Isolation of *Mycobacterium tuberculosis* protein antigens ES-31, ES-43 and EST-6 of diagnostic interest from Tubercle Bacilli by affinity chromatography

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Immunodiagnostically useful *M. tuberculosis* H₃₇Ra protein antigens ES-31, ES-43 and EST-6 were isolated from detergent soluble sonicate (DSS) antigen using monospecific antibodies by affinity chromatography and compared with similar antigens isolated from *M. tuberculosis* culture filtrate for seroreactivity in tuberculosis sera by Indirect Enzyme Linked Immunosorbent Assay. Recovery of affinity purified ES-31, ES-43 and EST-6 antigen from DSS antigen was approximately 3, 3.5 and 4% respectively, compared to 10, 9 and 6.3% from culture filtrate. Affinity purified ES-31, ES-43 and EST-6 antigens from both culture filtrate as well as DSS antigen showed similar seroreactivity with overall sensitivity 85, 80 and 75% respectively and specificity of 85% at optimum concentration of 50 pg protein of each antigen. The results suggest that DSS antigen may be a promising antigen source for isolating antigens of diagnostic interest obviating the need for cumbersome, time-consuming culture techniques of mycobacteria.

**Keywords:** Affinity chromatography, DSS antigens, ELISA, *Mycobacterium tuberculosis*, Pulmonary tuberculosis

Tuberculosis (*TB*) is a chronic infectious disease killing nearly 2 million people every year and developing into active disease in 8.7 million people. The rising incidence both of multidrug-resistant TB and of human immunodeficiency virus (HIV) coinfection poses serious challenges to the control efforts¹. A curable and preventable disease, *TB* continues to be a leading cause of mortality and morbidity worldwide. Early detection and treatment of infectious cases reduces spread of TB. Therefore, rapid and accurate identification of infected individuals is mandatory²,³ and has created much scientific interest in developing new antimycobacterial agents. The major goal of Biomedical TB research is to lessen the public health burden of this epidemic by developing improved, affordable diagnostic strategies. Because of low-sensitivity of staining of acid-fast bacilli and time-consuming culture techniques, a fast etiological confirmation is needed for management of patients⁴ and in this context serology of TB is proved to be useful adjunct tool for early diagnosis. Study of mycobacterial antigens from tubercle bacilli and their immunogenic response is of considerable interest in developing serological test for TB. *Mycobacterium tuberculosis* (*M. tuberculosis*) Excretory-secretory (ES) protein antigens obtained from short term cultures should be ideal targets for study.

Isolation of *M. tuberculosis* ES-31, ES-41 and ES-43 protein antigens of immunodiagnostic potential from culture filtrate using salt precipitation, sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) and fast protein liquid chromatography (FPLC) fractionation has been reported⁵-⁸. These antigens were shown to be diagnostically useful in detecting tuberculous antibody. The phosphate transport protein (38 kDa) was also found to be immunodominant and useful in diagnosis⁹. EST-6 antigen isolated from culture filtrate containing 38 and 41 kDa proteins was explored and found to be useful in antibody detection¹⁰. Culture techniques for isolation of ES antigens are cumbersome and time consuming. Hence, in this study an attempt has been made to isolate mycobacterial antigens from DSS antigen of *M. tuberculosis* bacilli and compare seroreactivity of detergent soluble sonicate (DSS) antigens with that of ES antigens isolated from culture filtrate.

**Materials and Methods**

*Sera* — The study group was composed of patients with confirmed pulmonary TB (PTB) attending...
Isolation of DSS antigen — *M. tuberculosis* H$_{37}$Ra

DSS antigen, was prepared from *M. tuberculosis* H$_{37}$Ra bacilli$^{11}$. Briefly, bacilli were phenol-inactivated in 0.5 M Phosphate buffer saline (PBS, pH 7.2) and incubated with sodium dodecyl sulphate (SDS) extraction buffer. The supernatant was dialysed against 0.01 M PBS, pH 7.2 and used as an antigen source.

Isolation of goat anti-DSS antibodies — Goat anti-DSS IgG antibodies were raised in goat by immunizing intramuscularly with 500 μg protein/ml DSS-antigen with 1 ml Freund’s incomplete adjuvant on days 0, 20, 33 and 45. Immune sera were collected on days 0, 32, 44, 57, 60 and thereafter fortnightly. Anti-DSS IgG was isolated from normal and immune sera by 33% saturation with ammonium sulphate on days 0, 20, 33 and 45. Immune sera were collected, pooled and dialyzed overnight in 0.01 M PBS, pH 7.2, concentrated by freeze drying and labelled as anti-DSS IgG antibody.

Isolation of monospecific Anti-ES-31, ES-43 and EST-6 antibodies by affinity chromatography — In present study, *M. tuberculosis* monospecific anti-ES-31 antibody was isolated from anti-DSS IgG by affinity chromatography$^{11}$. Briefly, cyanogen bromide-activated Sepharose-4B beads were coupled with purified ES-31 antigen. Anti-DSS IgG (1mg/ml) was passed through the column and purified anti-ES-31 antibody was eluted with glycine-HCl buffer (0.01 mol/l, pH 2.5) and neutralized by adding Tris. Similarly, anti-ES-43 and anti EST-6 antibodies were isolated from anti-DSS IgG by affinity chromatography using ES-43 and EST-6 antigen-coupled Sepharose-4B beads.

Isolation of ES-31, ES-43 and EST-6 antigens from DSS antigen — *M. tuberculosis* ES-31 antigen was isolated from DSS antigen by affinity chromatography. Briefly, cyanogen bromide-activated Sepharose-4B beads were coupled with purified anti-ES-31 antibody. DSS Ag (1mg/ml) was passed through the column and purified ES-31 antigen was eluted with glycine-HCl buffer (0.01 mol/l, pH 2.5) and neutralized by adding Tris. Similarly, ES-43 and EST-6 antigens were isolated from DSS Ag by affinity chromatography using anti-ES-43 and anti-EST-6 antibody-coupled Sepharose-4B beads.

ELISA — Indirect penicillinase enzyme-linked immunosorbent assay (ELISA) was performed individually for detection of IgG antibody using affinity purified ES-31, ES-43 and EST-6 antigens isolated from DSS antigen. Optimum antigen concentration of 50 pg protein of each antigen was used in this assay. In brief, 5 μl of optimally diluted antigen (10 ng protein/ ml) was applied to cellulose acetate membrane squares fixed to a plastic strip and used along with optimally diluted human serum (1:600). Serum incubation was done at 37°C for 1 hr. After washings, strips were incubated with anti-human IgG penicillinase conjugate (1:1000) at 37°C for 1 hr. Finally, strips were incubated with blue color starch-iodine-penicillin ‘V’ substrate. The sera samples showing complete decolorization of blue color of starch iodine penicillin ‘V’ substrate at least 5 min earlier than negative control were considered as positive.

Results and Discussion

Diagnostically important *M. tuberculosis* ES-31, ES-43 and EST-6 protein antigens were isolated directly from DSS antigen by affinity chromatography and recovery was compared with antigens isolated from culture filtrate. Recovery of affinity purified ES-31, ES-43 and EST-6 antigen from DSS antigen was approximately 3, 3.5 and 4% respectively, compared to 10, 9 and 6.3% from culture filtrate (Table 1). Further onwards, seroreactivity of DSS antigens from bacilli and ES antigens from culture filtrate were compared (Table 2). All the three antigen proteins obtained from DSS as well as culture filtrate (ES) antigen showed sensitivity of 75-90 % and specificity of 85% (Table 2).

Even after the development of specific immunodiagnostic assay, its widespread clinical use is hampered by non-availability of immunologic
reagents in particular purified TB antigens. It may be unreasonable to expect real success in antigen purification using physicochemical techniques. Very-high-resolution techniques such as acrylamide gel electrophoresis may present particular problems because they may resolve antigenically homogeneous materials into many components differing slightly in the physical structure of nonantigenic moieties. If the desired goal is antigenic purity and homogeneity, then techniques such as affinity chromatography may offer more rational approaches to mycobacterial antigen purification. Affinity chromatographic procedures have been explored by several workers to obtain purified antigens from the complex antigenic mixtures.

Nair et al.6,6 reported isolation of 31 kDa glycoprotein antigen (ESAS-7F) of immunodiagnostic potential in pulmonary TB from *M. tuberculosis* H37Ra culture filtrate using SDS-PAGE and FPLC fractionation. Similarly, *M. tuberculosis* 43 kDa antigen (ESAS-6) with immunodiagnostic potential was isolated from ES antigen by ammonium sulphate solubilization, SDS-PAGE and FPLC. *M. tuberculosis* EST-6 antigen was prepared by 6% trichloroacetic acid (TCA) solubilization, followed by SDS-PAGE fractionation of *M. tuberculosis* ES antigen. The eluate from the 6th gel fraction was labeled as *M. tuberculosis* EST-6 antigen10. Earlier isolation of ES-31, ES-43 and EST-6 from culture filtrate (ES) antigen by affinity chromatography has been explored6,11. But culture techniques of bacilli are cumbersome and time consuming. Hence, isolation of ES-31, ES-43 and EST-6 antigen was explored from DSS antigen of *M. tuberculosis* bacilli by affinity chromatography using affinity purified anti-ES-31, anti-ES-43 and anti-EST-6 antibody.

ES-31 and ES-43 antigens were glycoprotein in nature5,14 while 41 kDa antigen was a lipoprotein15. EST-6 antigen fraction was shown to be a mixture of lipoproteins of molecular weight 38 kDa and 41 kDa16.

Isolation of ES-31, ES-43 and EST-6 antigen by affinity chromatography from DSS antigen gave a decreased percentage recovery (3-4%) compared to culture filtrate (ES) antigen (10-6%). However, ES-31, ES-43 and EST-6 antigens isolated from DSS antigen of tubercle bacilli and ES antigen isolated from culture filtrate showed similar seroreactivity (optimum concentration of 50 pg protein) for tuberculous IgG antibody detection by Indirect ELISA. In the present study, DSS antigen showed promise as an antigen source for isolating antigens of diagnostic interest obviating the need for cumbersome, time-consuming culture techniques of mycobacteria. Evaluation of diagnostic potential of cocktail of *M. tuberculosis* antigens consisting of ES-31, ES-43 and EST-6 isolated from DSS antigen is in progress.

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**Table 1** — Percent recovery of ES-31, ES-43 and EST-6 antigen from DSS antigen and ES antigen of TB bacilli by Affinity Chromatography.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>DSS antigen</th>
<th>ES antigen</th>
</tr>
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<tbody>
<tr>
<td>ES-31</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>ES-43</td>
<td>3.5</td>
<td>9</td>
</tr>
<tr>
<td>EST-6</td>
<td>4</td>
<td>6.3</td>
</tr>
</tbody>
</table>

*Amount of DSS and ES antigen applied on the affinity column was 1mg/ml

**Table 2** — Comparative seroreactivity analysis of ES-31, ES-43 and EST-6 antigen from DSS antigen and ES antigen of *M. tuberculosis* in the patients of PTB by ELISA

<table>
<thead>
<tr>
<th>Group (sera)</th>
<th>Total no. Screened (n)</th>
<th>No. (%) showing positive reaction for antibody detection* using</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DSS antigens</td>
<td>ES antigens</td>
</tr>
<tr>
<td></td>
<td>ES-31</td>
<td>ES-43</td>
</tr>
<tr>
<td>PTB (S+)</td>
<td>20</td>
<td>18(90%)</td>
</tr>
<tr>
<td>PTB (S-)</td>
<td>20</td>
<td>16(80%)</td>
</tr>
<tr>
<td>Healthy control</td>
<td>20</td>
<td>3(15%)</td>
</tr>
</tbody>
</table>

Reactivity of individual antigens are standardized at 50 pg concentration.

*Serum showing positive reaction at 1:600 dilution.

PTB: Pulmonary tuberculosis; n: Total number of cases; (S+): Sputum positive; (S-): Sputum negative
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References