MATERIALS AND METHODS
This prospective study was carried out in the Department of Pediatrics, Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER), Pondicherry, India. JIPMER, a referral teaching hospital in southern India is located in an endemic area for tuberculosis.

Study Population, which included cases and controls, were children upto 12 years of age living in Pondicherry and neighbouring state of Tamil Nadu (peri-urban areas). The study was carried out from June 1987 to December 1991.

1.1 PATIENTS

Children (aged 6 months - 12 years) were randomly selected from among the admissions to the Pediatrics Ward of JIPMER Hospital, Pondicherry and recruited into the study prospectively based on the following criteria:

1.2 CRITERIA FOR SELECTING PATIENTS AS TUBERCULOUS MENINGITIS

(i) Clinical features in the form of fever for two weeks or more with loss of playful activity and or irritability, clouding of consciousness, convulsions, neck stiffness, cranial nerve or motor deficits.

(ii) CSF lymphocyte count at admission greater than $10 \times 10^6 /L$. 
(iii) CSF protein level at admission greater than 80 mgm/dl.

(iv) CSF glucose/blood glucose ratio at admission less than 0.5.

(v) Positive intrafamilial adult contact of pulmonary tuberculosis.

(vi) Induration of 10 mm or more on tuberculin testing with 1 TU of Purified Protein Derivative.

(vii) Positive radiological features of primary complex in the chest skiagram in the form of hilar lymphadenopathy and or consolidation with parenchymal infiltrate, segmental collapse, miliary mottling.

Every third child presenting with clinical features mentioned under 1.2 (i) and fulfilling ANY FOUR OF THE SIX remaining criteria mentioned under 1.2 (ii-vii) was selected as a case of TBM for the study. The combined clinical, diagnostic criteria adapted for the study were based on guidelines for diagnosis of tuberculosis in children (STEGEN & KENNETH, 1969).

1.3 CRITERIA FOR SELECTING CONTROLS

(i) Acutely ill children with bacterial meningitis proven by CSF culture.

(ii) BCG vaccinated healthy children admitted for febrile seizures or epilepsy recovering completely within 72 hours and in whom CSF was normal for cells, protein and sugar.

1.4 NUMBER OF STUDY AND CONTROL CHILDREN

The predictive value of a diagnostic test being dependant on the prevalence of the disease this randomised prospective study examined 91 children in the study group and 90 controls.
1.5 STUDY PROTOCOL

A total of 91 children selected as TBM as per criteria outlined under 1.2 and 90 CSF controls (30 culture proven bacterial meningitis and 60 children with febrile seizures/seizure disorders) were studied.

On admission, a detailed history of the presenting complaints, the duration of illness, recent past history of measles in the child and presence of household tuberculosis contacts were obtained from the parent or caretaker. The vaccination status of the child was confirmed by the presence or absence of BCG scar in the child. Nutritional status of the children was assessed by weight in kg / height in cm² (Quetlet Index) An index of more than 0.15 indicated normal nutrition in the child.

Clinical examination for the level of consciousness and extent of neurological deficits was done. The funduscopy changes were noted and confirmed by Ophthalmologist. The observations were recorded on a standard proforma (APPENDIX-II).

The severity of the disease (TBM) at admission was classified in accordance with the British Medical Research Council Report of 1948.

Stage I  Patients fully conscious with no definite signs.
Stage II Consciousness clouded and or cranial nerve lesions, hemiparesis
Stage III Patients comatosed with opisthotonus, decerebrate rigidity, involuntary movements.
Supportive evidence for tuberculosis was sought through the study of chest X-ray obtained in all cases at admission.

Tuberculin test was carried out with Purified Protein Derivative 1 TU in strength. The extent of induration was read in millimetres after 48 hours in all the cases.

CSF was obtained by lumbar puncture at admission in all the cases and controls. The samples were coded independantly.

CSF protein, glucose and blood glucose levels in all the cases were estimated by standard biochemical methods in the Department of Biochemistry. CSF cellular response (Number and type of cells) was studied (Improved Neubauer Chamber).

The pertinent clinical and preliminary data on CSF at admission were valuated independantly by another consultant and appropriate anti-tuberculous treatment started. The survivors were followed up for periods varying from 8 months - 6 years.

1.6 LABORATORY METHODS

Coded CSF samples obtained at the time of admission from the 91 cases were tested as follows.

(1) CSF cell count and morphology by improved Neubauer chamber.
(ii) In select cases with mixed cellular response (Polymorphs + Lymphocytes) mononuclear cells, the CSF cytosediment was stained by May-Grunwald Giemsa method. 100 cells were differentiated. (APPENDIX-VI).

(iii) Smear examination of the CSF samples was carried out by conventional Ziehl-Neelsen procedure (APPENDIX-IIb).

iv) CSF samples were stored in aliquots at -20°C to avoid multiple freeze-thaw cycles. The simultaneous detection of 17-kDa antigen and its corresponding antibody in CSF was done as per methods described in section on Immunological Methods 2.1.

Coded CSF samples were cultured for \textit{M. tuberculosis}. In brief centrifugation of CSF specimens at 2000 rpm was carried out and the sediment inoculated in duplicate slopes of Lowenstein-Jensen medium for mycobacterial culture at 37°C for 6-8 weeks with weekly observation for growth. Identification of the mycobacterial isolate was done according to KUBICA (1973). Help was extended at National Tuberculosis Institute (NTI), Bangalore for CSF mycobacterial cultures.

1.7 STUDY OF CONTROLS

Controls were children admitted during the study period for non-tuberculous CNS disorders from whom CSF could be obtained for analysis. They included children acutely ill with bacterial (pyogenic)
meningitis in whom CSF culture was positive (n = 30) and children with febrile seizures / epilepsy whose CSF was normal (n = 60).

CSF obtained from these controls selected randomly at admission were coded and stored at -20°C till assayed for antigen and antibody.

Clinical evaluation of cases and controls, CSF analysis viz., cell counts, smear examination and ELISA tests for 17-kDa antigen and antibody on all the coded CSF samples were carried out for the study.

2. IMMUNOLOGICAL METHODS

2.1 Bacterial Strain

*M. tuberculosis* (Strain H37 RV) was obtained from National Tuberculosis Institute, Bangalore. The standard reference strain of H37 RV used -(ATCC strain 35801 which is TMC 107 strain Erdman of H37 RV) - were fresh patient's isolates. This strain was used to identify and characterise the 17 KDa protein.

2.2 Purification of 17 KDa Protein

*M. tuberculosis* sonicates are rich in lipids and form intermolecular complexes. Consequently, individual proteins are difficult to purify using conventional methods of gel or affinity
chromatography. Thus in this study a method of extracting 17 KDa protein from polyacrylamide gel was used to purify the protein.

*M. tuberculosis* grown in Dubos broth for 2 weeks was harvested in log phase and washed thrice with saline. The suspension of bacilli in saline was ultrasonically disrupted using ultrasonic disrupter and the sonicate was kept frozen at -70°C. The whole cell sonicate was then loaded on to a semipreparative SDS-PAGE gel (Sodium Dodecyl Polyacrylamide Gel) (APPENDIX-III) and separated in a 12.5% gel. Electrophoresed gels were stained with Coomassie brilliant blue dye and protein bands visualised. 17 KDa antigen was a prominent band under these conditions (Fig. 1).

The gel band containing this antigen was then cut out. The protein was electroeluted from the gel by dialysis for 36 hrs against ammonium bicarbonate buffer containing 1% SDS. The eluted protein was then dried and extracted with cold acetone to remove trace SDS. The fractionated and purified protein showed a pure 17 KDa band on analytical SDS-PAGE (HUNKA PILLER & LUJAN, 1986). The purified protein was then reconstituted in saline and frozen in 100 ug aliquots at -70°C until use.

2.3 Specificity of 17 KDa Protein

The 17 KDa protein was used for immunisation of rabbits and a monospecific antiserum was raised as per standard methods
(WILLIAM & CHASE, 1967). Analysis of sonicates of many mycobacterial species using the Western blot method (MORIERTY, 1984) showed a single 17 kDa band only in M. tuberculosis but not in atypical mycobacteria like M. kansasii, M. avium, M. smegmatis and M. scrofulaceum. The 17 kDa protein which was thus found specific for M. tuberculosis was seen in preliminary studies to react with high specificity against sera from human tuberculosis patients but not with healthy control sera. It was thus selected for analysis of CSF in this study. Using this new M. tuberculosis specific 17 kDa protein, identified and standardised in 1987, CSF ELISA titrations were undertaken in this study. (APPENDIX-IV).

2.4 HRP Labelled Specific Antibody to 17 kDa Protein

The mono-specific serum was precipitated with 18% sodium sulfate to obtain an IgG fraction which was extensively dialysed and stored at -20°C. One aliquot of 10 mg of IgG antibody was labelled with horse radish peroxidase (HRP, Sigma, USA) according to standard methods (WILLIAM & CHASE, 1967) and stored at -20°C in 10% bovine serum albumin (BSA) in phosphate buffered saline (PBS), pH 7.2. The unlabelled antibody was stored as such at -20°C.
2.5 Antibody Detection Assay Based on ELISA

The ELISA procedure was developed as per the standard methods. Micro ELISA (Dynatech, Singapore) plates were coated with a 10 ug/ml solution of the 17 KDa antigen in PBS (100 ul/well) for 24 hrs at room temperature. Wells were blocked with 5% BSA in PBS and washed thrice with PBSt buffer (PBS with 0.2% tween-20). CSF samples were diluted in ¼ in PBSt containing 1% BSA (diluent) and dispensed to wells in duplicate at 100 ul/well. After incubation at room temperature for 2 hrs, wells were washed thrice with PBSt and added with HRP labelled anti-human Ig-polyvalent conjugate (Sigma, USA) diluted to 1/1000 in diluent. Plates were incubated further for 2 hrs and after another step of washing, O-phenylene diamine dihydrochloride substrate in phosphate-citrate buffer, pH 5.0 was added at 100 ul/well. The colour reaction developed in the dark was terminated after 15 min by addition of 3N sulfuric acid and the wells were read at 492 nm using a Dynatech micro ELISA reader. The optical densities of duplicate wells were taken for mean value representing the OD of the particular sample.

For interpretation of cut-off levels of OD, 25 samples of CSF were obtained from healthy adults or children during spinal anaesthesia. They were titrated against the 17 KDa antigen at ¼ dilution as above. The ODs were less than 0.1 in all the samples. Hence a cut-off OD of > 0.10 (which was mean + 2SD of OD from
25 healthy samples) or more was taken to indicate positive antibody binding to 17 KDa antigen. Substrate blank and enzyme labelled controls were always below 0.10.

2.6 Antigen Detection assay Based on Sandwich ELISA

Micro ELISA plates were coated with 10 μg/ml of rabbit monoclonal IgG antibody to 17 KDa (raised as described above) in PBS, pH 7.2 for 24 hr at 4°C. Plates were blocked with 5% BSA in PBS for 2 hrs and washed thrice with PBSt. CSF samples were dispensed at 100 ul per well in duplicates to antibody coated plates and incubated at room temperature for 2 hrs to allow capture of 17 KDa antigen by antibody from CSF. Plates were then washed thrice with PBSt and added with 100 ul per well of HRP labelled anti-17KDa antibody (10 μg/ml) and incubated for 1 hr. After another step of washing, plates were dispensed with OPD substrate and read for OD as above.

To determine cut-off ODs 25 samples of CSF from controls (collected as for antibody ELISA) were titrated and the mean OD was determined for 25 samples. The mean + 2SD of this OD was found to be < 0.1. Thus a cut-off of > 0.1 was used for antigen detection ELISA.

2.7 Immune Complex Assay Among CSF with Proven TBM

CSF samples from proven TBM (Group n_a) were treated with 7.5% polyethylene glycol solution and immune complexes were
precipitated at 4°C for 18 hrs. The immune complex precipitative was then dissolved in PBS, pH 7.2, heated at 56°C for 30 min and then analysed for antibody and antigen at 1/2 dilution for 17 kDa antibody and antigen as described above. Alternatively immune complexes were treated with glycine-HCl buffer, pH 2.8 for 15 min, neutralised with PBS and evaluated immediately in ELISA for antigen and antibody.

Technical, material facilities were provided in the laboratory of Dr. C. Jagannath (National Institute of Communicable Disease unit, National Tuberculosis Institute Campus, Bangalore) so that CSF ELISA titrations could be carried out for the study by the candidate.

3. ANALYSIS

The results of clinical evaluation, CSF analysis including cultures, 17 kDa antigen, antibody assays were decoded at the time of analysis in respect of the 91 cases (N) and 90 controls (C) studied. Based on the presence or absence of *M. tuberculosis* in CSF culture, the cases (N) were grouped as (i) Proven TBM (N_a) (ii) Suspect TBM (N_a) respectively.

The controls (C) were grouped as (i) culture proven bacterial meningitis (NC_1) and (ii) seizure disorders (NC_2).
The positivity of CSF 17 KDa antigen, antibody assays in the various groups \( (N_a, N_b, N_{C_1}, N_{C_2}) \) at admission was analysed.

Sensitivity, specificity of antigen, antibody assays were studied and compared with conventional tests like CSF cytology, biochemistry and indirect evidences like tuberculin test, chest X-ray.

The presence of 17 KDa antigen alone or antibody alone or both antigen and antibody in CSF at admission were analysed in relation to the stage of TBM.

4. STATISTICAL METHODS

4.1 Criteria for Assessment of the Test (FLETCHER, 1982)

a) Sensitivity of the diagnostic parameter was calculated by the proportion of children with TBM found positive by the test \( \frac{a}{a+c} \)

\( a = \) True positive
\( c = \) False negative

b) Specificity was calculated by the proportion of children without the disease found negative by the test \( \frac{d}{b+d} \)

\( d = \) True negative
\( b = \) False positive
c) **Positive Predictive Value** of each diagnostic parameter was defined as the number of children with TBM who are positive by the test divided by the total number of patients with the positive test \( \frac{a}{a+b} \).

d) **Efficiency or Accuracy** was calculated by the number of patients correctly picked up by the test divided by the total number of patients \( \frac{a + d}{a+b+c+d} \).

### 4.2 Tests of Significance

Analysis of the data was done with the SPSS/PC+ version 4.0 statistical package.

* **t test** (t independant sample test) was applied for difference between the means of age and weight in the study group and controls.

* Pearson Chi-square test for categorical variables was used to find out the association between variables.

### 4.3 Correlation Analysis

This was also applied to observe the linear association between two variables (antigen, antibody variables with respect to different stages).

**ANOVA** (Analysis of Variance) was conducted to see the difference between various means with respect to different factors (stage, antigen, antibody positivity).