Detection of *in vitro* and *in vivo* released antigens of diagnostic interest in *Mycobacterium tuberculosis* by immunoblotting

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Identification of *in vitro* and *in vivo* released mycobacterial antigens are of considerable interest in diagnosis of *Mycobacterium tuberculosis*. Isolation of *in vitro* released antigen from *M. tb* excretory-secretory culture filtrate protein and *in vivo* released circulating tuberculous antigen from smear positive pulmonary tuberculosis sera by ammonium sulphate precipitation is reported. The antigens were resolved by SDS–PAGE and immunoblotting was performed using pooled serum of smear positive, smear negative pulmonary tuberculosis sera and normal sera to identify reactive tuberculous antigens. *In vitro* and *in vivo* released mycobacterial antigens showed reactivity at 100, 31, 43 and 20 kDa with smear positive and smear negative pulmonary tuberculosis patients. Further, the *in vitro* released antigen showed strong reactivity exclusively at 55 kDa antigen with smear positive and 24 kDa antigen with smear negative pulmonary tuberculosis sera. *In vivo* released antigen reacted exclusively at 170 and 16 kDa with smear positive and 19 kDa antigen with smear negative pulmonary tuberculosis patients. Antigens of 24 and 19 kDa which are reactive with sputum negative sera will be of diagnostic interest and need further study in patients with low bacillary load. The *in vitro* and *in vivo* released mycobacterial 100, 31, 43 and 20 kDa antigens, reactive with patients sera are of diagnostic interest in tuberculosis.

**Keywords:** *In vitro* and *in vivo* released antigens, Immunoblotting, Pulmonary tuberculosis

The study of mycobacterial antigens has been of interest, as immunological methods have attained considerable attention due to limitations in conventional diagnostic methods for tuberculosis. The proteins released from growing mycobacteria into extra cellular medium have been an attractive source of candidate antigen, since they are closely associated with active infection and involved in the development of vigorous humoral immune response that is detectable even by least sensitive methods¹. Extensive work has been carried out using sonicate extract of *Mycobacterium tuberculosis* but reports have been scarce on the analysis of *in vitro* released excretory secretory (*ES*) antigens and their comparison with *in vivo* released antigens in body fluids. The protein profile expressed *in vivo* by *M. tuberculosis* may differ from those expressed during *in vitro* growth and as the antibodies are the markers of antigen expressed *in vivo*, it is necessary to analyse the *in vitro* and *in vivo* released antigens of *M. tuberculosis*. The recent advanced sepearative and probing techniques such as immunoelectrophoresis, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis have been useful to define the mycobacterial antigens². In the present study an attempt has been made to analyse the immunoblot reactivity pattern of excretory secretory antigens obtained from *M. tuberculosis* H₃₇Ra culture filtrate (*in vitro*) and circulating tuberculosis antigen (*CTA*) isolated from tuberculosis sera (*in vivo*) using pooled sera of sputum positive (S+) and sputum negative (S−) pulmonary tuberculosis and healthy normals.

**Materials and Methods**

*Sera*—Sera from the sputum positive and sputum negative but clinically suspected and X-ray suggestive or ATT responded tuberculosis patients were used in this study. Individuals without pulmonary, extrapolmonary TB or any other chronic infectious disease and with normal chest skiagram and without history of contact with TB patients served as healthy controls. Sera were stored at −20°C with preservative until further use.

**Isolation of in vitro released *M. tuberculosis* excretory secretory (*ES*) antigen**—In the present
study, more commonly explored avirulent lab strain H37Ra with less health risk was used for ES antigen isolation. Excretory secretory culture filtrate proteins were obtained from *M. tuberculosis* H37Ra bacilli grown in thyroxine supplemented Sautons’ liquid medium incubated at 37°C on an orbital shaker (150 rpm) for 10 days as described earlier.

Isolation of in vivo released circulating tuberculous antigen (CTA)—Circulating tuberculous antigen was isolated as described earlier. In brief, 1 ml volume from each of 10 bacteriologically confirmed pulmonary tuberculosis patients (PTB) sera were pooled and reactive fraction of CTA was obtained by 36-75% ammonium sulphate precipitation. The protein estimation was done by Lowry’s method and CTA was stored at –20°C with preservative, until further use.

**Immunoblotting**—The ES antigen and CTA were resolved by SDS-PAGE using 5-15% gradient gel. The antigens were transferred electrophoretically onto nitrocellulose membrane (NCP, BA 85 Schleicher and Schuell AG, Switzerland) at 0.6 amps (90 volts) for 90 min at 4°C as described by Towbin *et al*.

After transfer, the NCP was blocked with quench solution (5% skimmed milk powder (SMP) in 0.05M PBS), overnight at 4°C to saturate additional binding sites and then washed four times with PBS/T containing 2% SMP. The strips were then incubated with pooled sera (1:50 dilution) and peroxidase labelled antihuman IgG conjugate (1:1000 dilution). Antibody binding was revealed using freshly prepared peroxidase substrate consisting of 0.05% diamino benzidine and 0.03% of hydrogen peroxide in citrate buffer pH 5.0 for 15 min. The reaction was stopped after rinsing the strips in water.

**Results**

The reactivity pattern of Immunoblots of ES antigen and CTA probed with 3 serum pools viz sputum positive, sputum negative PTB and healthy control groups is shown in Figs 1 and 2 respectively. The antigens at 100, 70, 55, 43, 31, 20 kDa regions of ES and 170, 100, 48, 43, 31, 29, 20 and 16 kDa regions of CTA showed reactivity with sputum positive sera. Recognition of antigens of ES was very strong at higher molecular weight region i.e. 100, 70, 55, 43 compared to lower molecular weight region i.e. 20, 24, 26 kDa region. The antigens at 100, 70, 43, 31, 24 and 20 kDa regions of ES and 100, 48, 31, 43, 29, 20 and 19 kDa region of CTA showed reactivity with sputum negative sera. Smear positive and smear negative tuberculosis sera showed less reactivity below 14 kDa region of ES antigen without any distinct band pattern. The antigens of 100, 31, 43 and 20 kDa regions of ES and CTA were reactive with both sputum positive and sputum negative sera. Sputum positive sera showed strong and exclusive reactivity at 55 kDa antigens of ES and 170 and 16 kDa region of CTA while sputum negative sera recognized strongly antigens at 24 kDa region of ES and 19 kDa region of CTA. Healthy normal sera showed less reactivity at 24 and 70 kDa regions of ES and 48, 35 and 29 kDa regions of CTA.
Discussion

The understanding of the humoral response at various stages of infection and disease could help to identify antigens that may be important in conferring protection or in serodiagnosis. There are few studies on isolation of circulating antigens from TB sera which are secreted by living bacilli or contributed by dead bacilli in vivo. An attempt has been made to identify in vitro (ES) and in vivo (CTA) released M. tuberculosis antigens recognized by pooled sera of S+ and S-pulmonary tuberculosis patients by immunoblotting, to define the repertoire of antigens. It was observed that antigenic components of 100, 31, 43 and 20 kDa of ES and CTA were commonly reactive to both sputum positive and sputum negative PTB sera. In most of the previous studies, immunoblot analysis has been done using mycobacterial sonicate extract or saline extract or cell wall rich fraction and reported variable results. However to the best of our knowledge the comparison of immunoblot pattern of ES (in vitro) and CTA (in vivo) antigen with different groups (S+ and S-) of PTB patients has not been reported earlier. Studies are very few, which showed different patterns of serological response against mycobacterial antigen fraction at diverse stages of disease. Franco et al. observed heterogeneity in antibody response in smear positive and smear negative sera to M. tuberculosis sonicate antigen. It has been suggested that protein profile expressed in vivo by M. tuberculosis may vary from in vitro and also with local milieu in which the bacteria replicate in vivo. Samaniach et al. in Western blot analysis, observed that patients with cavitary lesion recognized the more number of culture filtrate antigens and several additional antigens compared to noncavitary patients and repertoire changed with disease progression. In this study, sputum positive and sputum negative sera did show differences in seroreactivity. Seroreactivity of 55 kDa of ES antigen and 170 and 16 kDa antigens of CTA with sputum positive and 24 kDa of ES and 19 kDa of CTA with sputum negative PTB sera was observed, possibly due to differences in antigens present in culture filtrate and serum. However, the differences in antigens detected in sera do show that it is possibly due to contribution by dead bacilli or secretion by H37Rv strain. Further, it is possible that individual sera may react differently, which needs further investigation. Antigens such as 55, 24, 19 and 16 kDa proteins, which showed reactivity in different groups of pulmonary tuberculosis sera has been evaluated individually by several groups. In vitro released antigen ES-31 (31 kDa) and ES-41 (41 kDa) have been identified and their antigenic similarity with the antigen present in vivo by inhibition ELISA have been confirmed. These studies helped in identifying the reactive target antigen ES-31 with potential in diagnosis of pulmonary TB and some forms of extra pulmonary like tuberculous meningitis and lymph node tuberculosis while ES-41 antigen in detection of bone and joint and abdominal tuberculosis. Thus, in the present study analysis of immunoblot profile of in vitro (ES) and in vivo (CTA) released antigens revealed the antibody response to particular antigens and offer potential for developing M. tuberculosis specific test. The immunoblot analysis in the present study revealed serologic response to TB sera at common reactive antigens at 100, 31, 43 and 20 kDa in ES and CTA which shows that these antigens may be useful in developing serodiagnostic test. Antigens of 24 kDa and 19 kDa which are reactive with sputum negative sera will be of diagnostic interest and need further investigation in patients with low bacillary load. Using probing techniques of SDS-PAGE and immunoblotting, adequately defined antigens could be detected, which allows the host immune response in disease state to be better understood and improve the serodiagnosis of tuberculosis. By focusing on virulent M. tuberculosis strain, one possibly may identify more candidate antigens of diagnostic interest.

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