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Immunoconfirmation of central nervous system tuberculosis by blotting: A study of 300 cases

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ABSTRACT

Tuberculous meningitis (TBM) is a serious form of disease of the central nervous system. Early and accurate diagnosis of the disease and effective treatment are key important factors to contain the disease. The disease presents as chronic meningitis where other partners such as fungal meningitis, neurosyphilis, cysticercal meningitis, carcinomatous meningitis and partially treated pyogenic meningitis share a similar clinical picture making the diagnosis complicated. Culturing of the pathogen Mycobacterium *tuberculosis* (MTB) from the cerebrospinal fluid (CSF) sample has shown a poor response. The main immunological method for the immunodiagnosis of TBM is the detection of an antibody response in the CSF. In the present study, total MTB sonicated extract antigen was used for ELISA and Western blot. ELISA shows overall immune response of the test sample, whereas Western blotting reveals the specific reactivity to a particular molecular weight antigen. This would also reveal the immunodominant antigen. A total of 300 CSF samples were analyzed by both ELISA and Western blotting. Of the 240 clinically suspected TBM cases, 111 samples were positive by ELISA and 81 samples by Western blot. A total of 76 CSF samples were positive by both ELISA and Western blot. None of the control samples showed positivity either by ELISA or by Western blot. TBM patients revealed major antibody reactivity to 30–40 kD region, followed by 14 kD region. ELISA is sensitive with mild non-specific binding, but Western blot is specific in detecting the immune response. The findings will be useful in definitive immunodiagnosis of TBM.

Introduction

Tuberculosis (TB) is the leading cause of death among communicable diseases, killing nearly 1.4 million people each year [1]. The clinical presentation of TB ranges from a few foci affecting the upper parts of the lungs to an intense tissue destruction and caseous necrosis that usually disintegrates forming cavity lesions, leading to bacteremia and further to

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Abbreviations: CNS, central nervous system; CSF, cerebrospinal fluid; HRP, Horseradish peroxidase; LJ, Lowenstein–Jensen; MTSE, *Mycobacterium tuberculosis* sonicated extract; TB, Tuberculosis; TBM, tuberculous meningitis.

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central nervous system (CNS) infection. CNS-TB is a serious condition. Major forms of the CNS-TB are tuberculous meningitis (TBM), intra-cranial tuberculomas and spinal tuberculous arachnoiditis [2]. The disease carries a high mortality rate and a distressing level of neurological morbidity. It majorly affects immunocompromised individuals like children and HIV-infected patients, subjects with malnutrition, recent measles-infected children, alcoholics, subjects with malignancies and immunosuppressive drug users [1,3]. A high rate of mortality and/or morbidity in TBM is due to misdiagnosis or late diagnosis and lack of adequate and appropriate treatment [4]. Clinically, TBM is presented with ill health lasting 2–8 weeks prior to the development of meningeal irritation. The classic presentations in adults are mild fever, headache and neck stiffness along with focal neurological deficits, behavioral changes and alterations in consciousness, nausea and vomiting from moderate to more severe. Improper treatment leads to lethargy and agitation and the patient may end up in a coma [5–8]. Due to its being a relatively rare condition and the protean nature of the symptoms, CNS-TB remains a formidable diagnostic challenge to both clinicians and laboratory scientists. In all forms of CNS-TB, brain damage is reported to be from an immune-mediated inflammatory response, which causes vasculitis, obstructive hydrocephalus and cranial nerve palsy [9,10]. The close differential diagnosis can be partially treated pyogenic meningitis, fungal meningitis and at times carcinomatous meningitis. In the case of TBM, CSF values become more abnormal over time, with increasing leukocyte counts and protein levels and decreasing glucose levels [11]. Microbiological detection of MTB in CSF by acid-fast staining (Ziehl-Neelsen) and culturing is very poor. CSF culture sensitivity reported is only up to 15–20% [12,13], although a marginal increase in positivity is noticed with a high volume of CSF samples. Recently, different types of liquid culture isolation techniques are also in use for the rapid diagnosis of TBM, apart from Lowenstein–Jensen (LJ) medium, the conventional solid medium.

To overcome the diagnostic lacunae, a number of immunological and molecular methods have been explored. The nucleic acid amplification tests developed for the diagnosis of the disease were also not fully satisfactory due to the fact that there is less bacterial load in CSF samples [14,15]. Another novel diagnostic method such as CSF interferon-gamma release assay by the mycobacterial antigen stimulation is being used as a biomarker in the diagnosis. However, this test requires further evaluation and validation to establish its utility as a diagnostic tool [16].

In view of the limitations of the above techniques, re-evaluation of immunodiagnostic methods was considered. Efforts were made to detect specific antibodies in CSF, which can act as potential immune markers for the diagnosis of TBM. Detection of mycobacterial antigens or antibodies in the CSF is specific, simple, inexpensive, and requires minimal training compared with other methods. The antibody-based ELISA technique has been employed over the period for the diagnosis of TBM [17]. A series of different antigens were also used for TBM diagnosis, such as lipoarabinomannan, early secretory antigen 6 (ESAT-6), A-60, Ag-5, 14 kD and MTB culture filtrate antigens [18–20]. However, all reports have a wide range of sensitivity and specificity. In an attempt to improve the sensitivity of the CSF ELISA for the immunodiagnosis of TBM, in the present study a total mixture of sonicated antigens of MTB are being used. Further, for confirmation of the immune response, Western blotting is performed to analyze individual immune reactivity to isolated antigens on nitrocellulose membrane.

Materials and methods

Samples

A total of 300 CSF samples were analyzed in the present study by ELISA and Western blotting. Informed consent was obtained from subjects before CSF was collected by lumbar puncture. Institutional ethical approval was sought for the study. Of the 300 CSF samples, 240 belonged to chronic meningitis categories which were suspected for TBM. Non-tuberculous cases ($n = 30$) involved Pyogenic meningitis, Cryptococcal meningitis, Neurocysticercosis and Viral meningoencephalitis. Non-diseased controls ($n = 30$) involved subjects undergoing spinal anesthesia for non-neurological causes. The inclusion and exclusion of subject selection were followed as per the criteria of Marais et al. [21].

Suspected TBM CSF samples ($n = 240$)

Patients with clinical signs and symptoms of meningitis including one or more of the following presentations like headache, irritability, vomiting, fever, neck stiffness, convulsions, focal deficits, altered consciousness over a period of one week and with typical radiological images showing meningeal involvement and/or CSF culture positive for AFB were classified as TBM cases.

In the present study, the initial presentation of cases is shown in Table 1. Patients were classified as 'highly probable' TBM, 'probable' TBM, 'possible' TBM and non-TBM. Confirmed TBM is based on a clinical presentation with bacterial isolation from CSF. In the present study, highly probable, probable and possible TBM are altogether grouped as suspected TBM for Western blot. The criteria followed for highly probable TBM ($n = 53$) are clinical diagnosis as TBM, radiological impression as TBM and/or CSF antibody positive by ELISA. Probable TBM ($n = 85$) is considered when clinical diagnosis presents as TBM, and doubtful radiological impression for TBM/antibody positivity by ELISA. Possible TBM ($n = 102$) is clinical suspicion of TBM or radiological impression of TBM or antibody positivity by ELISA.

Non-tuberculous meningitis controls ($n = 30$)

Cases in which an alternative diagnosis is established without a definitive diagnosis of TBM with convincing signs of other disease were categorized as non-tuberculous meningitis cases. Thirty CSF samples were taken as non-tuberculous meningitis cases which included cases with Pyogenic meningitis which were confirmed by isolation of *Diplococcus pneumonia*, Cryptococcal meningitis cases confirmed by isolation of *Cryptococcus neoformans*, and Neurocysticercosis cases confirmed by anticysticercal antibody in the CSF against *Taenia solium* cyst antigen. Viral meningitis cases were

Table 1 – Clinical presentation of 240 cases suspected for tuberculous meningitis.

Clinical presentation	Percentage of patients
Headache, vomiting and fever	33.3
Headache and vomiting	22.2
Vomiting	44.4

confirmed by the presence of antibodies to CNS viruses or positive by PCR.

*Non-infectious diseased controls (**n** = 30)*
Subjects undergoing spinal anesthesia for non-neurological causes like hernia, hydrocele, etc., served as non-diseased controls.

Other materials

MTB (H37Ra) culture, sonicator, other common laboratory reagents, apparatus for Western blot, and ELISA were the other materials used.

Preparation of antigen

MTB (H37Ra) was a kind gift from the National Referral Laboratory JALMA, Agra (India). The organism was grown on LJ media. Later the culture was transferred to 250 ml Sauton's liquid media and incubated at 37 °C with gentle agitation for 6 weeks. The above culture was centrifuged at 10,000 rpm at 4 °C for 30 min to separate culture filtrate. The pellet was inactivated by 5% phenol solution for one hour and re-pelleted. The pellet was re-suspended in PBS, and the suspension was sonicated in an ice bath for 15 min (Sonics vibra cells, U.S.A), followed by centrifugation to get clear sonicated supernatant rich in protein. The protein content was measured/adjusted and used for ELISA and Western blotting.

Indirect ELISA

ELISA was done according to the method described earlier [17]. Briefly, 96 well microtitre plates (Dynatech, U.S.A) were coated with MTB sonicated extract (MTSE) antigen (10 μg/mL) in PBS, 50 μl per well, by overnight incubation at 4 °C in a moist chamber. Unbound antigen was removed by washing with PBST. Nonspecific free binding sites of the wells were blocked with freshly prepared PBST-milk 1% at 37 °C for 2 h. After incubation, the plates were tap dried and stored at −20 °C in dry condition until further use. The CSF samples to be tested were diluted (1:10) in PBST milk 1% and 50 μl of the diluted samples were added to the wells in duplicates. The plate was incubated for 90 min in a moist chamber at 37 °C and then washed with PBST ×5 and tap dried; 50 μl of secondary antibody (anti-human IgG, Dakopatts, Denmark) conjugated with HRP (1:3000 dilutions in PBST-milk) was added to each well and incubated at 37 °C for 60 min. The plates were later washed with PBST and tap dried. Substrate (O-phenylene di-amine dissolved in phosphate citrate buffer with hydrogen peroxide) was added to each well (75 μl) and allowed for color development for 10–15 min. The reaction was arrested by adding 50 μl of 2N H_2SO_4. The readings were taken by ELISA reader (Sunrise-Magellan, Tecan AG, Austria) at 492 nm. The cutoff value was calculated on the basis of the absorbance (OD) value of controls ($n = 60$) by taking mean of it plus three standard deviations (SD).

Western blot

The antigens of MTB sonicated extract (MTSE) were subjected to SDS–PAGE. The antigens were first separated on polyacrylamide gel (12%) in 0.1% SDS, along with the protein marker with constant voltage in a discontinuous buffer system using Hoeffer electrophoresis apparatus (U.S.A.). The antigens thus separated were transferred to the nitrocellulose membrane (0.45 μm pore size, MDI, Ambala, India) by electro blotting (Hoeffer, U.S.A.). The blotted nitrocellulose membrane having MTSE antigen was cut into fine strips of 3 mm breadth (8–10 μg of MTSE/strip) and stored at −20 °C until further use.

The strips were washed with distilled water before use and later incubated with CSF diluted in 1% PBST milk (1:10) at room temperature with gentle overnight shaking. After washing for 5 times with tris-buffered saline (TBS), the strips were incubated with goat anti-human IgG immunoglobulin tagged HRP (Dakopatts, Denmark, 1:1000 in PBST milk) for 3 h at room temperature. After incubation, the strips were washed 5 times with TBS and were then developed with substrate (4-chloronaphthol) and later stored in distilled water. Immune reactivity to MTB antigens was also checked by using monoclonal antibodies obtained from Dr. J. Ivanyi, Wellcome Laboratories (UK).

Results

Anti-mycobacterial antibodies were detected by both ELISA and Western blotting. The cutoff value for ELISA (mean + 3 SD of controls) was around 0.200 OD. Hence, samples showing OD $\geqslant$ 0.200 were taken as positive by ELISA. Samples showing OD between 0.100 and 0.200 were taken as borderline positive (taken as antibody positive for the analysis) and OD < 0.100 was taken as negative by the assay. Among the 240 suspected TBM cases analyzed by ELISA for anti-TB antibodies, 111 (46.25%) cases showed antibody positivity (Table 2). The remaining 129 CSF samples categorized as chronic meningitis were found to be negative for anti-tuberculous antibodies by ELISA. Thirty CSF samples which were categorized as infectious non-tuberculous meningitis and another 30 CSF samples of non-diseased category were all negative for anti-mycobacterial antibodies by ELISA.

Western blot reactivity of TBM-suspected CSF samples ($n = 240$) with MTSE antigen showed 81 cases (33.75%) positive by the assay. The molecular mass of the visible band was calculated by the standard protein marker. The CSF reactivity was mainly noticed in the region of 30–40 kD followed by

Table 2 – Comparison of anti-mycobacterial antibody response in CSF samples by ELISA and Western blotting (WB).

Type of cases (N-number)	ELISA + Ve (%)	WB + Ve (%)	Predominant reaction
TBM suspected (N-240)	111/240 (46.25)	81/240 (33.75)	30–40 kD
Infectious cases other than TBM (N-30)	0/30 (0)	0/30 (0)	None
Non-infectious cases (N-30)	0/30 (0)	0/30 (0)	None

14 kD region (Fig. 1A). The remaining 159 CSF samples suspected for TBM were found to be non-reactive to MTB antigen. Among the non-tuberculous meningitis CSF samples ($n = 30$) and non-diseased CSF controls ($n = 30$), none showed reactivity by Western blot. Western blot reactivity using monoclonal antibodies against lipoarabinomannan (30–40 kD) and 14 kD region revealed strong reactivity to these antigens. However, the reactivity to the 38 kD region by MTB-specific monoclonal antibody TB-72 revealed weak reactivity (Fig. 1B). Comparison of ELISA with Western blotting revealed higher antibody positivity in ELISA compared with Western blotting. It is noticed that 76 CSF of suspected TBM were found to be positive by both ELISA and Western blot for anti-mycobacterial antibody. Five CSF samples suspected as TBM which were found to be borderline positive for anti-mycobacterial antibody by ELISA with the OD between 0.1 and 0.13 were shown reacting by Western blot with prominent band at the 30–40 kD region. Among the 5 samples, 1 was showing reactivity to both the 30–40 kD and 14 kD regions.

Western blot analysis of CSF from clinically confirmed and/or suspected TBM patients revealed a major antibody against mycobacterial antigen with a mass in the 30–40 kD region which corresponds to lipoarabinomannan antigen which is noticed to be highly immunogenic.

In the highly probable category ($n = 53$), 80% of the CSF were positive by ELISA and 62% by Western blot. Among the probable cases ($n = 85$), 50% of the CSF were positive by ELISA and 37% by Western blot. In the possible TBM cases ($n = 102$), 25% and 17% were positive by ELISA and Western blot, respectively (Table 3).

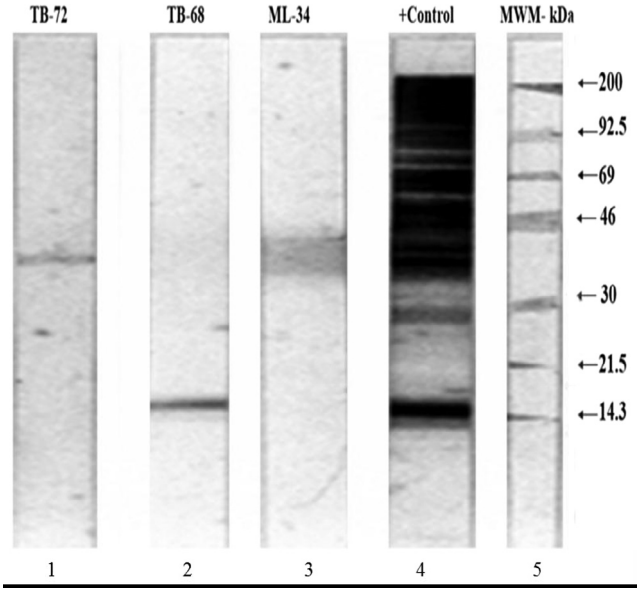

Fig. 1B – Western blot reactivity of monoclonal antibodies to *M. tuberculosis* antigens. Lane 1. *M. tuberculosis* specific monoclonal antibody (TB-72) against 38 kD protein. Lane 2. *M. tuberculosis* quasi specific antibody (TB-68) against 14 kD antigen of *M. tuberculosis*. Lane 3. Monoclonal antibody (ML-34) against mycobacterial common lipoarabinomannan at 30–40 kD region. Lane 4. Anti BCG antibody (polyclonal) reactivity with *M. tuberculosis*. Lane 5. Molecular weight markers.

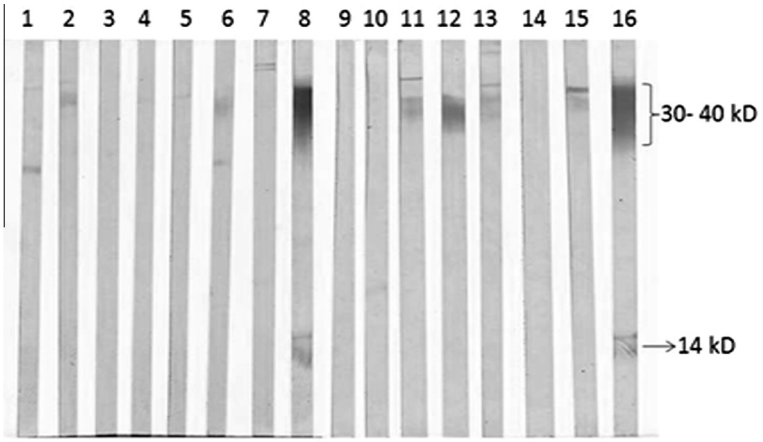

Fig. 1A – Lane 1–16. Western blot analysis for suspected TBM CSF samples ($n = 16$), showing reactivity at 30–40 kD and 14 kD region.

Table 3 – Category wise comparison of ELISA positivity and Western blot reactivity.

Category	No. of patients	Antibody positivity	
		ELISA (%)	Western blot (%)
Highly probable	53	42 (80)	33 (62)
Probable	85	43 (50)	31 (37)
Possible	102	26 (25)	17 (17)

Discussion

TBM is a serious form of TB with high morbidity and mortality. To contain the disease, early diagnosis and effective treatment is essential. The routinely practiced diagnostic techniques include isolation and staining of the causative organism MTB in the clinical specimen. The culture technique, though robust and the gold standard, is less sensitive in CSF and may not help in early diagnosis. According to previous reports from this center, the organism from the CSF could be isolated to the extent of about 15% in the TBM cases in spite of best efforts [12].

Biochemical analysis of CSF in TBM for measuring glucose and protein levels as an indirect indicator of the infection lacks specificity for CNS-TB [22]. Elevated CSF Adenosine deaminase (ADA) activity has been observed in patients with tuberculous effusions. However, it has a low sensitivity (44%), and ADA analysis cannot differentiate TB infection from other bacterial infections [23]. Interferon gamma release assay is another technique in which interferon gamma will be released by the immune cells after stimulation by mycobacterial antigens which can be measured and compared with controls for the diagnosis [24]. However, this test does not effectively distinguish between the present and the past infection. PCR, another molecular technique, is found to be less satisfactory in CSF samples due to low bacillary load and also due to PCR amplification inhibitory components noticed in occasional CSF samples [25]. A recent report by Jongeling and Pisapia [26] revealed the late appearance of molecular markers in TBM which led to the delay in the treatment and ultimately the death of a patient. Hence, there is an urgent need for the rapid diagnostic confirmation of this serious disease. As antibody response to the antigens in CSF will be much quicker compared with the detection of other biomarkers, the antibody response would be a promising early indicator.

Human immune response to MTB varies from individual to individual and population to population and antigen to antigen. In view of this, various workers have used multiple mycobacterial antigens as TB diagnostic markers [27,28]. Cell-mediated immune response also varies with different antigens of MTB in TB patients [29]. In the present study, the total MTSE extract was used as the antigen to have multiple antigenic epitopes for the test in both ELISA and Western blot. Usefulness of the mycobacterial strain H37Ra in preparation of the antigen of diagnostic significance in TB is well established in many laboratories [30]. The present study also reports the use of H37Ra sonicate antigen for the diagnosis of TBM by Western blot. In one of the earlier preliminary studies, the Western blot procedure with H37Rv sonicate antigen was reported [18], and that a vast majority of the proteins were similar in both the strains of MTB (H37Ra and H37Rv). These strains are very close biochemically [31], except that the H37Rv is virulent and may contribute to handling complications.

These results using monoclonal antibodies reveal that the antibody responses are induced to multiple MTB antigens, including the highly immunodominant lipoarabinomannan (30–40 kD). Increased reactivity to 30–40 kD components is also reported in the closely related mycobacterial infection, leprosy [32]. The immunogenic nature of LAM is also reported in TBM [18], and also in the sera of pulmonary TB cases [33,34] and in leprosy [35]. This indicates that the lipoarabinomannan molecule is highly immunogenic in CSF as well as in serum. Further, the antibody response to this polysaccharide antigen is noticed to be disease specific.

It is observed in the present study that few of the CSF samples which were antibody positive by ELISA were non-reactive on Western blot. This could be due to some degree of non-specificity in ELISA. Further, five of the CSF samples which were marginally positive (borderline positive) by ELISA showed reactivity by Western blot, highlighting appropriate reactivity to the presence of antibodies with reasonable sensitivity.

The comparison of both ELISA and Western blot in the present study has highlighted the specific binding in Western blotting, which can be used as an immunoconfirmatory test for TBM. Some degree of non-specificity in ELISA could be attributed to the non-specific binding components in CSF which bind passively to the ELISA plate. Such CSF samples when tested do show non-specific reactivity to any antigen coated or even to a blank ELISA plate itself.

The comparison of ELISA and Western blotting done in the present study signifies the evaluation of the definitive antibody response by Western blotting, whereas in ELISA there is likely to be nonspecific reactions. The present study is the largest in the series to report the comparison of ELISA with Western blot in the CSF of TBM cases. The predominant reactivity to the lipoarabinomannan and 14 kD protein of MTB in CSF needs to be strongly considered while developing any new diagnostic tests for TBM. Further, whenever the clinical suspicion is not strong for TBM and there is antibody positivity by ELISA, it is recommended that Western blot be done for confirming the infection by ruling out the ambiguity.

Disclosure

The authors do not have any commercial or other association which may pose a conflict of interest.

Conflict of interest

We have no conflict of interest to declare.

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