,25,27] and are difficult to prepare.[1,2,4,9,15,24,25] However, commercially available reagents were recently reported to be valuable for the detection of mycobacterial antigens in cerebrospinal fluid of patients with tuberculous meningitis.[12] With the use of generally available reagents, the test would be suitable as a routine diagnostic test of tuberculosis, provided that it has acceptable sensitivity and specificity.

In this study, we have evaluated the use of commercially available anti-BCG antibodies and anti-BCG peroxidase conjugate, in a double antibody sandwich ELISA for the detection of mycobacterial antigen. The assay has given promising results, as a routine diagnostic test for pulmonary tuberculosis.

MATERIALS AND METHODS

Specimens

1. Pleural fluid

A total of 37 pleural fluid specimens were obtained from patients attending the Central Chest Hospital, Nonthaburi.

Twenty six specimens were obtained from patients with tuberculous pleurisy. The diagnosis was based on the results of either smear and stain positive for acid fast bacilli

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(AFB) or culture positive for *M.tuberculosis*, in bronchial washing or pleural fluid. The other criteria which were also used as the gold standard for the diagnosis of these tuberculous patients included positive histopathological findings of lymph nodes or pleural biopsy specimens, and the improvement of the disease upon treatment with anti-tuberculous drugs.

Eleven specimens were obtained from patients with malignancy and used as the control group. The diagnosis of these patients was based on histopathological findings of bronchial or pleural biopsy specimens. Malignant cell types were found to be adenocarcinoma in 9 patients, squamous cell carcinoma and undifferentiated cell carcinoma respectively in the other 2 patients.

2. Serum

Sera were obtained from each of the 37 patients with tuberculous pleurisy or malignancy described above.

Other control sera were also used. These included 41 samples obtained from patients with bacteremia and 86 from normal healthy individuals. Bacteria found in hemoculture of these 41 patients included *Salmonella typhi*, Salmonella group A and group C, *Pseudomonas aeruginosa, Klebsiella pneumoniae, Enterobacter cloacae, Escherichia coli, Staphylococcus aureus, Streptococcus viridans*, group A streptococci, *Neisseria gonorrhea* and non fermentative gram negative rod. Candida species were also found in hemocultures of patients in this group. These 41 sera were kindly donated by Dr. Benjawan Rungpitarangsi, Department of Clinical Pathology and the hemocultures were the responsibility of Dr. Podjanee Komolpit, Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University.

3. Urine

A total of 123 urine specimens were used in this study. They comprised 86 specimens obtained from normal healthy individuals, 26 from tuberculous patients and 11 from patients with malignancy described above.

4. Sputum

A total of 385 sputum specimens, sent to the Department of Microbiology, Faculty of Medicine Siriraj Hospital, for routine microbiological examination, were used in this study.

Twenty five specimens were positive for staining of AFB but negative for culture of *M.tuberculosis*. Sixteen specimens were negative for the former but positive for the latter and 69 were positive in both tests. All of these were considered as specimens of patients with pulmonary tuberculosis.

One hundred and sixty four specimens which were negative for both staining of AFB and culture of *M.tuberculosis* were used as the control group, for the calculation of various parameters contributing to the validity of the ELISA performed with sputum.

The other 111 sputum specimens were also subjected to mycobacterial antigen detection by ELISA. They were positive for culture of other organisms, including Candida spp., *Staphylococcus aureus, Streptococcus pneumoniae, Hemophilus influenzae, Acinitobacter anitratus, Proteus vulgaris, Klebsiella pneumoniae, Escherichia coli, Enterobacter cloacae Pseudomonas aeruginosa*, and non-fermentative gram negative rods. These specimens and the results of bacterial culture were kindly provided by Professor Dr. Sophon Kongsamran, Department of Microbiology, Faculty of Medicine Siriraj Hospital.

Preparation of specimens for use in the ELISA

Pleural fluid and urine specimens were centrifuged to remove cells. The supernatant was collected and stored at -20° C until used. Sera were also stored at -20° C.

Sputum specimens were autoclaved at 121° C, 15 lbs/sq.inch for 15 minutes, then sonicated with the microtip probe (1/8 inches diameter) of the SONICATOR® (Heat Systems-Ultrasonics, Inc., model W-380). The sonicated sputum specimens were then centrifuged to remove cell debris. The supernatant was collected and stored at -20° C until used.

ELISA for the detection of mycobacterial antigens

A double antibody sandwich ELISA was used for the detection of mycobacterial antigens. Either 100 μl of rabbit anti-BCG immunoglobulin (Dakopatts, DK-2600, Glostrup, Denmark) or 100 μl of normal rabbit immunoglobulin (diluted in 0.05 M carbonate buffer, pH 9.8, containing 0.1% sodium azide) were added to each well of an ELISA plate (Nunc-Immuno Module Maxisorp F16; Nunc, Kamstrup-DK4000, Roskilde, Denmark). The plate was incubated at 37° C, overnight. After being washed 3 times with 0.01M phosphate buffered saline (PBS), pH 7.1 containing 0.05 percent Tween 20 (PBST) and tapped dry, the unoccupied binding sites of each well were blocked with 5 percent fetal calf serum (FCS) in PBS. The incubation conditions of this step were 37° C, 1 hour. After washing, the test samples were added and incubated at 37° C, 2 hours. Each specimen was assayed in duplicate, in both rabbit anti-BCG and normal rabbit immunoglobulin coated wells. The conjugate used was peroxidase labelled rabbit anti-BCG Ig (Dakopatts) diluted 1:750 in PBS containing 5 percent FCS. The conjugate incubation was at 4° C for 2 hours. The substrate solution used was 1,2-phenylenediamine dihydrochloride (OPD) (Dakopatts) dissolved to a concentration of 0.66 mg/ml in 0.1M citric acid phosphate buffer, pH 5.0, containing 0.0125 percent H_2O_2. The reac-

tion was allowed to take place at 37° C for 1 hour before terminated by the addition of 50 µl 4 M H_2SO_4. The absorbance of the colour developed was read spectrophotometrically at 492 nm with a Titertek Multiskan® (Flow Laboratories, Gmbh Bonn, West Germany).

The wells with various concentrations of purified protein derivatives of tuberculin (PPD) (Statens Serum Institut, Tuberculin Department, DK-2300 Copenhagen, Denmark) were used as positive controls, while wells without antigen (DCC) and those coated with normal rabbit immunoglobulins were used as negative control wells.

The absorbance values of DCC performed in the anti-BCG and in the normal immunoglobulin coated wells were both subtracted from those of the test specimens assayed in the corresponding wells. The values of the test specimens assayed in the well coated with normal immunoglobulin were again subtracted from those assayed in the wells coated with anti-BCG. This final absorbance value, designated as optimized absorbance value, was then used for calculating the result of mycobacterial antigen detection in each specimen. The result was expressed as absorbance index (AI) by relating the optimized absorbance value of each specimen with that of a PPD concentration 0.05 µg/ml, which was the lowest detection limit of the ELISA.

Statistical Methods

The indices were calculated as follows : sensitivity, $[a/(a+c)] \times 100$; specificity, $[d/(b+d)] \times 100$; efficiency, $[(a+d)/(a+b+c+d)] \times 100$; positive predictive value, $[a/(a+b)] \times 100$; negative predictive value, $[d/(c+d)] \times 100$; where a is the number of true-positive samples, b is the number of false positive samples, c is the number of false negative samples and d is the number of true-negative samples. [28]

Positive results obtained from the sputum specimens which were either smear and stain positive for AFB or culture positive for *M.tuberculosis* were considered as true positive while negative results obtained from samples which were negative for both staining and culture were considered as true negative.

RESULTS

Sensitivity limit of the ELISA

In order to evaluate the sensitivity limit of the ELISA, the assay was carried out 14 times, with various concentrations of PPD ranging from 0.001 to 20 µg/ml. A typical dose response curve of the assay is shown in Fig. 1. The absorbance value which was higher than the mean absorbance value of DCC + 3 standard deviations (SD) was obtained from the assay of PPD concentration $\geqslant 0.05$ µg/ml (Table 1). Thus, the lowest detection limit of the ELISA was found to be equivalent to 0.05 µg/ml of PPD.

Reproducibility of the ELISA

To evaluate the reproducibility of the ELISA, various concentrations of PPD ranging from 0.001 to 20 µg/ml were assayed on different occasions for a total of 14 times. The mean, standard deviation and coefficient of variation of the absorbance values obtained from these assays were calculated and are shown in Table 1. It can be seen that the coefficient of variation was lower than 10 percent for every PPD concentration ranging from 0.05 µg/ml (the lowest detection limit) to 20 µg/ml.

Detection of mycobacterial antigen in sputum

The results of ELISA for mycobacterial antigen detection in sputum are shown in Fig. 2. It can be seen that, at the cut-off level of AI $\geqslant 5.0$, positive results were obtained from 24/25 specimens which were positive

Table 1. Inter–run reproducibility of the ELISA

PPD concentration (µg/ml)	Absorbance value (n=14)[a]	
	$\bar{X}$ ± SD	% CV
20.0	1.249 ± 0.038	3.08
10.0	1.192 ± 0.046	3.84
5.0	1.141 ± 0.051	4.46
2.5	1.041 ± 0.061	5.83
1.0	0.855 ± 0.054	6.35
0.5	0.722 ± 0.054	7.42
0.25	0.555 ± 0.040	7.15
0.1	0.416 ± 0.032	7.79
0.05	0.349 ± 0.027	7.64
0.025	0.331 ± 0.035	10.62
0.01	0.281 ± 0.036	12.71
0.005	0.297 ± 0.039	13.29
0.001	0.292 ± 0.039	13.42
0 (DCC)	0.246 ± 0.031	12.44

[a]Abbreviations : $\bar{X}$, arithmetic mean of absorbance values; SD, standard deviation of absorbance values; CV, coefficient of variation of absorbance values.

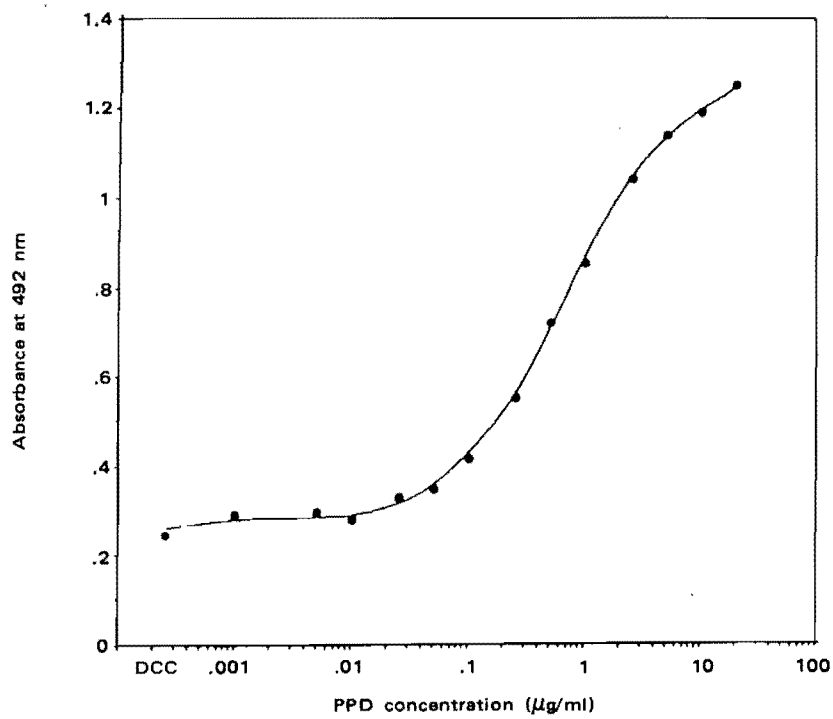

Fig. 1 A typical dose response curve of PPD as assayed by the ELISA.

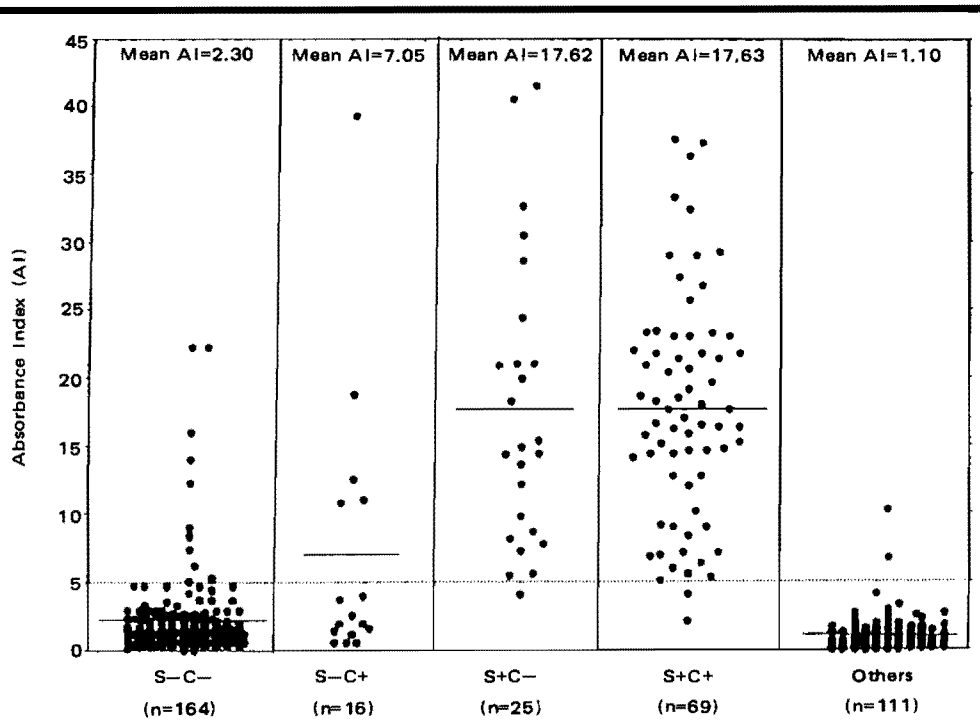

Fig. 2 Absorbance index obtained from the assay of sputum specimens by ELISA for mycobacterial antigen detection. S, smear and stain of acid fast bacilli; C, culture of *M. tuberculosis;* −, negative result; +, positive result; others, culture positive for organisms other than *M. tuberculosis.*

only for staining of AFB, 5/16 specimens positive only for culture of *M.tuberculosis* and 67/69 specimens positive for both tests. Positive results were also obtained from 11/164 specimens which were negative for both staining of AFB and culture of *M.tuberculosis*. By using either positive staining of AFB, or culture of *M.tuberculosis* as the gold standard, then, the sensitivity, specificity, efficiency, positive predictive value and negative predictive value of the ELISA for mycobacterial antigen detection in sputum were 87.27, 93.29, 90.88, 89.72 and 91.62 percent respectively.

In comparison with either AFB staining or *M.tuberculosis* culture alone, the ELISA was found to have higher sensitivity, as those of the formers were 85.45 (94/110) and 77.27 (85/110) percent respectively.

In addition, among the 11 sputum specimens which were negative for the gold standard but gave a positive ELISA, 4 of them were obtained from patients who actually had pulmonary tuberculosis. One of them was obtained from a patient who had a sputum culture positive for *M.tuberculosis* 3 months previously and was on treatment during the time of this specimen collection. One specimen was obtained from a patient whose repeat sputum sample, collected on the following day, was found to be culture positive for *M. tuberculosis*. Two were obtained from patients who had chest roentgenograms positive for pulmonary infiltration and were on treatment with anti-tuberculous drugs.

Only 2 of 111 sputum specimens, which were culture positive for other microorganisms, were found to have positive results with the ELISA. The organisms found in culture of these 2 specimens were Candida spp and *Pseudomonas aeruginosa*. However, these specimens were not subjected to either smear and stain of AFB or culture of *M.tuberculosis*.

Detection of mycobacterial antigens in other biological fluids

Only 3 of 26 pleural fluid specimens obtained from patients with tuberculous pleurisy gave the results of ELISA with AI >1. The remaining 23 specimens obtained from this group of patients and also 11 specimens obtained from patients with malignancy all had undetectable levels or did not contain any mycobacterial antigens (AI < 1). AI values less than 1 were also obtained with all the sera and urines tested.

DISCUSSION

Tuberculosis is still a major health problem in many areas of the world, especially developing countries. Since standard diagnostic tests for this disease, i.e. staining and culturing techniques, are cumbersome and time-consuming, various attempts have been made to develop new rapid immunodiagnostic tests for tuberculosis.[1] However, these new techniques have not come into widespread clinical use. One major obstacle is their dependency on the antigen or antibody, which is not generally available.[5,7,15,22,24,25,27] Therefore, we planned to evaluate the validity of an ELISA carried out with commercially available anti-BCG and anti-BCG peroxidase conjugate, as a possible routine diagnostic test for pulmonary tuberculosis.

With sputum specimens, the ELISA performed was found to have sensitivity, specificity, efficiency, positive predictive value and negative predictive value of 87.27, 93.29, 90.88, 89.72 and 91.62 percent respectively. The sensitivity was higher than that of either smear and staining of AFB or culture of *M.tuberculosis* alone which were 85.45 and 77.27 percent respectively.

In addition, among 11/164 sputum specimens which were negative for both staining and culture but gave positive ELISA for mycobacterial antigen detection, 4 of them were obtained from patients who definitely had pulmonary tuberculosis. This confirms the value of the ELISA used, as a dignostic test of pulmonary tuberculosis.

Although we considered that positive results found in 2 of 111 sputum specimens, which were positive for culture of other organisms, were false positive, these specimens were not subjected to the examination of *M.tuberculosis* by either staining or culture. Therefore, the possibility that they might also contain *M.tuberculosis* cannot be excluded.

Furthermore, the results of genomic DNA hybridization studies which have indicated that *M.tuberculosis* and *M.bovis* BCG share greater than 95 percent DNA homology overall,[29] also lend support to our findings in this study, that anti-BCG can be used efficiently as a reagent in ELISA for the diagnosis of pulmonary tuberculosis.

In comparison with a similar study recently reported by others,[6] we found a higher sensitivity (87.27% versus 57.58%) but similar specificity (93.29% versus 91.43%). However, it should be noted that, while we tested a total of 110 specimens with positive and 164 specimens with negative gold standard results, they tested only 33 of the former and 35 of the latter. This difference in numbers might contribute to the discrepancy of the results obtained.

With other biological fluids tested, i.e. sera, urine and pleural fluid specimens, positive ELISA results were obtained in only 3/26 pleural fluid specimens of patients with tuberculous pleurisy. Such a low sensitivity could be due to the very low amount of soluble antigen in such a specimen. Our unpublished data with the use of bronchial lavage specimens have shown that the amount of antigen detected in the supernatant of the specimen was much lower than that in the same specimen which still contained cells and was subjected to sonication.

This finding, therefore, could possibly explain our negative findings with sera, urine and the majority of pleural fluid specimens of patients with pulmonary tuberculosis in this study.

Although others have reported a high sensitivity of mycobacterial antigen detection by ELISA in pleural fluid specimens,[8,10] some points from these studies should be brought into consideration. In one study, although the test had a high sensitivity, its specificity was very low, i.e. 38 percent. Thus, the test would appear to be useless for clinical application.[8] In addition, false positive reactions could occur in the assay employed in their study,[8] as the first and second antibodies used in the double antibody sandwich-anti-globulin ELISA were obtained from the same species.[30,31]

Another study in which ELISA inhibition was employed, only 5 pleural fluid specimens obtained from patients with tuberculosis were tested and no negative controls for the same type of specimens were included.[10] However, antibodies specific to mycobacterial antigens can be found in pleural fluid obtained from both tuberculous and non-tuberculous patients.[26] The specific antibody can give rise to a false positive reaction in ELISA inhibition for antigen detection.[32,33] Thus, it is nessary to include the same type of specimens obtained from a negative control group in studies using ELISA inhibition, as well as when using other assays, in order to confirm the validity of positive results obtained.

In conclusion, our study has shown that an ELISA, using commercial anti-BCG and anti-BCG peroxidase conjugate, for mycobacterial antigen detection in sputum is useful as a routine diagnostic test of pulmonary tuberculosis. This is due to its high efficiency and to the general availability of the reagents used. The only draw back of this test is the need of a sonicator for the preparation of specimens before testing.

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