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A LOW MOLECULAR WEIGHT ES-20 PROTEIN RELEASED IN VIVO AND IN VITRO WITH DIAGNOSTIC POTENTIAL IN LYMPH NODE TUBERCULOSIS

N Shende, V Upadhye, S Kumar, *BC Harinath

Abstract

Purpose: To determine role of antigens released in vivo and in vitro in immunodiagnosis of tuberculosis (TB).

Methods: In vivo released circulating tuberculosis antigen (CTA) was obtained from TB sera by ammonium sulphate precipitation and in vitro released excretory-secretory (ES) antigens from Mycobacterium tuberculosis culture filtrate. CTA and ES antigens were fractionated by SDS-PAGE and electro-eluted gel fractions were analysed for antigen by ELISA. Results: Low molecular weight proteins CTA-9 and ES-9 showed high titre of antigen activity. To explore the diagnostic potential of low molecular weight ES antigen, M. tuberculosis ES antigen was further fractionated by gel filtration chromatography followed by purification on anion exchange column using fast protein liquid chromatography and a highly seroreactive ESG-5D (ES-20) antigen was obtained. Competitive inhibition showed that CTA-9 and ES-9 antigens inhibit the binding of ES-20 antigen to its antibody. Seroanalysis showed sensitivity of 83 and 80% for ES-20 antigen and antibody detection, respectively, in pulmonary TB and 90% in lymph node TB. Conclusions: Seroreactivity studies using M. tuberculosis ES-20 antigen showed usefulness in detection of TB; in particular, lymph node TB.

Key words: Circulating tuberculosis antigen, diagnosis, ELISA, excretory-secretory antigen, tuberculosis

Tuberculosis (TB) is a leading cause of morbidity and mortality in the world. The diagnosis of active TB still largely depends upon initial clinical suspicion and radiographic findings, with subsequent laboratory confirmation by bacteriological studies. Owing to limitations in conventional methods for TB diagnosis, efforts to explore and establish serological means for detection of TB have been taken for a long time.

The demonstration of antigen in patients’ serum is a better marker for confirming the presence of active infection. Studies showed that concentration of circulating antigen correlates with disease status and their levels are elevated mainly in blood. There are studies on isolation and characterization of antigens released in vivo from body fluids and their diagnostic use in other diseases, but studies are few on exploring isolation and analysis of in vitro and in vivo released antigens in TB diagnosis.

Recently, overwhelming importance for an immunological test for TB has been observed, since a serodiagnostic test can satisfy the need for optimal use in the field conditions. It has also been suggested that surface proteins and proteins actively secreted from Mycobacterium tuberculosis are important targets of immune system during the early phase of infection and are capable of generating specific antibodies in TB patients. Culture filtrate proteins are therefore an attractive source of candidate antigens for developing immunodiagnostics for TB, as the antigens actively secreted by mycobacteria are more or less similar to the in vivo released mycobacterial antigen in TB patients. However, very few reports are available on studies exploring in vivo and in vitro released antigens in TB diagnosis. In the present study, circulating antigen was isolated from TB serum and seroreactive fraction was purified by SDS-PAGE. An attempt was made to isolate and identify the reactive antigen component of circulating antigen by purifying M. tuberculosis excretory-secretory (ES) antigen using fast protein liquid chromatography (FPLC) and competitive inhibition assay. Further, seroreactive studies were carried out using purified ES antigen by ELISA for evaluating its use in diagnosis of TB.

Materials and Methods

Sera from smear-positive (S+, n = 15) and smear-negative (S−) pulmonary TB (n = 15) patients and fine needle aspiration cytology (FNAC) proven lymph node TB (n = 30) patients were screened in the study. The smear negative pulmonary TB patients were diagnosed either by culture or by chest X-ray with a high degree of clinical suspicion and response to ATT treatment. Sera from non-tuberculous pulmonary and extra-pulmonary disease served as disease controls (n = 35) and healthy individuals (n = 20) as healthy controls. Sera were stored at −20°C with 0.1% sodium azide.

Circulating tuberculosis antigen (CTA) was prepared as described earlier. One millilitre volumes of sera from
S+ pulmonary TB patients (n = 10) were pooled from which CTA was isolated by 36-75% ammonium sulphate precipitation. The CTA was resolved by SDS-PAGE on 10% non-gradient slab gel (12 x 12 cm) and sliced horizontally at 1 cm intervals (12 slices designated as CTA-1 to CTA-12) and the protein in each slice was electro-eluted. Antigenic activity of each eluted fraction was tested by indirect ELISA.

_Mycobacterium tuberculosis_ ES culture filtrate proteins were obtained from _M. tuberculosis_ H_{37}Ra cells grown in thyroxine-supplemented Sauton liquid medium incubated at 37 °C on an orbital shaker (150 rpm) for 10 days as described earlier. Similarly, _M. tuberculosis_ ES antigen was resolved by SDS-PAGE and the protein in each gel slice was electro-eluted as described above.

_Mycobacterium tuberculosis_ ES antigen was fractionated by FPLC using Superdex 200 HR (high resolution) 10/30 gel filtration column (Pharmacia Biotech, Sweden) as described earlier, following the manufacturer’s protocol. _Mycobacterium tuberculosis_ ES antigen (600 µg/0.5 mL) was applied in 250 µL sample loop and elution was carried out at a flow rate of 0.5 mL/minute. Fraction size of 1 mL volumes were collected and seven protein peaks (designated as ESG-1 to ESG-7) were obtained. Elution was monitored at 280 nm. The serologically active fraction ESG-5 was further fractionated by anion exchange Resource ‘Q’, 1 mL column as described earlier. Six protein peak fractions were obtained (designated as ESG-5A to 5F). The protein content of each fraction was measured by Lowry’s method and the antigenic activity was analysed by indirect ELISA.

Polyclonal antibodies to _M. tuberculosis_ sonicate antigen were raised in goat serum. Specific antibody against ESG-5D (ES-20) antigen was isolated from immune sera using ESG-5D antigen coupled with CNBr-activated Sepharose-4B column. Goat anti-sonicate IgG antibody was applied to the column and washed with 0.01 M phosphate buffer saline (PBS). Bound antibody (anti-ESG-5D) was eluted with glycine-HCl buffer, pH 8.6.

Inhibition ELISA was carried out as described earlier. Briefly, optimal concentration of ESG-5D antigen (100 ng/100 µL/well) was coated onto flat polystyrene microplates (Nunc, Denmark) and incubated overnight at 4 °C and blocked with 2% BSA. Hundred microlitres (starting concentration 10 µg/mL and serial 10-fold dilution) of reactive CTA-9 and ES-9 antigens were incubated separately with affinity purified goat anti-ESG-5D penicillinase conjugate (1:500) for 30 min. After centrifugation, supernatant was added to the ESG-5D antigen sensitized plate and incubated for 1 hour at 37 °C. After washing the plate with PBS/T, the enzyme activity on the plate was revealed by adding blue-coloured starch-iodine-penicillin ‘V’ substrate. The persistence of blue colour indicated a positive reaction.

Indirect ELISA was carried out for tuberculous antibody detection as described earlier. A 5-µL volume of optimally diluted ESG-5D antigen (1 ng/stick) was applied on cellulose acetate membrane (CAM) square fixed onto a plastic strip. Antigen-coated sticks were incubated with 0.5 mL optimally diluted serum (1:600) in PBS/T at 37 °C for 1 hour. Antihuman IgG penicillinase conjugate (1:1000) was used as tracer in this assay. The sera showing complete decolourization at least 5 minutes earlier than negative control of blue-coloured substrate (Starch-Iodine-Penicillin ‘V’) denoted positive reaction.

Sandwich ELISA was performed for detection of circulating antigen as described earlier. A 5-µL volume of optimally diluted goat anti-ESG-5D antibody (1 µg/stick) was applied onto CAM sticks. Antibody-coated sticks were further incubated with 0.5 mL optimally diluted serum (1:300) in PBS/T at 37 °C for 1 hour. Goat anti-ESG-5D penicillinase conjugate (1:500) was used as tracer in this assay. The sera showing complete decolourization at least 5 minutes earlier than negative control of blue-coloured substrate (Starch-Iodine-Penicillin ‘V’) denoted positive reaction.

**Results**

Among the ammonium sulphate precipitated fractions (0-33, 34-75 and >75%) of TB sera, the 34-75% fraction showed antigenic activity and was labelled as circulating tuberculous antigen (CTA). Further, on fractionation of CTA by SDS-PAGE and electro-elution, 12 protein eluates were obtained. On analysis for antigenic activity of eluates by indirect ELISA, the CTA-2 and CTA-9 antigen fractions were observed to be highly antigenic showing a titre of 1000. Similarly, fractionation of ES antigen resulted in isolation of highly antigenic low molecular weight _M. tuberculosis_ ES-9 (20-25 kDa) antigen fraction (Table 1). In view of the seroreactivity of low molecular weight excretory-secretory (ES) culture filtrate antigens, the _M. tuberculosis_ ES antigen was further fractionated by FPLC using gel filtration column, which yielded seven protein peaks (Fig. 1). The antigenically active fractions, _M. tuberculosis_ ESG-2 and ESG-5, were evaluated for antibody detection in sera samples of pulmonary TB (S+ and S−) patients using indirect ELISA. ESG-5 antigen showed sensitivity of 86% and specificity of 90% (Table 2). The seroreactive _M. tuberculosis_ ESG-5 antigen was further purified by FPLC using anion exchange column, which yielded six protein peaks (designated as ESG-5A to 5F) as shown in Fig. 2. On analysis by Inhibition ELISA, the CTA-2 and CTA-9 antigen fractions were evaluated for antibody detection in sera samples of pulmonary TB (S+ and S−) patients using indirect ELISA. ESG-5 antigen showed sensitivity of 86% and specificity of 90% (Table 2). The seroreactive _M. tuberculosis_ ESG-5 antigen was further purified by FPLC using anion exchange column, which yielded six protein peaks (designated as ESG-5A to 5F) as shown in Fig. 2. On analysis by Indirect ELISA, _M. tuberculosis_ ESG-5D antigen fraction was observed to be highly antigenic. SDS-PAGE analysis of _M. tuberculosis_ ESG-5D antigen using 15% non-gradient gel followed by silver staining showed a 20-kDa molecular weight protein band (Fig. 3). Henceforth, it is referred to as _M. tuberculosis_ ES-20 antigen as a synonym to ESG-5D antigen.

Seroreactivity analysis by Inhibition ELISA showed that low molecular weight circulating antigen CTA-9 and in vitro released ES-9 antigen fraction bind to anti-ES-20 antibody and inhibited the binding of affinity purified anti-ES-20...
antibody penicillinase conjugate to ES-20 antigen applied on plate at a concentration 10 ng/mL and above (Table 3). On analysis by indirect ELISA, ES-20 antigen showed a sensitivity of 80 and 90% for antibody in pulmonary and lymph node TB samples, respectively. However, antigen detection using anti-ES-20 antibody by sandwich ELISA showed sensitivity of 83 and 90% in pulmonary and lymph node TB samples respectively. The overall specificity of both the assays was observed to be 89% (Table 4).

### Table 1: Fractionation and reactivity of CTA and ES antigens by SDS-PAGE and ELISA

<table>
<thead>
<tr>
<th>Fraction number (CTA/ES)</th>
<th>Approximate molecular weight (×10⁻³ kDa)</th>
<th>Antigenic titre of ES fraction showing positive reaction*</th>
<th>Antigenic titre of CTA fraction showing positive reaction*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>&gt;160</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>160-95</td>
<td>100</td>
<td>1000</td>
</tr>
<tr>
<td>3</td>
<td>95-68</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>68-56</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>56-45</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>45-37</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>7</td>
<td>37-29</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>8</td>
<td>29-25</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>25-20</td>
<td>1000</td>
<td>1000</td>
</tr>
<tr>
<td>10</td>
<td>20-17</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>11</td>
<td>17-14</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>14-12</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*The reciprocal of the highest dilution of fraction showing positive reaction. Initial protein concentration of 1 µg/mL and serial dilutions (10, 100, 1000 times) were made and coated on the sticks, CTA - Circulating tuberculosis antigen, ES - Excretory-secretory

### Table 2: Comparative analysis of seroreactivity of *M. tuberculosis* ESG-2 and ESG-5 antigen fractions for detection of antibody in PTB sera by Indirect ELISA

<table>
<thead>
<tr>
<th>Serial number</th>
<th>Groups</th>
<th>Number screened</th>
<th>Positive reaction for antibody* using</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>ESG-2 antigen, n (%)</td>
</tr>
<tr>
<td>1</td>
<td>Pulmonary TB</td>
<td>35</td>
<td>28 (80)</td>
</tr>
<tr>
<td></td>
<td>S+ PTB</td>
<td>20</td>
<td>16 (80)</td>
</tr>
<tr>
<td></td>
<td>S– PTB</td>
<td>15</td>
<td>12 (80)</td>
</tr>
<tr>
<td>2</td>
<td>Healthy controls</td>
<td>20</td>
<td>2 (10)</td>
</tr>
</tbody>
</table>

*Sera showing positive reaction at 1:600 dilution, PTB = Pulmonary tuberculosis; S+ = Smear positive; S– = Smear negative

Figure 1: Elution profile of FPLC separation of *M. tuberculosis* ES antigen on Superdex 200 HR 10/30 gel filtration column. *Mycobacterium tuberculosis* ES antigen (300 µg/mL) was applied on column. For Elution, 0.05 M phosphate buffer, pH 7.2 was used at a flow rate of 1 mL/min. Fractions of 1 mL were collected

antibody penicillinase conjugate to ES-20 antigen applied on plate at a concentration 10 ng/mL and above (Table 3). On analysis by indirect ELISA, ES-20 antigen showed a sensitivity of 80 and 90% for antibody in pulmonary and lymph node TB samples, respectively. However, antigen detection using anti-ES-20 antibody by sandwich ELISA showed sensitivity of 83 and 90% in pulmonary and lymph node TB samples respectively. The overall specificity of both the assays was observed to be 89% (Table 4).

Figure 2: Elution profile of FPLC separation of *M. tuberculosis* ESG-5 antigen fraction on Resource ‘Q’ 1 mL anion exchange column. *Mycobacterium tuberculosis* ESG-5 antigen (250 µg) was applied on column. For elution, 20 mM Tris-HCl, pH 8.0 and gradient of 0-0.7 M NaCl were used at a flow rate of 0.5 mL/min. Fractions of 0.5 mL were collected

**Discussion**

Several different approaches have been explored in searching for ideal antigens for use in TB diagnostics. Our laboratory has focussed on the ES proteins present in the culture filtrate of *M. tuberculosis* and isolated several
target antigens ES-31 (31 kDa), ES-41 (41 kDa) and ES-43 (43 kDa) which confirm active infection in different forms of pulmonary and extra-pulmonary TB.\[11,13,14\]

The mycobacterial antigens secreted into the culture medium are more or less similar to the antigens released \textit{in vivo}. However, there are very few studies on isolation and analysis of \textit{in vivo} and \textit{in vitro} released antigens of \textit{M. tuberculosis}. In the present study, \textit{in vivo} released CTA and \textit{in vitro} released ES antigens were fractionated separately by SDS-PAGE and the gel fractions were electro-eluted and analysed by indirect ELISA. This has helped to identify the highly antigenic low molecular weight CTA-9 and ES-9 (20-25 kDa) antigen fractions. An attempt was made to isolate and identify CTA-9 equivalent antigen protein in culture filtrate. The secreted protein antigens of \textit{M. tuberculosis} provide the first stimulus in \textit{vivo} for humoral and cell-mediated immune response to mycobacteria and thus play a useful role in serodiagnosis. The diagnostic potential of low molecular weight ES culture filtrate antigens (ESAT-6 and CFP-10) of \textit{M. tuberculosis} has been reported in earlier studies.\[15,16\] Keeping this in view, ES antigen was fractionated using FPLC for isolation of low molecular weight antigen resulting in identification of a 20-kDa protein (ES-20). The ES-20 antigen was observed to be similar to circulating antigen CTA-9 by inhibition studies, indicating \textit{in vivo} released CTA-9 and \textit{in vitro} released ESG-5D (ES-20) sharing identical antigenic determinants. Earlier studies from our laboratory identified \textit{in vitro} released antigens ES-31 (31 kDa) and ES-41 (41 kDa) and confirmed their presence \textit{in vivo}.\[13,14\] These studies helped in identifying the reactive target antigen ES-31 with potential in diagnosis of pulmonary TB and ES-41 antigen with potential in diagnosis in sera of bone and joint and abdominal TB. Seroreactivity studies using ES-20 antigen by indirect ELISA for antibody detection showed sensitivity of

**Table 3: Antigenic similarity of \textit{M. tuberculosis} ESG-5D (ES-20) and CTA-9/ES-9 antigen fraction by competitive Inhibition ELISA**

<table>
<thead>
<tr>
<th>Concentration (ng) of CTA-9 and ES-9</th>
<th>Reaction with antigen incubated separately with anti-ESG-5D antibody penicillinase conjugate and reacted with ESG-5D*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000</td>
<td>Inhibition</td>
</tr>
<tr>
<td>100</td>
<td>Inhibition</td>
</tr>
<tr>
<td>10</td>
<td>Inhibition</td>
</tr>
<tr>
<td>1</td>
<td>No inhibition</td>
</tr>
</tbody>
</table>

*M. tuberculosis* ESG-5D (100 ng/well) was coated to plate

**Table 4: Analysis of seroreactivity of ESG-5D (ES-20) antigen for detection of antibody and anti-ES-20 antigen for antigen detection in TB sera**

<table>
<thead>
<tr>
<th>Group</th>
<th>Serum screened</th>
<th>Positive reaction for antibody* using ES-20 antigen, n (%)</th>
<th>Positive reaction for antigen** using anti-ES-20 antibody, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pulmonary tuberculosis</td>
<td>30</td>
<td>24 (80)</td>
<td>25 (83)</td>
</tr>
<tr>
<td>S+ PTB</td>
<td>15</td>
<td>12 (80)</td>
<td>13 (87)</td>
</tr>
<tr>
<td>S– PTB</td>
<td>15</td>
<td>12 (80)</td>
<td>12 (80)</td>
</tr>
<tr>
<td>Extra pulmonary tuberculosis</td>
<td>30</td>
<td>27 (90)</td>
<td>27 (90)</td>
</tr>
<tr>
<td>Tuberculous lymphadenopathy</td>
<td>30</td>
<td>4 (11)</td>
<td>4 (11)</td>
</tr>
<tr>
<td>Disease controls</td>
<td>35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>COAD</td>
<td>5</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Pneumonia</td>
<td>3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Asthma</td>
<td>5</td>
<td>-</td>
<td>-</td>
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<tr>
<td>PUO</td>
<td>4</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Leprosy</td>
<td>3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Non-specific lymphadenopathy</td>
<td>15</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Healthy controls</td>
<td>20</td>
<td>2 (10)</td>
<td>2 (10)</td>
</tr>
</tbody>
</table>

*Sera showing positive reaction at 1:600 dilution, **Sera showing positive reaction at 1:300 dilution, PTB - Pulmonary tuberculosis; S+ - Smear positive; S– - Smear negative*
80 and 90% in pulmonary (S+ and S–) TB and lymph node TB patients respectively. Further, on exploring the utility of affinity purified ES-20 antibody for detecting TB, we observed that 87% (S+ PTB), 80% (S– PTB) and 90% (lymph node TB) of the patients were positive for the antigen, which is a better indicator for confirming the diagnosis of active TB infection.

Thus, the study helped in isolating the ES-20 antigen, identical to in vivo released CTA-9 antigen. Serological studies further showed that in vitro released ES-20 antigen could be a potential antigen for diagnosis of active infection in TB in particular, lymph node TB.

Acknowledgements

This study was supported by Tropical Disease Research grant from Kasturba Health Society, Sevagram and partly by CSIR, New Delhi. Our thanks to Dr Kalsait, District Tuberculosis Officer, Dr Hivrale (medical officer) and the staff at Civil Hospital, Wardha and Dr. N. Gangane, Head, Department of Pathology and his senior residents for extending help in this study.

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