A dot-immunobinding assay (Dot-Iba) for rapid diagnosis of pulmonary tuberculosis

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IgG antibody to Mycobacterium tuberculosis from the sera of patients with ‘definite’ pulmonary tuberculosis (PT) was isolated and coupled with Cyanogen bromide-Sepharose 4B. Using an immunoabsorbent affinity chromatography, 14 kDa antigen was recovered from the culture filtrates of M. tuberculosis. With this mycobacterial antigen, a dot immunobinding assay (Dot-Iba) was developed for the detection of specific antibody to M. tuberculosis in the sera of patients with PT and controls. The assay gave positive results in all the 12 sputum-smear positive [acid fast bacilli (AFB)] patients with PT and gave negative results in the 50 sera from control groups. The Dot-Iba as described in this study, is simple, rapid and specific for laboratory diagnosis of PT.

At present a confirmative laboratory diagnosis of pulmonary tuberculosis (PT) depends on isolation of Mycobacterium tuberculosis in the clinical specimens, a process that can take up to 8 weeks to yield a result because of slow growing nature of the mycobacteria. Radiometric liquid (BACTEC) and biphasic (MB-Chek) culture systems have definitely improved, both the recovery rates and the speed of isolation of M. tuberculosis. However these new methods still cannot replace or influence the initial bed-side decision making in a patient with suspected PT. Thus alternative rapid modes of laboratory diagnosis are relevant as well as essential particularly in those geographic zones where the incidence and prevalence of human tuberculosis is still high.

The present study describes a simple, inexpensive, reproducible dot-immunobinding assay (Dot-Iba) for rapid laboratory diagnosis of PT. The sensitivity of this method has been evaluated with the sputum acid fast bacilli (AFB) positive patients with PT and the specificity of the assay has been critically evaluated in non-tuberculous subjects.

Materials and Methods
Sera were collected from 50 patients admitted to the Tuberculosis and Chest Diseases Hospital, Thiruvananthapuram. The age of the patients ranged from 4-78 years. These included 31 male and 19 females. Diagnosis of PT in all these patients was done on the basis of presenting clinical features and supported by positive intradermal tuberculin test, elevated erythrocyte sedimentation rate and radiological evidence of the disease in the lungs. The radiological extent of the disease in these patients ranged between apical lesions, hilar lymphadenopathy, cavitations and infiltrative lesions with or without pleural effusion. All these patients gave a history that they were admitted earlier at an outlying hospital and received anti-tuberculosis chemotherapy (ATT) consisting of rifampicin 450 mg, isoniazid 300 mg, streptomycin 700 mg and ethambutol 80 mg daily for 2-8 weeks. At the time of admission in this hospital, AFB was demonstrated in the Ziehl-Neelsen stained sputum smears in 12 patients. The conventional culture of sputum specimens in Lowentstein and Jensen medium also confirmed the growth of M. tuberculosis in these 12 patients. Hence they were regarded as ‘definite’ cases of PT. In the remaining 38 patients, sputum examinations on more than three different occasions were reported to be negative for AFB. The sputum cultures on three different occasions also did not reveal the growth of M. tuberculosis in these 38 patients. PCR in sputum specimens also did not establish the diagnosis in these
38 patients. However all these patients had radiological features, suggestive of PT and showed optimal clinical response to ATT. Hence they were regarded as ‘probable’ cases of PT. In 26 out of 38 patients with ‘probable’ PT, two to three sera samples were collected during follow up period. In 12 patients serial sera samples could not be collected, as these patients did not report in the follow-up clinic. Twenty four sera samples were collected from (A) 50 patients with chronic pulmonary diseases [bronchiectasis, n=24; bronchial asthma, n=16; chronic bronchitis with emphysema, n=10; (disease control)]. The age of these patients ranged from 4-76 years. These included 34 males and 16 females. (B) 50 healthy voluntary blood donors attending blood transfusion services of this Institute were collected from (A) 50 patients with ‘definite’ PT and voluntary healthy donors were similarly prepared from pooled sera from (B) 50 healthy voluntary blood donors. Gamma globulins both from patients with ‘definite’ PT and voluntary healthy donors were individually passed through the Immunoabsorbent affinity columns (Sigma; St.Louis; Mi) for 1 hr. The NCM discs were washed thoroughly and the NCM discs were blotted dry. The NCM discs were stored at +4°C until used. Sera from patients with PT and control groups were serially diluted (1:200-1:800) in PBS-TBA. Serially diluted sera samples (5 µl) were placed over the NCM discs. After incubation for 1 hr at room temperature, the NCM discs were washed thrice with PBS-T and blotted dry. The NCM discs were then immersed in (1:2000) antihuman IgG-biotin conjugate (Sigma; St.Louis; Mi) for 1 hr. The NCM discs were washed thoroughly with PBS-T. This was followed by incubation with (1:200) extravidin alkaline phosphatase (Sigma). Substrate (5 ml) consisting of tetrazotized-o-dianisidine (0.25mg/ml), β-naphthyl acid phosphate (0.25 mg/ml), 0.6 M sodium borate/MgSO₄, 7 H₂O buffer (pH 9.7), was poured onto NCM discs and incubated for 10 min. The reaction was stopped by pouring off the substrate and washing NCM first in a solution containing methanol: acetic acid: water (5:1:5) and followed by a final rinse in distilled water. A positive reaction was indicated by the development of a purple to purple-pink circle in the NCM discs. All the sera samples were tested on two occasions to assess the reproducibility of the assay. Variations in the colour reaction in the same serum sample tested on two different occasions were seldom encountered. To eliminate observer-bias, the assay was performed on coded sera samples on two different occasions and the result were recorded without the knowledge of the clinical status of the patient initially but subsequently correlated with the clinical diagnosis in every patient.

**Results**

The SDS-PAGE of culture filtrate antigen (CFA) and affinity column purified antigen are shown in Fig. 1. Table 1 represents the results of Dot-Iba. In all the patients with ‘definite’ PT, the assay gave positive...
reaction at all dilutions (1:200-1:800). Thus the sensitivity of Dot-Iba in ‘definite’ cases of PT was 100%. Out of 38 patients with ‘probable’ PT, 32 showed positive colour reaction at 1:800 dilutions. In the remaining 6 patients, colour reaction appeared between 1:200-1:400 dilution. In the 50 sera from both disease and healthy control groups, the antibody titer ranged between 1:200-1:400 dilutions and none of the sera samples showed a colour reaction at 1:800 dilution. Thus a ‘cut-off’ point was set at 1:800, as this gave the best discrimination between tuberculous and control subjects. In order to evaluate the specificity of the 14 kDa antigen in Dot-Iba, the results of Dot-Iba were compared with crude CFA of M. tuberculosis in controls. With CFA, 26 out of 50 sera from disease control and 22 out of 50 sera from healthy control group gave positive colour reaction at 1:800, indicating that with non-specific antibodies in the sera from disease and healthy control groups reacted with CFA. However at 1:1600 dilution the sera from both the control groups did not react with CFA. With the 14 kDa antigen, the non-specific antibodies in the sera of disease and healthy control did not give positive colour reaction at 1:800 dilution. Therefore, 14 kDa antigen of M. tuberculosis was considered specific for the detection of anti-mycobacterial antibody. The Dot-Iba was performed in the sera samples of 26 out of 38 probable PT patients on two-three occasions during the course of the disease. The antibody titer remained the same 12 out of 26 probable PT patients and in some patients (6/26), the antibody titer was even found to be increased. These 26 probable patients also showed positive clinical and radiological evidences of recovery during ATT.

Discussion
Because the conventional bacteriological technique carry low sensitivity, the ability of any newly proposed test is to detect M. tuberculosis or its constituents, directly or indirectly in the clinical specimens, help immensely in making the diagnosis of pulmonary tuberculosis. The most recent advances in the diagnosis of pulmonary tuberculosis have been the introduction of PCR\textsuperscript{46}. However PCR method cannot be applied for routine application in most of the clinical laboratories particularly at peripheral centers in developing world, because it requires technical expertise and laboratory resources.

Several antibody based immunoassays for detection of M. tuberculosis by using polyvalent and monoclonal antibodies has been described\textsuperscript{7-9}. Improvement in the antibody based diagnostic assays require specific antibody reagent with high affinity for mycobacterial antigen. In order to achieve this object, in the present study an attempt has been to isolate a specific immunoreactive antigen from the culture filtrates of M. tuberculosis. Human IgG antibody to M. tuberculosis was isolated from the sera of patients

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Fig. 1—SDS-PAGE showing (A) molecular weight standard, (B) culture filtrate antigen (CFA), (C) 14 kDa antigen isolated from CFA by specific IgG to M. tuberculosis, and (D) control column (no binding to 14 kDa antigen).

| Table I—Sera antibodies against mycobacterial antigens by Dot-Iba |
| --- | --- |
| | 14 kDa Ag | CFA |
| | 1:200 | 1:400 | 1:800 | 1:200 | 1:400 | 1:800 | 1:1600 |
| Definite PT-12 | — | — | 12 | — | — | 12 | 0 |
| Probable PT-38 | — | 6 | 32 | — | 2 | 36 | 12 |
| Disease control-50 | 38 | 12 | 0 | — | 24 | 26 | 36 |
| Healthy control-50 | 44 | 6 | 0 | — | 28 | 22 | 0 |
with 'definite' PT. This human IgG antibody to M. tuberculosis was coupled with Cyanogen bromide-Sepharose 4B and an immunoabsorbent affinity column was made. The culture filtrates of M. tuberculosis containing several immunoreactive antigens were passed through the affinity column. Specific mycobacterial antigen bound to immunoabsorbent was recovered. The antigen recovered by the above technique possessed a molecular weight of 14 kDa and was thermostable at 65°C. It has been reported that 14 kDa antigen of M. tuberculosis belongs to the alpha-crystalline family of low-molecular heat-shock proteins. This antigen has shown considerable promise as a serodiagnostic tool for the detection of anti-mycobacterial antibody in the sera of M. tuberculosis.

PT is a potentially curable disease. The mortality and morbidity rates currently associated with this disease could be decreased, if an early laboratory diagnosis is achieved and appropriate ATT is administered. The Dot-Iba as described in this study can be utilised for the rapid diagnosis of PT in those laboratories in developing countries where the laboratory resources and technical expertise is limited.

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References


