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Confirmation of antibody response to Mycobacterium tuberculosis and Cysticercus cellulosae in cerebrospinal fluid by western blotting

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ABSTRACT

In India TB and NCC are the predominant endemic CNS infections with high mortality and morbidity. The clinical presentation usually overlap with each other and other CNS infections, causing dilemma in diagnosis. The study aims to assess significance of western blot of antibody response to both antigens of MTB and Cysticercus cellulosae. We prospectively evaluated 19 CSF samples showing reactivity by ELISA for both MTSE and Cysticercal antigens. Western blot was utilized for confirmation of the immune response. Out of the 19 CSF samples, 13 were reactive and 6 were non- reactive for MTSE antigens by western blot. Positive samples showed reactivity to one or more region at 14 kDa, 18-25 kDa, 30-40 kDa and 67 kDa. All the 19 samples were non-reactive for Cysticercal antigens by western blot although they showed positivity by ELISA. Four of the patients had past history of tuberculosis and one patient had contact with TB case. HIV infection was present in three patients. Low CD4 count was seen in one patient. The study reveals that ELISA can be used as screening test and western blot would serve as a confirmatory test for both CNS TB and NCC.

1. Introduction

The chronic infection of central nervous system (CNS) can be caused by viruses, bacteria, fungi and parasites [1]. India is endemic to many diseases, Tuberculosis (TB) and Neurocysticercosis(NCC) are predominant among them [2]. Both of these diseases have certain common clinical manifestations and radiological findings, hence there is dilemma in diagnosis [3].

Tuberculosis (TB) of central nervous system has varied clinical manifestations like meningitis, vasculopathy, encephalopathy, brain abscess, Pott's spine and many others. Tuberculous meningitis (TBM) is the most severe form of all the CNS manifestations. Early diagnosis, prompt treatment will help in

overcoming the disease [4], [5]. In the year 2019, there were 10 million new cases of TB worldwide, one million (10%) among the children and 1.5 million deaths [6]. Prevalence of TBM in developed countries is 5 % and in developing countries 10 % respectively [4], [5], [7]. Diagnosis of the TBM is challenging, though various tests are available. *Mycobacterium tuberculosis* (MTB) was the leading single most common organism causing death worldwide in the same year [7]. MTB is considered as the predominant causative agent of death even among HIV (Human immunodeficiency virus) patients [8].

The global prevalence of NCC is 2.5-8.30 million [9]. Cysticercal larvae can develop in muscles, eye, skin and CNS. Cyst in CNS can present symptoms such as head ache, convulsions and epileptic seizures, which at times mimics TBM [10]. NCC accounts for 30% of all cases of treatable epilepsy in endemic region [8], [10], [11], [12]. People living in under privileged regions with unavailability of proper sanitation has risk of faeco-oral contamination of *Taenia solium* egg [11- 13]. World Health Organization in the year 2010 recognized NCC as a neglected tropical disease [9].

In the department of Neuromicrobiology, NIMHANS, cerebrospinal fluid (CSF) of patients attending neurology service with clinical diagnosis of chronic meningitis are subjected to analysis, which comprises of CSF cell count and typing, India ink preparation, ELISA(enzyme linked immunosorbent assay) for detection of anti TB and anti NCC antibodies, VDRL (venereal disease research laboratory) test, fungal culture and Mycobacterial culture.

At times we have found ELISA test showing reactivity for both TB and NCC with similar OD values, which would be considered positive if tested for single infection. In these situations, there is dilemma with laboratory diagnosis. The following study was undertaken for confirmation of the infection by western blot (WB) analysis of CSF samples which were reactive for both anti TB and anti NCC antibodies by ELISA.

2. MATERIALS AND METHODS

2.1 Overview

The present prospective study was carried out in the Department of Neuromicrobiology, NIMHANS, Bangalore, India for one year duration. A total of 19 patients CSF samples showing both anti TB antibodies and anti NCC antibodies by ELISA formed the study group. Mycobacterium tuberculosis sonicate extract (MTSE) and cysticeral antigen were used for ELISA test for detecting anti TB and anti NCC antibodies respectively. The antigens were prepared as per the methods described by [14] (MTSE) and [15] (Cysticercus cellulosae). The CSF samples were subjected for repeat testing by ELISA before subjecting for immunoblot confirmation by western blot technique. Both MTSE and cysticercal antigens were utilized for Western blot testing. Detailed clinical history of the patients was collected from the medical record section. The following study was conducted on the CSF samples left out after performing the routine laboratory examination of chronic meningitis, so the study was exempted from ethical clearance.

Inclusion criteria:-

1. CSF samples positive for both anti-TB and anti-NCC antibodies by ELISA.

Exclusion criteria:-

1. CSF samples positive only for anti TB antibody by ELISA.
2. CSF samples positive only for anti NCC antibody by ELISA.
3. CSF samples negative for both anti- TB and anti-NCC antibodies by ELISA.

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Immunological procedure:-

MTSE Antigen preparation [14]-

MTB antigen was prepared as mentioned in [14] article. Irradiated H37RV strain of M. tuberculosis culture is homogenised and centrifuged to get the supernatant rich in protein and polysaccharide. Thus obtained antigen is used for ELISA and Western blot.

Cysticercus cellulosae antigen preparation [15]-

NCC antigen is prepared according to [15] article. The NCC antigen is the homogenised solution obtained by grinding cysticercal cyst with phosphate buffer solution.

Enzyme linked immunosorbent assay [14] -

Coating of the ELISA plate with MTSE and NCC antigen and test procedure was performed as described elsewhere [14]. All test samples were analysed in duplicates.

Western Blot Assay [16]-

The MTSE and Cysticercus cellulosae antigens were separated by using Sodium dodecyl-sulfate polyacrylamide gel electrophoresis technique. Thus obtained antigens were blotted to nitrocellulose paper by electroblotting. The WB procedure was performed by following the procedure mentioned by [16].

3. RESULTS

Out of the 19 CSF samples analysed, 13 CSF samples were reactive to MTSE antigen and all were non-reactive for cysticercal antigens by WB. Table-1 Summarizes the demographic, clinical and diagnostic details. All the samples which were reactive by WB to MTSE antigen showed reactivity to one or more regions at 14 kDa, 18-25 kDa, 30-40 kDa and 67 kDa. Refer figure1 and figure 2 for Western blot analysis of NCC antigen and MTSE antigen for CSF samples respectively.

Ten of the patients had clinical diagnosis of TBM. Samples of patients with clinical diagnosis TBM (10) and brain abscess (1) showed immune reactivity in the above mentioned all 4 regions. Non TB bacterial meningitis (1) and seizure disorder (1) cases showed bands at 30 -40 kDa. Among the patients who were reactive to MTSE antigen, only two of the patients were tuberculosis culture positive. All the patients in our study were adults. Nine (47.36%) patients were between the age group of 31-40 years. (Refer table 1 for Age distribution of the patients). Males were predominant in our study with male to female ration 2.8:1. Males were 73.6% and females were 26% in our study.

Predisposing factors were present six of the patients in our study, all of them were among the WB reactive group. Past history of tuberculosis was seen in four patients and TB contact history was seen in one of the patients. HIV infection was seen in three patients. One patient showed low CD4 count. Presence of single predisposing factor was observed four patients and two predisposing factors in two of the patients respectively. Out of 19 patients analysed for CSF glucose and CSF protein, twelve had hypoglycorrhachia and all 19 patients had high protein respectively. Only seven patients showed lymphocytosis on CSF analysis in our study. Refer Table 2 for CSF Analysis details.

Table 1 Summarizes the demographic, clinical and diagnostic details.

Case no	Age (Y)	Sex	WB MTSE	WB NCC	AFB Culture	Clinical Diagnosis
1	28	M	Pos	Neg	Neg	Seizure disorder
2	36	M	Neg	Neg	Neg	Neuropathy
3	38	F	Pos	Neg	Pos	TBM
4	61	M	Pos	Neg	Neg	Brain abscess

5	28	F	Pos	Neg	Neg	TBM with hydrocephalus
6	19	M	Neg	Neg	Neg	Chronic Meningitis
7	41	F	Pos	Neg	Neg	Bacterial meningitis
8	24	F	Pos	Neg	Neg	Retroviral disease with TBM
9	56	M	Neg	Neg	Neg	Pituitary macroadenoma
10	24	F	Neg	Neg	Neg	Bacterial meningitis
11	45	M	Neg	Neg	Neg	Chronic meningitis
12	40	M	Pos	Neg	Neg	TBM
13	41	M	Pos	Neg	Pos	TBM
14	33	M	Pos	Neg	Neg	TBM
15	62	M	Neg	Neg	Neg	Cryptococcal meningitis
16	55	M	Pos	Neg	Neg	TBM with Retropositive
17	24	M	Pos	Neg	Neg	TBM
18	45	M	Pos	Neg	Neg	TBM
19	38	M	Pos	Neg	Neg	TBM

Keys for the table

1. No.- Number
2. Y -Years
3. WB- Western blot
4. MTSE- Mycobacterium tuberculosis sonicate extract
5. NCC- Neurocysticercal antigen extract
6. Pos -Positive
7. Neg -Negative
8. TBM- Tuberculous meningitis

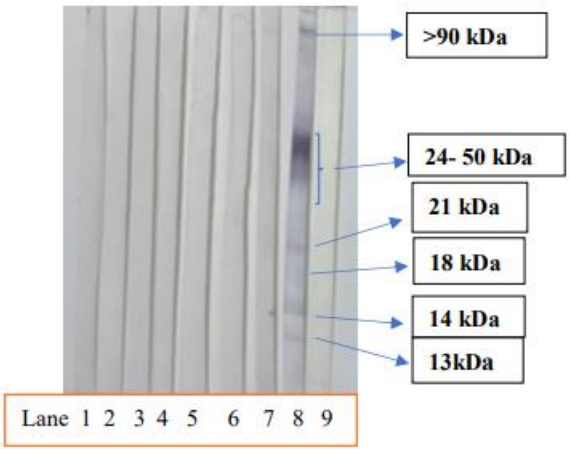

Fig 1. Lane 1-9 Western blot analysis for NCC antigen for CSF samples

Lane 1-7 Test samples, Lane 8 Positive control showing (6 important reactive bands), Lane 9 Negative control

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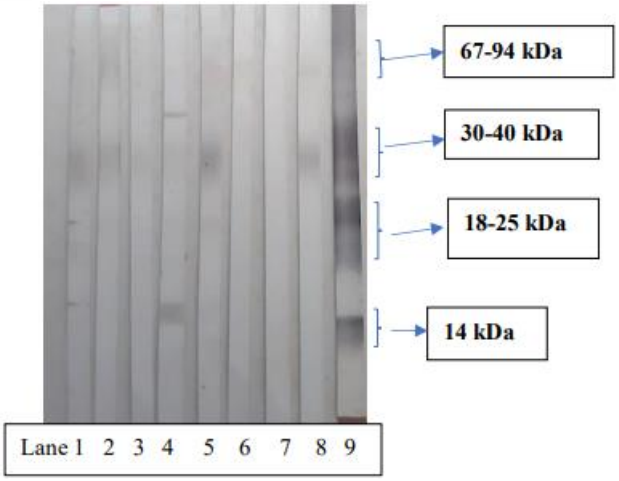

Fig 2. Lane 1-9 Western blot analysis for MTSE antigen for CSF samples, Lane 1-6 and 8 Test sample, Lane 7 Negative Control, Lane 9 Positive Control (4 important bands).

TABLE 2 CSF Analysis Details

Case no	**Cell count**	**Glucose (mg/dl)**	**Protein (mg/dl)**
1	CC 4, P3, L1	129	780
2	Nil cells	78	66
3	CC 192, L 90%, P 5%, D 5%	16	159
4	CC 120, P10%, D 90%	93	95
5	CC 44, P2,L42	40	77
6	CC 190, P40%, L60%	23	278
7	CC 600, P80%,L15%,D5%	24	784
8	CC 110, L95%, P5%	25	573
9	CC 150, P5%, L95%,	60	505
10	CC 139 P4,L130,D5	8	257
11	CC 640, P70%,L30%	19	248
12	Nil cells	34	600
13	CC 168, P55%,L25%, D-20%	19	470
14	CC 50, L-45, P-5	38	540
15	CC 50 P2,L13,D35	28	183
16	CC not possible as the sample was blood stained	24	717
17	Nil cells	64	623
18	Nil cells	39	4815
19	CC 6, L6	42.9	94.6

Keys to the table

1. no- number
2. CC No- Cell count number, P-Polymorphs, L-Lymphocyte, D- Degenerated cell, RBC – Red blood

cell,

4. DISCUSSION

Diagnosis and management of TBM is a challenging task. Culture and microscopy for MTB bacilli are less sensitive. Laboratory tests which are rapid and sensitive are not available yet. Molecular diagnosis for TBM by Gene Xpert MTB/RIF which uses real time PCR (polymerase chain reaction) to detect MTB and identify drug resistance appears promising [17]. But the sensitivity of Gene-Xpert in TBM diagnosis is only 50-60%, more over it is expensive and less available for the diagnosis in high burden regions. In such situations antibody detection assays can aid in diagnosis [7], [18].

For definitive diagnosis of NCC, histological, fundoscopy findings and radiological evidences are utilized [13]. The non-cystic stage of NCC is asymptomatic and cannot be diagnosed by imaging [19]. Serology, which is directed to the detection of anticysticercal antibodies will be helpful in these cases. Enzyme-linked immunoelectrontransfer blot assay which detects antibody has sensitivity of 98% in cases with more than one viable larval cyst and a specificity of 100% [20].

In the present study the discordance experienced in interpretation of ELISA findings in few of the patients with both anti-TB and anti-NCC antibodies, was overcome by western blot assay.

Thirty one percent of samples which were positive by ELISA were non-reactive by WB for both MTSE and NCC antigens. Immune reactivity at 30-40 kDa region corresponds to lipoarabinomanan (LAM) in all the MSTE reactive cases. Mycobacterial cell wall is made up of polysaccharide LAM, which is a major component constituting 25-40% of it [21]. LAM is a vital virulence factor, detection of antibody against it makes diagnostically important [21], [22].

Anti-NCC antibodies were seen in clinically diagnosed TBM patients and both anti-TB and anti-NCC antibodies among Non TBM/NCC patients in the CSF sample by ELISA.

The possible explanation for this particular phenomenon would be because there is likely hood of some degree of passive binding of CSF components to the ELISA plate. Similar observation is been made by previous studies in our institution [16].

We observed reactive anti-TB antibodies to MTSE antigen by western blot in patients with clinical diagnosis of seizure disorder (1), brain abscess (1) and bacterial meningitis (1). CSF culture was negative for MTB in both brain abscess and bacterial meningitis patients, but still the possibility of tuberculosis would have been present. Mycobacterial culture by both conventional solid medium (Lowenstein Jensen agar) and automated liquid culture (mycobacterium growth indicator tube) was positive only among two patients (10.5%). Both these cases were correlating with clinically diagnosis of TBM. Data from previous studies and our institute suggests CSF culture for MTB is less sensitive (25%-70%) [7], [23]. Our study emphasises the importance of antibody detection tests in such paucibacillary condition. Extrapulmonary samples such as CSF where the Mycobacterial culture sensitivity is very low antibody detection can play vital role. Possible reasoning was not possible in seizure disorder patient.

Thirty percent (3/10) of patients with clinical diagnosis of TBM had HIV Co-infection in our study. For patients infected with MTB, HIV infection is the strongest risk factor for the development of active TB either drug-susceptible or drug-resistant TB [25- 27].

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Among 25% of HIV patients, MTB has become predominant causative agent of death. MTB bacilli which are acquired recently or present latently is stimulated by HIV to manifest with active disease [8].

One patient had low CD4 count in our study. T cells play pivotal role in defense mechanism. Any decline in CD4 + cells, will invite the opportunistic pathogens. The risk of developing TB doubles as the CD 4 + T cell counts reduce lower than 200 cells/µl [8].

Forty percent (4/10) of the patients in our study were either of default pulmonary tuberculosis or TBM on follow up. Contact history to TB patient was seen with one of the patient. About 10% of patients who have pulmonary TB develop CNS disease [7], [14]. Majority of the patients in theour study showed hypoglycorrhachia and high protein. The usual CSF glucose is 60 to 80% of the plasma glucose. Values under 45 mg/dl can usually be considered abnormal; values under 40 mg/dl are almost without exception abnormal [3], [11]. With the values of CSF glucose and protein levels along with cell count, we will only be able to differentiate acute from chronic meningitis cases.

The nonspecific reaction attributed by the ELISA can be overcome by the specific binding by western blotting test. Western blot can be used as the confirmatory test to overcome the discordance results by the ELISA [19].

There is need for low cost and point of care test for CSF samples. Gene X-pert is promising but the availability limited in the high burden countries. Finding from our study shows western blot can be used for early diagnosis of MTB in CSF, if modifications are made, the MTSE antigen can be used for point of care testing also.

5. CONCLUSION

Tubercular meningitis and Neurocysticercosis are the most common chronic meningitis presentation seen in NIMHANS. TBM has varied clinical manifestations. At times it is difficult to diagnose. In such situations patient succumb to illness within an year. Most of the present available diagnostic modalities are less sensitive to diagnose MTB in CSF. In such situation presence of antibody in CSF will help in diagnosis. Even in NCC, immune diagnosis plays a major role. Present study shows that in situations where reactivity for both MTSE and NCC is observed, majority of the cases will be TBM. Most of the TBM cases will have past history of TB. HIV-coinfection to be screened for. It also confirms that CSF samples showing antibody response by ELISA does not necessarily always mean there is a disease entity. There is likely hood of some degree of passive binding of CSF components to the ELISA plate. The study reveals that ELISA can be used for initial screening test and western blot as confirmatory test for both CNS TB and NCC. There is no cross reaction between both the organism.

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