CULTURE FILTRATE ANTIGENS IN TUBERCULOSIS DIAGNOSIS

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Abstract: Tuberculosis displays all of the principal characteristic features of a global epidemic disease and is rampant throughout the world. More than a century after its original development, the microscopic examination of sputum is still the only widely available diagnostic tool for identifying TB in most developing countries. Hence there is need for a rapid, sensitive and simple technique for the detection of \textit{Mycobacterium tuberculosis}. Immunodiagnosis appears to be a promising approach for the diagnosis of pulmonary as well as extra-pulmonary tuberculosis. The sensitivity of a diagnostic test based on immunological approach depends on the nature of the diagnostic reagent viz., antigen or antibody employed, kind of diagnostic technique employed. A good serological assay should perform well in specific targeted populations, especially childhood TB, HIV positive and extrapulmonary TB. Unfortunately, the sensitivity of most serological tests falls with smear negativity - a finding attributed to the lower burden of organisms. In addition, the fact that large number of environmental bacteria have cross-reactive antigens and the antigens used so far are not very species-specific contrive to confuse the diagnosis. Hence the use of culture filtrate antigens 85 kDa antigens which is known to be non-cross reactive even with the environmental mycobacterium is being used for the diagnosis of pulmonary and extra pulmonary tuberculosis. Culture filtrate of \textit{M. tuberculosis} H37Rv is highly enriched with secretions, metabolites and degradative products of \textit{M. tuberculosis} which may be proteins, lipids, carbohydrates and other metabolites. The metabolites of \textit{M. tuberculosis} H37Rv in the culture filtrate are strong candidates for superior diagnosis of tuberculosis. The protein fraction was first separated from the CF based on the precipitation procedures. The protein fractions were then separated on SDS-PAGE with subsequent immunoblotting. Liposomes interchelated with CF 85 kDa and 38 kDa protein antigen complexes were employed in slide agglutination test for detection of specific antibodies in patient sera. The results are compared with that of smear test. The sensitivity and specificity of the test are of high degree and varied with the type and nature of specimens. Use of liposome agglutination technology employing culture filtrate immunodominant antigens has simplified the currently followed diagnostic procedures and considerably made the test economical, hence this test could be recommended even at primary health centres that lack in automation and skilled technicians.

Keywords: \textit{Mycobacterium tuberculosis}, culture filtrate antigens, liposome, specificity, sensitivity
1 Introduction

Tuberculosis is a chronic granulomatous disease caused by tubercle bacterium Mycobacterium tuberculosis and is responsible for the highest mortality [1]. Diagnosis of pulmonary tuberculosis as well as extra-pulmonary tuberculosis in its early stages, is difficult because of nonspecific clinical features. The conventional method of AFB staining and culture methods are being used as gold standards for TB diagnosis, these tests lack sensitivity, often gives false negative results when the bacilli content is too low (less than 8 bacilli). Modern approaches explored for detection of tuberculosis bacterium include Bactec culture method [2,3], Immuno chromatography based tests [4] Mass spectrometric analysis of the antigens [5], GC profiling of the Mycobacterium specific fatty acids in the infected sample and nucleic acid amplification techniques. However the rapidity of the detection methods, complexity of the tests, cross reactivity with other bacterial species, latent Tb infection cases are the setbacks of these modern detection methods. The hurdles in accurate diagnosis of tuberculosis could be minimized by the use of appropriate mycobacterial antigens coupled with the use of simple, rapid and hassle free technique. The use of culture filtrate antigens such as ESAT-6, HSP, Ag 85 complex and 38 kDa in the diagnosis is wide, since they exhibit less cross reactivity with closely related bacterial species and high degree of specificity. The sensitivity and detection efficacy of a test can be enhanced by the technique being used, hence the present study is focused on exploring the use of liposomes with immunogenic antigens derived from culture filtrate in agglutination based tuberculosis diagnosis.

2 Immunodiagnostic potential of culture filtrate antigens

The availability of better diagnostic reagents from culture filtrates of M. tuberculosis such as more species-specific skin test antigens and serological markers, has been a driving force in the efforts devoted to diagnosis of M. tuberculosis. The M. tuberculosis release culture filtrate proteins (CFPs) that stimulate host immune system [6] and many CFPs are recognized by the sera of tuberculosis subjects. The M. tuberculosis secretes active culture filtrate proteins, which are missing in non-tuberculous Mycobacteria which serve as diagnostic markers. Many such culture filtrate proteins identified and characterized from the M. tb complex include PPD, antigen 5(38kda), antigen A60, 45/47 kDa , 30/31 kDa, 40kda, 42 kDa, SOD, 30 kDa, 31 kDa, 85 B, ESAT-6, ESAT-7, CFP-10, kP90, glycolipid, sulholipids, lipopolysaccharides, P32 antigen, Lipoarabinomannan (LAM), Cord factor (trehalose -6',6' dimycolate) and phenolglycolipid- lipid antigen (PGL Tb1)[7,8]. Culture filtrate protein antigens are reported to be detected in various types of body fluids at a minimum concentration of 3-20µg /ml. Many of the other protein antigens found in the culture filtrate [9] viz, Sod A, Kat G and Gln A (glutamine synthase), do not have leader sequence that are usually involved in protein secretion [10-12] but are very stable [13]. The presence of many proteins in culture filtrates, especially those with missing leader sequences, caused by bacterial leakage or lysis serve as potential source of diagnostic reagents. The CFP antigens of M. tuberculosis have been exploited in various immunodiagnostic techniques such as sandwich ELISA, Inhibition ELISA, latex agglutination and reverse passive haemagglutination with sensitivity and specificity in the range of 93% and 95% respectively. ELISA is found useful for early and sensitive diagnosis of all forms of tuberculosis. The 16-kDa cytosolic antigen of M. tuberculosis showed significant diagnostic potential by ELISA. The IgG, IgA, and IgM antibodies against 16-kDa antigen were detected with sensitivities of
62%, 52% 11% and with specificities of 100%, 97% and 95% respectively [14]. Antibodies to 31/30 kDa antigen fibronectin binding proteins, which are cross reactive with other species of mycobacteria, have been demonstrated in the sera of tuberculosis patients and lepromatous leprosy patients. The 31 kDa antigenic proteins in the culture filtrate was the most promising one with varying sensitivities (48-94%) and specificities (87-100%) [15]. The 32kDa antigen is abundantly secreted into the culture supernatant of various species of mycobacteria [16] and antibodies (IgG) against it could be detected in 70% of tuberculosis patients. Measurement of antibodies to 38 kDa protein showed a sensitivity of 36.1%, and specificity of 91.6% [17]. Serological sensitivity of 38 kDa antigenic protein ranges between 16% to 94% and specificity ranges from 93% to 100% [18-19] and this antigenic protein is the diagnostic reagent in various commercially available serological tests [Pathozyme TB complex kit (Omega Diagnostics and IgA (Omega Diagnostics); Rapid TB test (Quorum Diagnostics, Vancouver British, Columbia, Canada); ICT Tuberculosis AMRAD-ICT (Amrad, Sydney, Australia)] [19,21]. The sensitivity of IgA- and IgM-based assays appeared to be significantly lower than that of IgG assays [19]. Culture filtrate of the M. tuberculosis generated various types of heat shock proteins with significant diagnostic potential. The indirect ELISA employing hsp 65 yielded 84% sensitivity and 90% specificity [22]. The A60 antigen based kit (Anda Biologicals, Strausbourg, France) has been widely used for estimation of IgA, IgG and IgM antibodies in serum, cerebrospinal fluid and pleural fluid [25-27] with sensitivity and specificity of 74% 84% and 88% 100% respectively [28-29]. The main limitation of this antigen is not specific for mycobacteria alone and is also present in Norcodia and Corynebacteria species. Early secreted culture filtrate protein antigen viz., 6-kDa (ESAT-6) antigen are used in ELISA to measure anti-ESAT antibody in sera. It was reported that ESAT-6 antigen is cross reactive with sensitivity of 94% and specificity of 88% [30]. The 33 kDa antigen fraction (ESAS-7) showed a sensitivity and specificity of 90% and 92% respectively in the diagnosis of pulmonary as well as extra-pulmonary tuberculosis[30]. The sensitivity of ESAS-7 antigen fraction is comparable with that of 30 kDa antigen but with better specificity. Limited studies are available on culture filtrate derived lipid antigens and due attention is needed on non-proteinaceous antigens of the M. tuberculosis culture filtrate for their use in simple diagnostic assays.

Table 1

<table>
<thead>
<tr>
<th>Antigen used</th>
<th>Antibody class</th>
<th>Test</th>
<th>Sensitivity %</th>
<th>Specificity %</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>A60 (ERBALI SA testkit)</td>
<td>IgG</td>
<td>ELISA</td>
<td>72.9</td>
<td>60</td>
<td>Banerjee et al., 2005 [15]</td>
</tr>
<tr>
<td>A31 kda (SEVA TB test)</td>
<td>IgG</td>
<td>ELISA</td>
<td>91.6</td>
<td>78</td>
<td>Banerjee et al., 2005 [15]</td>
</tr>
<tr>
<td>A60(Immunozyme, Austria)</td>
<td>IgG</td>
<td>Sandwich ELISA</td>
<td>100</td>
<td>96.6</td>
<td>Malati et al., 1995 [20]</td>
</tr>
<tr>
<td>A60 (Anda biologicals)</td>
<td>IgG, IgM</td>
<td>ELISA</td>
<td>69.6, 10.5</td>
<td>92.1, 99.4</td>
<td>Luh et al., 1996 [26]</td>
</tr>
<tr>
<td>HSP 65</td>
<td>Indirect ELISA</td>
<td>84</td>
<td>90</td>
<td></td>
<td>Mudaliar et al., 2006 [22]</td>
</tr>
</tbody>
</table>
2.1 Culture Filtrate Protein Antigens in Tb diagnostics

The CFP antigens of \textit{M. tuberculosis} have been exploited in various immunodiagnostic techniques such as sandwich ELISA, Inhibition ELISA, Latex agglutination and reverse passive haemagglutination with sensitivity and specificity in the range of 93% and 95% respectively. ELISA is found useful for early and sensitive diagnosis of all forms of tuberculosis. The 16-kDa cytosolic antigen of \textit{M. tuberculosis} showed significant diagnostic potential by ELISA. The IgG, IgA, and IgM antibodies against 16-kDa antigen were detected with sensitivities of 62%, 52%, 11% and with specificities of 100%, 97%, and 95% respectively [14]. Antibodies to 31/30 kDa antigen fibronectin binding proteins, which are cross reactive with other species of mycobacteria, have been demonstrated in the sera of tuberculosis patients and lepromatous leprosy patients. The 31 kDa antigenic proteins in the culture filtrate was the most promising one with varying sensitivities (48-94%) and specificities (87-100%) [15]. The 32kDa antigen is abundantly secreted into the culture supernatant of various species of mycobacteria and antibodies (IgG) against 32 kDa protein could be detected in 70% of tuberculosis patients [16]. Measurement of antibodies to 38 kDa protein showed a sensitivity of 36.1%, and specificity of 91.6% [17]. Serological sensitivity of 38 kDa antigenic protein ranges between 16% to 94% and specificity ranges from 93% to 100% [17-19] and this antigenic protein is the diagnostic reagent in various commercially available serological tests [Pathozyme TB complex kit (Omega Diagnostics and IgA (Omega Diagnostics); Rapid TB test (Quorum Diagnostics, Vancouver British, Columbia, Canada); ICT Tuberculosis AMRAD-ICT (Amrad, Sydney, Australia)] [19,21]. The sensitivity of IgA- and IgM-based assays appeared to be significantly lower than that of IgG assays [19]. Culture filtrate of the M. tuberculosis generated various types of heat shock proteins with significant diagnostic potential [30]. The indirect ELISA employing hsp 65 yielded 84% sensitivity and 90% specificity [30]. The A60 antigen based kit (Anda Biologicals, Strausbourg, France) has been widely used for estimation of IgA, IgG and IgM antibodies in serum, cerebrospinal fluid and pleural fluid [25-29] with sensitivity and specificity of 74%-84% and 88%-100% respectively. The main limitation of this antigen is not specific for mycobacteria because it is also present in Norcodia and Corynebacteria species. Early secreted culture filtrate protein antigen viz., 6-kDa (ESAT-6) antigen are used in

<table>
<thead>
<tr>
<th>Glycolipid</th>
<th>IgA</th>
<th>ELISA</th>
<th>88</th>
<th>88-100</th>
<th>Bezerra et al., 2008</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBF6/DP EP</td>
<td>IgA</td>
<td>ELISA</td>
<td>58</td>
<td>90</td>
<td>Bezerra et al., 2008</td>
</tr>
<tr>
<td>PPD</td>
<td>IgA</td>
<td>ELISA</td>
<td>38.7</td>
<td>90</td>
<td>Araujo et al., 2004</td>
</tr>
<tr>
<td>38kda</td>
<td>sIgA</td>
<td></td>
<td>36.1</td>
<td>97.1</td>
<td>Araujo et al., 2004</td>
</tr>
<tr>
<td>HSP60</td>
<td>IgG</td>
<td>ELISA</td>
<td>89</td>
<td>97</td>
<td>Meena LS et al 2002</td>
</tr>
<tr>
<td>20 kDa</td>
<td>FD-ELISA</td>
<td>90.8</td>
<td>89.6</td>
<td>El-Masry et al 2008</td>
<td></td>
</tr>
<tr>
<td>55-kDa molecular weight</td>
<td>dot-ELISA</td>
<td>87</td>
<td>93</td>
<td>Attalla &amp; et al 2003</td>
<td></td>
</tr>
</tbody>
</table>
ELISA to measure anti-ESAT antibody in sera. It was reported that ESAT-6 antigen is cross reactive with sensitivity of 94% and specificity of 88% [30]. The 33 kDa antigen fraction (ESAS-7) showed a sensitivity and specificity of 90% and 92% respectively in the diagnosis of pulmonary as well as extra-pulmonary tuberculosis [30]. The sensitivity of ESAS-7 antigen fraction is comparable with that of 30 kDa antigen but with better specificity. Limited studies are available on culture filtrate derived lipid antigens and due attention is needed on non-proteinaceous antigens of the M. tuberculosis culture filtrate.

### 2.2 Nonproteinaceous culture filtrate antigens in Tb diagnosis

Culture filtrate antigen analysis of *M. tuberculosis* H37Rv revealed that three lipoprotein antigens of M. wt 38 k Da was present among the 20 protein antigens identified (Rv0934), (LppZ,Rv3006). The third lipoprotein that is closely related to the 38-kDa antigen, both involved in phosphate transport, pstS2 (Rv0932c), was also identified as a good serodiagnostic antigen in this study [34].

#### 2.2.1 Culture filtrate polysaccharides antigens in Tb diagnosis

Lipoarabinomannan is detected in culture filtrate of a 3-week-old culture of *M. tuberculosis*. The culture filtrate contained approximately 100 µg of LAM/ml [33]. LAM is a major culture filtrate polysaccharide antigen present in cell wall of all mycobacteria. Purified LAM from *M. tuberculosis* in its native acylated state was first used for serodiagnosis of leprosy [35]. The diagnostic ELISA kit developed using LAM exhibited good specificity (91%) and sensitivity (72%). A commercially available test (Mycodot; Genelabs Switzerland) specific for *M. tuberculosis* detects IgG antibodies to lipoarabinomannan antigen. The assay has achieved a high degree of specificity (84–100%) but the sensitivity was low (16–56%).

#### 2.2.2 Advantages of using culture filtrate Antigens

- The lipoprotein antigens have enough advantages in therapeutics as well as in diagnostics.
- High titre of these antibodies were raised against these secretory proteins hence the antibody based diagnostics could be developed with high sensitivity and specificity.
- These CF antigens are always the soluble cleavage products or proteolytic digests or post translation modification and these epitopes have got a high seroreactivity with Tb positive sera when compared with the other cell disrupt antigens.
- The lipoproteins which are present as a CF antigens are known to have conformations of surface exposed phosphate transport protein which exclusively enhances the Tb diagnosis.

### 3 Liposome Technology in diagnosis

Interchelation of *M. tuberculosis* specific lipids in liposomes results in simple and rapid diagnostic means [2, 22]. The conformationally modified epitopes are spiked in the lamellae of the liposomes for the enhanced specificity and sensitivity of the test (Table 2).

**Table 2**

<table>
<thead>
<tr>
<th>Technique</th>
<th>Sensitivity %</th>
<th>References</th>
</tr>
</thead>
</table>

Comparative analysis of different techniques used for Tb diagnosis
4. Conclusion

The serodiagnostic potential of culture filtrate antigens/ conformationally modified epitopes is found superior when employed in appropriate diagnostic assay. The liposome agglutination test which is considered as one of the simplest test, rapid economical and highly sensitive technique can be used for Tb diagnosis using cocktail of culture filtrate antigens for achieving the maximum diagnostic potential.

References


