Abstract

Background: - Tuberculous meningitis (TBM) remains a major global health problem. Difficulty in diagnosis of tuberculous meningitis (TBM) from partially treated cases of pyogenic meningitis (PTPM) remains an obstacle despite some newly developed diagnostic methods. Delay in starting appropriate medication for TBM and PTPM worsens prognosis. In our earlier study we have demonstrated the presence of 30 Kd protein antigen in CSF of TBM patients.

Method: - A cell enzyme linked immunosorbent assay (Cell ELISA) has been developed for the quantitative measurement of anti 30 Kd protein antibody production derived by B cells from CSF of TBM and PTPM patients.

Objective: - Our study is intended to develop an assay, which can discriminate the TBM cases from the cases of PTPM where other sophisticated diagnostic methodologies do not work.

Results: - The sensitivity and specificity of measurement of anti-30Kd protein antibody production were 91% and 93% respectively for the differential diagnosis of TBM from PTPM patients by Cell ELISA method.
Conclusion: - Cell ELISA assay using 30 Kd protein antigen is a promising tool for differentiating TBM from PTPM and give confidence to the neurophysician for diagnosis of TBM.

Keywords: 30 Kd protein antigen, tuberculous meningitis, partially treated cases of pyogenic meningitis, Cell ELISA.

Running title: - Diagnosis of TBM

Background

Tuberculous meningitis (TBM) is the infection of the Central Nervous System (CNS) and is prevalent in under-developed and developing countries and there is resurgence of TBM due to growing number of people infected with Human immunodeficiency Virus (HIV). Diagnosis of TBM remains a problem despite the many new and advanced technologies [1,2]. Previous clinical studies of TBM have clearly demonstrated that the timing of treatment is the most critical factor affecting the ultimate outcome, which stresses the importance of early diagnosis of TBM [3]. The laboratory confirmation for the diagnosis of TBM is based on the detection of Acid-fast Bacilli (AFB) in the Cerebrospinal fluid (CSF) and by culturing CSF for Mycobacterium tuberculosis bacilli (MTB) [4]. The sensitivity of direct AFB smear in the CSF is only 5-10% and culture of MTB takes 4-6 weeks. Recently it has been
reported that from higher amount of CSF the presence of acid–Fast bacilli can be detected by staining in 50% of TBM cases but the amount of sample required is very high [5].

The clinical as well as the CSF features of TBM are helpful indicator for the diagnosis of TBM and differentiate with other infectious and non-infectious disorders [6-7], but clinicians often have difficulty in differential diagnosis of TBM from partially treated case of pyogenic meningitis (PTPM). The biochemical and pathological analysis of CSF and clinical picture in TBM can often be similar to that of PTPM, which results in frequent diagnostic confusion.

In earlier study we have reported the presence of diagnostic 30 Kd protein antigen in CSF of confirmed and suspected cases of TBM patients and immunological method such as antibody capturing ELISA have been devised for the diagnosis of TBM. [8,9]. However the sensitivity and specificity of the method is sometime is debatable in differential diagnosis of TBM from PTPM. Cellular immune function is characterized by the existence of various types of lymphoid cells. Immunological activities for thymus derived (T) and bone marrow derived (B) lymphocytes have been established. B-lymphocytes take part in the production of Humoural mediated immunity, in addition are important in the regulation of the immune response. Because of the important
functions of B cells in the production of immunity, it is of interest to study these cells in response to our 30 Kd protein antigen in tuberculous meningitis and pyogenic meningitis cases.

On the above principle a Cell Enzyme Linked Immunosorbent assay (Cell ELISA) method has been developed to study the response of B cells derived from TBM and PTPM cases by challenging with 30Kd protein antigen. It is of interest to see whether this method is useful to avoid diagnostic confusion between TBM and PTPM patients. By using Cell ELISA the above problem might be circumvented and sensitivity and specificity might be enhanced by evaluating the antibody response at a cellular level. The present study evaluates the antibody response to 30 Kd protein antigen in CSF of TBM/PTPM patients by Cell ELISA.

**Subjects and Methods**

**Patients:** CSF samples were collected from 24 individuals having signs and symptoms of chronic meningitis hospitalized at Central India Institute of Medical Sciences (CIIMS), Nagpur, India who had a diagnostic L.P. Routine investigations for CSF such as cell count, total and differential cell count and smears for grams, India ink, acid-fast bacilli staining.
Collection of samples:
Approximately 2-5 ml of CSF was obtained from each patients. A part was used for routine biochemical and pathological analysis and one ml was used for the cell ELISA study.

1. TBM: 12 patients were diagnosed as having TBM on the basis of following:
   Group A – TBM proved by bacteriological culture and AFB staining (2 cases).
   Group B – TBM strongly suggestive on biochemical and pathological analysis of CSF and clinical profiles. All these patients responded to anti-TB medication (10 cases).

2. PTPM: -12 patients were diagnosed as cases of pyogenic meningitis. These patients responded to treatment for pyogenic meningitis with broad-spectrum antibiotics.

3. Control group: The peripheral blood samples from six healthy volunteers were also analyzed and included as negative control.

Laboratory studies:
Sodium dodecyl sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE):
CSF samples obtained from confirmed and suspected cases of TBM were subjected to SDS-PAGE. SDS-PAGE was performed with vertical slab gel electrophoresis system (BROVIGA, INDIA) by using standard Laemmli method
(10). 4% stacking gel and 10% running gel were used. The electrophoresis was carried out at 250 volts at 50 mAmps. The gel were developed by staining with coomassie brilliant blue GR-250 and the patterns were studied. The band size (molecular weight) was measured by comparing with molecular weight markers (Geni, Banglore, India) run alongside the sample.

**Preparation of antigen (30Kdpa):** After the separation of protein from CSF of confirmed TBM patients by SDS-PAGE, the 30 Kd protein bands was sliced from the gel and preequilibrated in the elution buffer (0.15M PBS, pH-7.4). The gel was electroeluted in a whole gel eluter system (BIOTECH, INDIA) for 90min, at 30 volts (11), and harvested from the unit and dialyzed against PBS and the protein content was measured by Bio Lab KIT. The purity of protein was checked by running Native PAGE and was used in the study for the evaluation of antibody response of B cells derived from CSF of TBM and PTPM cases.

**Preparation of CSF Cells:** One ml of CSF samples collected from both type of patients (Tuberculous meningitis/partially treated pyogenic meningitis) was centrifuged at 400 rpm for about 20 min. The supernatant was discarded and cell pellet was washed twice with PBS and then diluted in RPMI 11640 tissue culture medium containing 10% of fetal calf serum.

**Preparation of Blood Cells:** - Heparinized blood samples were obtained from six healthy volunteers. Peripheral blood mononuclear cells (PBMC) were
isolated from heparnized blood by standard Ficoll-Hypaque gradient centrifugation. The PBMC were dissolved in PBS and centrifuged at 400 rpm for about 15-20 min, PBMC were diluted in RPMI 11640 tissue culture medium containing 10% of Fetal Calf Serum.

**Cell ELISA:** 96 well ELISA plates with a flat bottom were coated with 10ugm of 30Kdpa /ml diluted in phosphate buffer saline (PBS) pH 7.2. After overnight incubation the plates were washed with PBS and then coated with 5% BSA-PBS for 4 hrs. The plates were again washed five times with the PBS. 200 ul of the cell preparation derived from CSF of Patients with TBM and PTPM was then added in the wells and coded. Each sample was prepared in duplicate. Plates were kept overnight at 37°C in 5% CO₂ in a carbon dioxide incubator. Next day plates were washed with PBS and Horseradish peroxidase (HRPO) conjugated rabbit anti human IgG (1:10,000) was then added to the plate. After 2 hrs of incubation at 37°C the plates were washed again with the PBS and 100 ul of TMB/H₂O₂ was added. The TMB/H₂O₂ act as substrate for the HRPO enzyme. After 15 minutes of incubation stop solution (2.5 N Sulphuric acid) was added and the plates were then read with an ELISA reader at 450nm (12).

**Results**
The CSF finding profile of TBM and PTPM patients are presented in Table-1, table-1 and table-2 respectively. Out of the 12 patients of PTPM in 2 cases microorganism were seen and cultured (gm+ve cocci I one case and gm-ve bacillus in the another case). Among the 12 patients who fulfilled the criteria for TBM, 2 were definite cases of TBM on AFB positive staining which were also confirmed on culture and were considered as confirmed cases of TBM and 10 were suspected/probable cases of TBM. A brief perusal of the Figure 2 indicates the presence of 30 Kd protein band in CSF of confirmed and probable cases of TBM. This band was markedly absent in the PTPM.

The ELISA absorbance value of IgG to 30 Kd protein antigen in CSF of TBM and PTPM patients are presented in Table-3. Out of 12 patients of TBM in 11 cases high titer of IgG antibody production against 30Kd protein antigen were observed however in pyogenic meningitis the titer was very low as compared to the cases of TBM patients in 48 hrs. IgG antibody production, expressed as ELISA absorbance value ranged from 0.8 to 2.0 for Cells derived from CSF of TBM patients and from 0.05 to 0.38 for cells derived from CSF of pyogenic meningitis cases. The sensitivity and specificity of IgG antibodies for the diagnosis of TBM patients by cell ELISA was 92 % and 93% respectively. However the sensitivity and specificity in two confirmed cases of TBM was
100%. No IgG antibody production to 30 Kd protein antigen was produced by peripheral blood lymphocytes from 6 health blood donors within 48 hrs.

Discussion

During the past decade, several conventional immunoassays such as ELISA, Dot Immunobinding assay, immunoblot assay, and various molecular methods such as Polymerase chain reaction (PCR) have been reported as an adjunct in the diagnosis of TBM [13,14,15]. However all the aforementioned techniques have been reported to have problems in differentiating TBM from PTPM. CSF Total leukocyte counts (TLC), Differential leukocyte counts (DLC), protein and glucose estimation are helpful parameter for diagnosis of TBM and for differentiating other infectious and non infectious neurological disorders but are non-specific and cannot differentiate from PTPM whose organism are not seen and grown. Delay in diagnosis and treatment are regarded as major contributing factors in the high mortality and morbidity. Delay in starting appropriate medication for TBM and PTPM worsens outcome.

In our laboratory studies we have demonstrated by SDS-PAGE, a 30Kd protein antigen marker in CSF of TBM patients. The presence of this marker in CSF of TBM patients consistently would indicate that the selected protein
band (marker) carries the candidate protein marker antigen, which is specific to M.tuberculosis and could be considered as diagnostic marker for TBM. 30 Kd protein antigen was chosen as the antigen for evaluation of the IgG antibody response of B cells derived from CSF of TBM, PTPM and blood samples from six healthy volunteers. A Cell Enzyme Linked Immunosorbant Assay was developed for the quantitative measurement of anti-30Kd protein antigen antibody production by these cells. High titer of IgG antibody production were observed in TBM as compared to the cases of PTPM patients. The cells obtained from CSF of TBM patients give an early response since they are already sensitized against the MTB antigens. However the cells obtained from PTPM patients and healthy volunteer when challenged with the 30 Kd protein antigen give a delayed response since they are not sensitized against this antigen. Therefore on time scale an early response indicates TBM.

The Cell ELISA has been shown to be a sensitive technique for the differential diagnosis of TBM from PTPM. This method involves the demonstration of active antibody production by cells, particularly those derived from the affected site [16]. Earlier in our laboratory we have standardized Cell ELISA methodology using standard culture filtrate protein of M.tuberculosis of H37Rv strain received from Colorado State University U.S.A (Data not shown). The
only limitation of this study is time period for the outcome of the results, which takes 24-30 hours, but this is the only sensitive method, which can discriminate the TBM from PTPB where other rapid diagnostic method have failed.

The sensitivity and specificity of IgG antibody for the differential diagnosis of TBM from PTPM using 30 Kd protein antigen by Cell ELISA was 92% and 93% respectively. The sensitivity in the two confirmed cases were 100%. We have demonstrated that antibody production against 30 Kd protein antigen is higher in CSF of patients with TBM than in those from Partially treated pyogenic meningitis (PTPM).

Various methods were developed in our laboratory for the diagnosis of TBM showing high specificity and sensitivity but in few cases false positive results were often observed in case of pyogenic meningitis especially in partially treated pyogenic meningitis cases [17,18]. The Cell ELISA method developed in our laboratory using 30 Kd protein antigen marker will be very useful in such circumstances and gives confidence to the treating physician to differentiate TBM cases from cases of PTPM.

The other advantage of the Cell ELISA method for the diagnosis of TBM is based on the assumption of local synthesis of humoral antibody against MTB antigen. Various workers showed that CSF derived cells had a significantly
higher proliferative response to Purified protein derivative (PPD) in patients with TBM suggesting an intrathecal immune response [11,19].

In summary, the data of present study highlights three important relevant observations. Firstly Cell ELISA method is a promising method for differentiating TBM from PTPM using 30 Kd protein antigens. Secondly the method of challenging the B-lymphocytes from the CSF of suspected TBM with 30 Kd protein antigen can be helpful in confirming the diagnosis of TBM. Thirdly the assays allows several samples to be analyzed simultaneously. Hence we advocate that this assay is very useful in differential diagnosis of TBM from PTPM.

**Conclusion**

The presence of 30 Kd protein antigen in CSF of TBM patients indicates that this protein carries the candidate protein marker antigen, which is specific to M.tuberculosis. We have demonstrated that by using Cell ELISA method one can differentiate TBM from PTPM patients and gives confidence to the neurophysician for diagnosis of TBM. In addition our data provide new information about the immune response of CSF cells. The magnitude of the response of CSF lymphocytes from patients with TBM to 30 Kd protein antigen appeared specific. It is possible, therefore that a response of this degree may be of value in the early diagnosis of TBM.
Competing Interest statement

The authors declare that they have no competing financial interests.

Author’s contribution

RSK carried out study design, data collection, statistical analysis, data interpretation, literature search, and manuscript preparation, NPA, RPK, and RMS assisted in data analysis collection, NHK assisted in data collection, statistical analysis, data interpretation, HJP participated in the preparation of manuscript, data interpretation and study design, GMT provided his assistance in preparation of manuscript, data interpretation, study design and funds collection and HFD supervised the study design, statistical analysis, data interpretation manuscript preparation and literature search.

Reference:


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15. Katti MK. Assessment of antibody responses to antigens of mycobacterium tuberculosis and Cysticercus celluloseae in cerebrospinal fluid of chronic meningitis patients for definitive diagnosis as TBM/NCC by passive


### Table-1.

Presentation of CSF findings of tuberculous meningitis (TBM)

<table>
<thead>
<tr>
<th>S.no</th>
<th>Age/sex</th>
<th>CSF Findings</th>
<th>Glucose</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TLC#</td>
<td>Lymphocytes (%)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>39/m</td>
<td>200</td>
<td>96</td>
<td>38</td>
</tr>
<tr>
<td>2</td>
<td>30/m</td>
<td>30</td>
<td>75</td>
<td>31</td>
</tr>
<tr>
<td>3</td>
<td>68/m</td>
<td>30</td>
<td>65</td>
<td>42</td>
</tr>
<tr>
<td>4</td>
<td>20/m</td>
<td>150</td>
<td>99</td>
<td>38</td>
</tr>
<tr>
<td>5</td>
<td>8/m</td>
<td>70</td>
<td>96</td>
<td>16</td>
</tr>
<tr>
<td>6</td>
<td>40/m</td>
<td>60</td>
<td>17</td>
<td>29</td>
</tr>
<tr>
<td>7</td>
<td>8/m</td>
<td>90</td>
<td>86</td>
<td>21</td>
</tr>
<tr>
<td>8</td>
<td>48/m</td>
<td>05</td>
<td>98</td>
<td>31</td>
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<tr>
<td>9</td>
<td>16/f</td>
<td>400</td>
<td>69</td>
<td>44</td>
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<tr>
<td>10</td>
<td>38/m</td>
<td>70</td>
<td>98</td>
<td>45</td>
</tr>
<tr>
<td>11</td>
<td>50/f</td>
<td>03</td>
<td>98</td>
<td>27</td>
</tr>
<tr>
<td>12</td>
<td>17/f</td>
<td>270</td>
<td>69</td>
<td>29</td>
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</tbody>
</table>

* #Cu.mm (mgm/dl)
Table-2

Presentation of CSF findings of Partially treated Pyogenic Meningitis (PTPM)

<table>
<thead>
<tr>
<th>S.no</th>
<th>Age/sex</th>
<th>CSF Findings</th>
</tr>
</thead>
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<td></td>
<td></td>
<td>TLC#</td>
</tr>
<tr>
<td>1</td>
<td>65/m</td>
<td>30</td>
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<tr>
<td>2</td>
<td>35/m</td>
<td>50</td>
</tr>
<tr>
<td>3+</td>
<td>66/m</td>
<td>300</td>
</tr>
<tr>
<td>4</td>
<td>1/m</td>
<td>225</td>
</tr>
<tr>
<td>5</td>
<td>63/f</td>
<td>300</td>
</tr>
<tr>
<td>6</td>
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<tr>
<td>7</td>
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<tr>
<td>8</td>
<td>25/m</td>
<td>50</td>
</tr>
<tr>
<td>9++</td>
<td>25/f</td>
<td>750</td>
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<tr>
<td>10</td>
<td>63/f</td>
<td>200</td>
</tr>
<tr>
<td>11</td>
<td>25/m</td>
<td>50</td>
</tr>
<tr>
<td>12</td>
<td>18/f</td>
<td>45</td>
</tr>
</tbody>
</table>

* (mgm/dl)  # Cu.mm

+ ------ Few gm-ve bacilli seen
++++++Few gram+ve cocci in pairs (non capsulated)
Table-3.

Prevalence of Anti CFP IgG antibodies between TBM and partially treated pyogenic meningitis by Cell ELISA.

<table>
<thead>
<tr>
<th>Subjects</th>
<th>No. Assayed</th>
<th>Positivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tuberculous meningitis</td>
<td>12</td>
<td>11 (91.5%)</td>
</tr>
<tr>
<td>Confirmed on AFB positive And culture</td>
<td>02</td>
<td>2 (100%)</td>
</tr>
<tr>
<td>Suspected TBM</td>
<td>10</td>
<td>9 (90%)</td>
</tr>
<tr>
<td>Pyogenic Meningitis</td>
<td>12</td>
<td>1 (8%)</td>
</tr>
</tbody>
</table>
Figure 1

SDS-PAG Electrophoretogram of CSF from control (lanes B, C, E) and suspected TBM subjects (lane D) along with Molecular weight marker (lane A). The arrow indicates the 30 Kd band (30 Kd protein antigen). Presence of this protein was noted in 84 out of 92 (91%) clinically suspected TBM patients.
B Cell response (IgG reactivity) to 30 KD protein antigen in CSF cells derived from (Tuberculous meningitis) TBM and partially treated pyogenic meningitis (PTPM) and blood cells from control subjects.