IMMUNODIAGNOSTIC SYSTEM FOR NEUROTUBERCULOSIS - SUITED TO LABORATORIES IN DEVELOPING COUNTRIES

A thesis presented
by

SUMI MARY GEORGE

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in partial fulfillment of the requirements
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Doctor of Philosophy

SREE CHITRA TIRUNAL INSTITUTE
FOR
MEDICAL SCIENCES & TECHNOLOGY

TRIVANDRUM

August, 2001

Certificate

I, **Sumi Mary George** hereby certify that I had personally carried out the work depicted in the thesis entitled **"IMMUNODIAGNOSTIC SYSTEM FOR NEUROTUBERCULOSIS - SUITED TO LABORATORIES IN DEVELOPING COUNTRIES"** except where external help sought and acknowledged.

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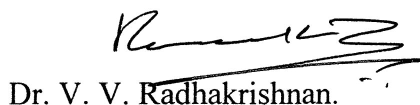

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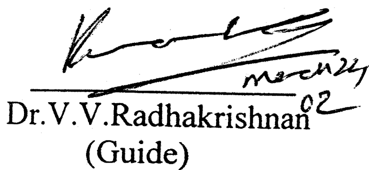

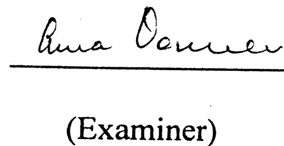

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SYNOPSIS

SYNOPSIS

I. BACKGROUND INFORMATION

Human tuberculosis, caused by acid-fast bacilli *Mycobacterium tuberculosis* continues to remain as one of the major public health problem throughout the world. In developing countries incidence, mortality and morbidity rates have not shown any decline, while in developed countries there is an upward trend, which may be attributed to the increasing prevalence of HIV infection as well as due to the emergence of drug resistant mycobacteria. An estimated one third of the world's population is currently infected with *M tuberculosis.* Tuberculosis hit an all-time high in 1999 with 8 million new cases, 95% of them occurring in developing countries and also caused 2 million deaths[1]. Being a potentially curable disease, there is a need for rapid laboratory diagnosis and appropriate effective antituberculosis chemotherapy.

II. INTRODUCTION

Tuberculous meningitis (TBM): It is one of the common clinical and morphological manifestations of extra-pulmonary tuberculosis. Early confirmative laboratory diagnosis and institution of effective anti-tuberculosis chemotherapy is essential in reducing the mortality rate and neurological sequelae in TBM. In a vast majority of patients, the diagnosis of TBM is usually made on the presenting clinical features such as neck rigidity, positive Kernig's sign and supported by elevated total leukocyte - cell counts, proteins and reduced glucose concentration in the cerebrospinal fluid (CSF) specimens.

An absolute criterion i.e. "Gold standard" for establishing the laboratory diagnosis of TBM depends upon the demonstration of *M tuberculosis* in CSF specimens. Conventional bacteriological method (culture on L-J medium) is cumbersome, time consuming and also lacks sensitivity. Therefore, isolation of *M tuberculosis* from CSF specimen, in patients with TBM by the conventional bacteriological method is often infrequent. There are several factors that could account for the infrequent isolation of *M tuberculosis* in the CSFs of patients with TBM. The CSFs from patients with TBM are usually obtained through the lumbar route and the lumbar CSF contains lower concentration of *M tuberculosis* than ventricular or cisternal CSF[2]. Secondly, tubercle bacilli get embedded in the dense exudates in basal cisternae or basal leptomeninges and the exudates form a barrier, preventing tubercle bacilli from circulating into lumbar CSF. Thirdly and perhaps more importantly, most patients with TBM would have received a course of antituberculosis chemotherapy before being referred to tertiary referral centres for neurological diseases.

The clinical manifestations of TBM are usually classical but atypical manifestations are also equally common. Problems in the diagnosis of TBM arises when

- Clinical features are atypical.
- CSF cytological and biochemical parameters are non-specific.
- CSF smears and culture results are negative.
- Clinical and cytological features of partially treated pyogenic meningitis (PTPM) resemble TBM.

Development of several biochemical[3], immunological[4] and molecular biological techniques[5] during the past decade, as an alternative method in the laboratory diagnosis of TBM, has evoked considerable interest among the clinicians and laboratory investigators. However, these methods need elaborate instrumentation and technical expertise, which are not generally available in most of the laboratories in the developing world. It needs to be emphasised that any newly proposed method should be reliable, rapid and should differentiate patients with TBM from other causes of meningitis. The sensitivity of the proposed test must be compared with the conventional bacteriological methods. The specificity of the test must also be critically evaluated in a large number of control specimens. A high degree of specificity is essential in order to eliminate the potential risk of false positivity.

Intracranial tuberculoma: Central Nervous System Tuberculosis (CNS) constitutes 15% of extra pulmonary tuberculosis[6] and is the second most common clinical manifestation of CNS tuberculosis after TBM. Intracranial tuberculoma clinically manifest either as single or multiple space-occupying lesions. However, problems in diagnosis occur when the neuroradiological features in MRI scan did not distinguish tuberculoma from solitary enhancing lesion i.e. abscess or metastasis. Bacteriological confirmation of tuberculosis is often negative and histopathological features such as caseating granulomatous lesions caused by other microbial agents such as fungi (*Aspergillus*), mimic that of caseating granulomatous lesions, characteristic of mycobacterial infections. In an attempt for an accurate laboratory diagnosis, for the institution of appropriate chemotherapy in patients with intracranial granuloma,

immunohistochemical methods (IHC) and polymerase chain reaction (PCR) have been utilized for the diagnosis of intracranial tuberculoma.

III. OBJECTIVES

1. **To establish a sensitive, rapid, reproducible, and reliable immunodiagnostic system for the laboratory diagnosis of tuberculous meningitis (TBM) suited to the laboratories in the developing world. This immunodiagnostic system will carry a high degree of specificity and will differentiate patients with TBM from other causes of meningitis.**

2. **To compare the newly proposed immunodiagnostic system with molecular biological technique (Polymerase chain reaction) for the diagnosis.**

3. **To evaluate the utility of PCR for the detection of *M tuberculosis* DNA in the formalin fixed, paraffin-embedded tissues for the diagnosis of intracranial tuberculoma and its comparison with immunohistochemical methods.**

IV. RESEARCH METHODOLOGY

Sample collection:

TBM patients- Lumbar CSF was collected from 40 patients with TBM admitted in the Neurology unit of this hospital between August, 1996- December, 2000. Five of them

were classified as 'confirmed' cases of TBM as *M tuberculosis* was isolated from their CSFs while the remaining 35 were diagnosed as TBM based on the clinical features and they showed compatible cytological features and biochemical parameters in the CSF. However CSF cultures were negative for *M tuberculosis* and other microbial agents including atypical mycobacteria. Hence the patients were classified as 'probable' cases of TBM.

Control patients- Lumbar CSFs from 40 patients with non-tuberculous neurological diseases were collected. This included bacterial meningitis (n=10); partially treated pyogenic meningitis (n=15); fungal meningitis (n=5); viral encephalitis (n=8) and non-disease control (n=2).

Technical Aspects:

Isolation of specific mycobacterial antigen in the culture filtrate by immunoabsorbent affinity chromatography. An immunoabsorbent affinity column was prepared as earlier described[7], by coupling the specific human IgG antibody to *M tuberculosis* (3mg/ml) with activated Cyanogen bromide -Sepharose 4B. Cell- free unheated culture filtrates of $H_{37}Ra$ *M tuberculosis* (5mg/ml) was passed through the immunoabsorbent column and specific mycobacterial antigen present in the culture filtrate was eluted by the addition of 4M Urea. In SDS-PAGE, the elute gave a single band and the molecular weight was found to be 14 kDa. The IgG from control CSF did not bind to the 14 kDa antigen. Antibody to 14 kDa antigen was raised in rabbits and the

IgG fraction in the immune rabbit serum was recovered by Protein-A- Sepharose column chromatography. The elute was dialyzed, concentrated and stored in aliquots (1mg/ml) at -20°C.

Rapid immunodiagnostic dot immunobinding method (D0t-Iba) for diagnosis of tuberculous meningitis.[8, 9] The assay was standardized to measure the circulating mycobacterial antigen in the cerebrospinal fluid (CSF), for the laboratory diagnosis of tuberculous meningitis (TBM). The antibody to 14 kDa antigen was used for the detection of circulating mycobacterial antigen. A dot-immunobinding assay (Dot-Iba) was also standardised to measure circulating antimycobacterial antibody against 14 kDa antigen in cerebrospinal fluid (CSF) samples from TBM patients.

Standardisation of PCR technique for the diagnosis of tuberculous meningitis using primers that amplified a 123bp sequence of IS6110 insertion sequence[10], which is specific for *M tuberculosis* complex. Standardization of PCR protocol was carried out using dilutions of *M tuberculosis*, H_{37} R_a bacilli and different dilutions of *M tuberculosis* DNA.

Immunohistochemical detection of mycobacterial antigens in tuberculous granuloma tissues: Immunohistochemical studies for the demonstration of

mycobacterial antigen in tuberculous lesions were performed using streptavidin-biotin method[11].

Standardisation of PCR technique for the detection of *M tuberculosis* DNA in the paraffin embedded formalin fixed tissues of intracranial tuberculoma specimens. DNA was extracted from tuberculous and non-tuberculous paraffin embedded tissue sections[12]. A 123 bp region from the *M tuberculosis* complex specific insertion sequence IS6110 was used as target sequence for amplification.

A new and novel approach to establish the rapid diagnosis of TBM was attempted by using the cytospin smears of CSFs from patients with TBM. The cytospin smear contained macrophages and transformed lymphocytes. IHC studies of these smear using polyvalent rabbit antibody to *M tuberculosis* and antibody to 38 kDa mycobacterial antigen was performed.

V. MAJOR FINDINGS

Using an immunoabsorbent affinity chromatography, 14 kDa antigen in the culture filtrate of *M tuberculosis* was isolated. Antibody to the 14 kDa mycobacterial antigen was raised in rabbits. Standardisation of Dot-Iba indicated that the assay is sensitive to detect the antigen at a concentration of 50 ng/ml.

Table I. represents the data showing the results of PCR and Dot-Iba in CSF. The Dot-Iba detected mycobacterial antigen in all the five culture positive patients with TBM and 26 out of 35 CSF specimens from patients with probable TBM. While the PCR gave positive results in 3 out of 5 patients with 'confirmed' TBM and 13 out of 35 patients with probable TBM. Thus the sensitivities of Dot-Iba in 'confirmed' and 'probable' TBM group were 100% and 74.3% and that of PCR was 60% and 37% respectively.

Table II. represents the results of IHC, PCR and bacteriological methods in ten cases of intracranial tuberculoma. Mycobacterial antigens were characteristically localized within the cytoplasm of several macrophages and Langhan's giant cells. They appeared as diffusely stained granular brownish-pink material and they were not demonstrated in tissues with extensive caseation. Only 6 out of 10 intracranial tuberculoma gave positive results by PCR method. All positive cases depicted a single band that corresponded to the mycobacterial DNA standard, used as positive control in PCR.

Using a polyvalent rabbit antibody to *M tuberculosis,* we have attempted to demonstrate mycobacterial antigen in the cytoplasm of macrophages and plasmacytoid lymphocytes from patients with TBM.

VI. RESEARCH OUTCOME

Using human CSFs, IgG to *M tuberculosis,* a specific mycobacterial antigen from the culture filtrate of *M tuberculosis* was isolated. Dot-Iba developed in our laboratory is simple, rapid and specific for the detection of mycobacterial antigen in CSF. More

importantly, it can be readily performed in any routine clinical laboratory and does not require sophisticated equipment and the results can be easily interpreted by visual examination of the NCM discs. The entire procedure required only 6h after the receipt of the specimen in the laboratory.

Our data shows that sensitivity of PCR in 'confirmed' and 'probable' patients with TBM to be 60% and 37% respectively. The low sensitivity of PCR for TBM could be due to several factors a) Pausibacillary status of CSF particularly the lumbar CSF b) The presence of host factors in CSF which may inhibit the PCR, c) The methodology used d) partial treatment with antituberculous chemotherapy (ATT), may effect the bacterial load which in turn result in reduced yield of DNA. Dot-Iba, in contrast to PCR is a rapid and relatively easier method. More importantly, Dot-Iba is suitable for routine application for the laboratory diagnosis of TBM and therefore best suited to laboratories in the developing world.

PCR has also been applied for the identification of mycobacterial DNA in the formalin fixed paraffin sections. Published reports regarding the utility of PCR in formalin fixed paraffin sections of intracranial tuberculoma are only few and most of them are case reports[13-15]. In our data, PCR yielded positive results in 6 out of 10 surgical specimens of intracranial tuberculoma. The effectiveness of PCR for detection of *M tuberculosis* DNA, in formalin fixed paraffin embedded tissue is dependent on a) fixation time, b) DNA extraction procedure, c) length of PCR target, d) concentration of target DNA to be amplified and e) the PCR protocol itself. Some of the above factors may be responsible

for the relatively low sensitivity of PCR in intracranial tuberculoma. On the contrary, IHC method is simple, reproducible, and possesses operational advantage over PCR

Table I. Data showing the results of, PCR and Dot-Iba in CSF.

Sl No	Patient group	PCR	Dot -Iba
1	TBM (n=40) Definite (n=5)	3 (60)	5 (100)
	Probable (n=35)	13(37)	26(74.3)
2	Disease Control	0	0
3	Overall specificity (%)	100	100

Table II Comparative analysis of IHC and PCR

	IHC	PCR
Sensitivity (%)	80	60
Specificity (%)	100	100
Duration of assay	12-16h	48-72h
Cost effectiveness	Yes	No
Application in developing countries	Yes	No
Laboratory resources and Technical expertise	Not required	Required

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INTRODUCTION

CHAPTER-I

Human tuberculosis still remains as one of the major public health diseases in most of the developing countries. Inspite of worldwide tuberculosis control programmes, as well as emergence of newer diagnostic and therapeutic modalities, human tuberculosis continues to carry high mortality and morbidity rates. During the past two decades the incidence and prevalence of human tuberculosis showed a global increase due to the emergence of human immunodeficiency virus (HIV) infection. Every year, 3 million deaths and 8 million newer cases of tuberculosis are estimated. (142).

Human tuberculosis is caused by *Mycobacterium tuberculosis*, an acid-fast bacterium. In the spring month of 1882, Robert Koch (80) isolated a rod-shaped bacilli from crushed tubercles. Robert Koch then inoculated these bacilli in guinea pigs and reproduced tuberculous lesions. He also isolated and characterised this bacilli from the lesion of the recipients with features resembling that of *M tuberculosis*. Subsequently, in 1891 Robert Koch observed an enhanced immunological response, when *M tuberculosis* was reinoculated into a host, inoculated earlier with the same bacteria. These observations were the forerunner of what we recognise today as immunity and hypersensitivity phenomenon in tuberculosis. Despite the fact that more than one hundred and nineteen years have passed since Robert Koch isolated tubercle bacilli, precise immunological response in patients with tuberculosis is not well understood. Considerable lacunae still exist in our concept regarding the host immunological mechanism involved in tuberculosis.

1.1 PATHOGENESIS OF HUMAN MYCOBACTERIAL INFECTION

In a susceptible host, the primary mycobacterial infection usually manifest in the lungs and this is often acquired by the inhalation of infected droplets containing *M tuberculosis* bacilli. The infected droplets containing viable *M tuberculosis* bacilli traverse down the upper respiratory tract to the bronchial tree and reach the alveoli of the lungs through the bronchioles. Alveolar macrophages may phagocytose the bacilli at this stage, but the mycobacteria may remain viable and can replicate within the intracellular environment of alveolar macrophages (68). Whenever the host surveillance mechanism gets impaired, the tubercle bacilli replicate and enter the circulatory system as well as the regional lymphatic system from the lungs. Tubercle bacilli then reach the regional and distal visceral organs by haematogenous and lymphatic routes. Tuberculous lesions are usually found in the apical region of the lungs and mediasternal lymphnodes. Less frequently renal parenchyma, central nervous system and skeletal system are also involved in the primary mycobacterial infection. At the onset of host immunity, the primary site of tuberculous infection usually gets healed. A significant proportion of individuals who develop immunity after a successful recovery from the primary tuberculous infection, do not eliminate the bacillus and thus remain at the risk of reactivation. The precise risk factors for reactivation of dormant tuberculous lesions are not well understood, but these may include prolonged and recent corticosteroid treatment, immunosuppression, malnutrition and ageing (107). In general, the risk is about 8% during the first two years following primary infection.

1.2 CENTRAL NERVOUS SYSTEM TUBERCULOSIS

In general, the incidence of central nervous system tuberculosis in a geographic zone is directly proportional to the prevalence of tuberculosis. Among the extrapulmonary sites, 15% of tuberculous infection occurs in central nervous system (CNS) (126). The CNS involvement in tuberculous infection may clinically manifest in four forms *viz* a) tuberculous meningitis, b) tuberculoma as intracranial space occupying lesion and c) cerebrovascular accident in the form of stroke, d) encephalopathy. CNS tuberculosis constitutes approximately 45% of all forms of tuberculosis in paediatric age group. This assumes greater significance when one considers the fact that there is very high incidence of tuberculosis in paediatric age group inspite of the emergence of new and potent anti tuberculosis treatment (ATT), during the last two decades.

1.3 TUBERCULOUS MENINGITIS (TBM)

TBM is usually caused by *M tuberculosis*. However, *M avium, M intracellular* (MAC), *M kansasii* and *M fortuitum* have also been isolated in patients with TBM (61).

Cranial form of tuberculous meningitis is by far the most common manifestation of neurotuberculosis and constitutes 80% of clinical neurotuberculosis (127). TBM can manifest at any age but it is uncommon under the age of six months. The incidence of TBM is highest during the first five years of life. In the paediatric age group, TBM is usually a complication of primary complex. In adults, disease mainly occurs either as

reactivated form of an old lesion or as an isolated form or in association with pulmonary tuberculosis. The latter is more often seen in patients with HIV infection.

1.4 PATHOGENESIS OF TBM

Until the decisive study of Rich and McCordock (137), it was believed that, TBM was a direct and immediate result of haematogenous spread of the infection. Rich and McCordock have observed that a) in many instances, TBM occurs in the absence of milliary tuberculosis. b) Meningitis may be absent in most cases of milliary tuberculosis, c) When milliary tuberculosis and TBM occur together, the age and morphological features characteristic of milliary tuberculosis do not correspond with morphological features of meningitis. d) Introduction of large number of tubercle bacilli into blood stream in a susceptible animal invariably produces milliary tubercle but fail to produce meningitis even though the inoculum has been made directly into carotid artery. e) Typical diffuse form of cranial tuberculous leptomeningitis can be produced when tubercle bacilli is introduced directly to the subarachanoid space of a susceptible animal.

On the basis of the above observation, Rich and McCordock postulated that TBM occurs in two stages. Initially, tuberculous lesions are formed in the brain or in the meninges due to the haematogenous dissemination of tubercle bacilli from the primary site of the infection. Meningitis develops due to the discharge of tubercle bacilli from the cerebrocortical focus (Rich foci) directly into the subarachanoid space. Alternatively, caseous focus may also be located within the choroid plexus of the lateral ventricle and

the discharge of tubercle bacilli from the choroid plexus, results in dissemination of tuberculous bacilli along the cerebrospinal pathway. This can also result in diffuse leptomeningitis.

1.5 SALIENT NEUROPATHOLOGICAL FEATURES OF TBM

Three general processes results in the subsequent neuropathology of TBM; a) exudate formation, b) vascular lesion and c) encephalitic processes (41). Meningeal exudates are formed in the subarachanoid space. Exudates contain lymphocytes, plasma cells, macrophages and increasing quantities of fibrin. In the network of fibrinous matrix, these inflammatory cells and tuberculous bacilli are usually lodged and result in adhesion in the basal leptomeninges and basal cisterns. Adhesion around the interpeduncular fossa and related anatomical structures particularly, the supraclinoid segment of internal carotid artery, results in obliterative vasculitis. These may clinically manifest as cerebral infarcts, which account for an appreciable part of irreversible neurological sequelae of the disease. At times the extension of basal exudates into cerebral parenchyma may result in encephalitis. Edema occurring as a consequence can be severe, involving both the cerebral hemispheres. This will contribute to the raising intracranial pressure and clinical neurological deficits (155).

1. **Meninges:** A thick exudate is usually present in the basal leptomeninges of the brain. The exudates may extend to involve the basal cisterns (40). The convexities

of the leptomeninges over the supralateral surface of the brain are relatively unaffected. The exudate surrounds the cranial nerves and the major blood vessels at the base of the brain. The choroid plexus involvement within the lateral ventricle is almost always present.

2. **Blood vessels:** Vasculitis essentially involving the vessels in the circle of Willis constitutes an important feature of TBM; both from the clinical and pathological stand point. The vascular changes are diffuse and often involve large, medium and small arteries. The histopathological examination of involved artery depicts inflammatory proliferative and degenerative changes. The adventitial involvement represent an extension of tuberculous processes from the subarachanoid space and consists of inflammatory cellular infiltration with or without caseous necrosis. The media of the artery may sometimes show fibrinoid necrosis. The intimal lesions may include sub-endothelial inflammatory cellular infiltration, intimal proliferation, degeneration and fibrinod necrosis. Owing to the characteristic distribution of basal exudates, the middle cerebral arteries and their branches are most commonly involved. The meningeal veins traversing the inflammatory exudate show varying degree of phlebitis, which may lead to thrombosis with partial or complete occlusion of their lumen.
3. **Brain:** The degree of extent of brain involvement is variable. The usual parenchymatous lesions are a) border zone reaction, b) infarcts and ischaemic lesions and c) hydrocephalus (154).

1.6 CLINICAL MANIFESTATION OF TBM

The clinical manifestation of TBM are usually classical, but during the past decade, the clinical manifestation has become increasingly varied and atypical manifestations are much more common than the classical clinical presentation (79, 50). The factors responsible for the changing patterns of clinical manifestation of TBM are multiple and probably attributed to the dominant neuropathological processes in an individual patient. These factors include a) thick exudates in the base of the brain which may manifest as cranial nerve palsies and hydrocephalus, b) obliterative vasculitis may clinically present as infarcts, seizures and raised intracranial pressure, d) presence of tuberculoma may manifest as intracranial space occupying lesions.

As defined by British Medical Council (15), most cases of TBM progress through 3 stages.

Stage I → Prodromal stage

Stage II → Stage of meningeal irritation

Stage III →Stage of diffuse or focal cerebral involvement

In most patients with TBM, there is a history of vague ill-health lasting for two weeks, prior to the onset of meningeal irritation. These symptoms are non-specific. In paediatric age group, headache, loss of appetite, nausea, vomiting and abdominal pain are the usual presenting symptoms (152,91). Intermittent low-grade fever may also be present. In adults, lassitude, depression, confusion or behavioural abnormalities are

usually seen (114). With the onset of meningitis, headache and fever become the major symptoms. In children below 3 years, vomiting is more frequent while in adults headache is the predominant symptom. In most patients, neck stiffness and Kernig's sign are often present. Sometimes multiple and cranial nerve palsies may be the only presenting clinical features. The cranial nerve palsies may be unilateral or bilateral (91, 157).

The involvement of optic nerve may produce visual disturbancse and at times patient may present with visual blurring. Elevated intracranial pressure usually does not manifest in the initial stages of disease, but occasionally sign and symptoms of raised intracranial pressure may precede the features of classical meningitis (119). It is not uncommon in those geographic zones of high prevalence of tuberculosis, to find a child with hydrocephalus or an adult with suspected tuberculoma to have TBM. In these patients, gradually signs and symptoms of raised intracranial pressure become more prominent.

The clinical course of the disease may be punctuated by the sudden onset of neurological deficit. Hemiplegia may occur at the onset or at later stages and usually correlate with ischemic lesions and infarcts in the territory of middle cerebral artery (158). Less commonly, it may due to severe brain edema, secondary to bilateral cerebral infarction.

During the active stages of meningitis, extensive inflammation from cranial leptomeningitis spread to the spine, which may result in clinical features of spinal meningitis and myeloradiculopathy (163,39). Thick tenacious fibrous exudate encases the

spinal cord, resulting in complete or partial spinal block. This may result in paraplegia (79).

As the disease progresses, increasing evidences of cerebral dysfunction sets in, meningeal signs may disappear and the patient may manifest confusion and coma. Terminally, the patient may manifest decorticate postune, extensive rigidity and deep coma. Pupils may become dilated, fixed and respiration becomes irregular. Clinical features of brain stem dysfunction may occur due to infarcts, secondary due to obliterative vasculitis (154).

1.7 TUBERCULOMA

Tuberculoma are the next important clinical and morphological manifestation of CNS tuberculosis. Pathogenesis of CNS tuberculoma is somewhat similar to TBM. Tubercle bacilli lodge within the CNS during the haematogenous dissemination of the primary infection. Discrete small tubercles are formed (38). However instead of rupturing into the subarachanoid space, these tubercles continue to progress in size. Progressive enlargement of one or more than one meningeal tubercle lead to distinctive flat and adherent mass which is called as tuberculoma enplaque. Microscopically tuberculomas are composed of caseating granulomatous lesion, composed of macrophages, Langhan's giant cells and lymphomononuclear cells. These granulomatous lesions are bordered and have peripheral zone of reactive gliosis.

1.8 TUBERCULOUS ENCEPHALOPATHY

At times, the neuropathological studies may reveal cerebral edema, demyelination and perivascular aggregates of mononuclear cells. There are no histopathological evidences of tuberculous granuloma in the brain. This encephalitic process is regarded as a hypersensitivity phenomenon to tuberculous proteins (158).

1.9 LABORATORY DIAGNOSIS OF TBM

The laboratory diagnosis of TBM can be discussed under

a) Cytological and biochemical parameters in CSF

b) Bacteriological method

c) Sophisticated biochemical methods

d) Immunodiagnostic approaches

e) Molecular biological approaches

a) Cytological and biochemical parameters in CSF

The usual cytological picture in the CSF of patients with TBM is lymphocytosis. However cytological features can vary from patient to patient. In some patients with TBM the total leukocyte cell count may be normal. ***Polymorphonuclearcytosis*** can occur in the initial stages of the disease or can also manifest as a reaction to the release of tuberculoprotein into the subarachanoid space (75,121). ***Pleocytosis***: Composed of lymphocytes and plasmacytoid lymphocytes and macrophages. Occasionally several

macrophages or monocytes may aggregate and they are referred to as "wandering tubercles".

The biochemical parameters in CSF include elevated protein. The protein concentration can vary from 50-1000 mg % (91). Proteins are made up of albumin and gamma globulins. Some of these gammaglobulins contain specific antibodies against antigens of *M tuberculosis.* CSF glucose level is grossly reduced; usually less than 40 mg % (165). Chloride concentration is often reduced. (132).

b) Bacteriological methods

The 'Gold Standard' for establishing the laboratory diagnosis of TBM is the demonstration of acid-fast bacilli *M tuberculosis* in CSF by Ziehl Neelsen smear or by culture methods (69).

Acid-fast bacilli in CSF Smear

To demonstrate the acid-fast bacilli in CSF smears, the utility of cytospin is valuable, as it promotes the localisation of the bacilli within the inflammatory cells. It also promotes the preservation of morphological characteristics of the bacilli. AFB can be stained using basic fuchsin dyes such as Ziehl-Neelsen and Kinyoun method. *M tuberculosis* bacilli can also be demonstrated using fluorescent dyes such as auramine and rhodamine. (169)

CSF culture for *M tuberculosis*

In contrast to the other types of bacteria, mycobacteria has a longer generation time of 18-24 h. Conventional culture methods such as Lowenstein-Jensen medium (L-J)

usually require 8-10 weeks to grow *M tuberculosis*. This is not only cumbersome but also less sensitive and may lead to false negative results. As an alternative to LJ medium, several short-term culture methods are recently in use for the isolation of *M tuberculosis* (139).

BACTEC– 460 system (108)- radioactive estimation of $^{14}CO_2$, released from radiolabelled palmitic acid substrate in BACTEC 7H12 medium during mycobacterial growth. Positive culture results can be obtained within 7-11 days (135,12).

Biphasic culture (MB-Check, Septi-check)- Consists of a biphasic medium containing Middle brooke's 7H9 broth and an agar slope. The liquid phase consists of modified MB 7H9 broth with a supplement containing oleic acid, albumin, glucose, catalase, glycerol, pyridoxal hydrochloride, polyoxyethylene-40-stearate, polymyxin B, Amphotericin B, Naladixic acid, trimethoprin and azlocillin in a screw-capped glass bottle. The agar slope consists Middle brooke's 7H11 agar on one side and the opposite side has two portions.One portion contains MB7H11 media with p- nitro α- acetylamino-3-hydroxy propiophenone (NAP) and the other portion contains chocolate agar. By inverting the system during incubation with the clinical specimen, this broth culture gets flooded over the slope. Mycobacteria growing in the broth will form visible colonies on the Middlebrook agar whereas the microbial agent in clinical specimens does not belong to the tuberculosis complex, they will also grow on the Middlebrook agar containing NAP. Bacteria other than mycobacteria will grow on chocolate agar.

Mycobacterial growth indicator tube – A non- radiometric broth method consists of MB 7H9 broth and a silicon rubber impregnated with a ruthenium metal complex as fluorescence quenching based oxygen sensor. The growth of mycobacteria is accompanied by the consumption of oxygen, which allows the indicator to fluoresce under 365nm UV light.

MB Redox medium- Consists of 4ml modified serum supplemented Kirchner medium with a colourless tetrazolium salt as a growth indicator. During growth, this tetrazolium salt gets reduced to a pink, red or violet coloured formazan insoluble material and is secreted to the cell surface in a granular form. Media contains vitamin for the acceleration of the growth and formation of formazan.

c) Biochemical methods

Sophisticated biochemical parameters have been described in the literature for the laboratory diagnosis of TBM. These include measurement of metabolic products of *M tuberculosis* bacilli. These are as follows

i. **Tuberculostearic acid** (100,54)- It is a component of the cell wall. This can be measured by mass-spectroscopy and electron gas liquid chromatography.

ii. **3,2 ketohexyl indoline** (16,17)- Substance of unknown origin, related to seratonin. It is measured using mass spectroscopy and electron gas liquid chromatography.

iii. **Radiolabelled Bromide Partition Test** (98,96)- Measures the ratio of radioactive bromide in serum and CSF. Values less than 1.6 are usually characteristic and diagnostic for TBM.

iv. **Adenosine Deaminase (ADA) activity** (136,44)- It is an enzyme secreted by T lymphocytes. ADA has been used as a biochemical marker and values more than 6-8 U are diagnostic to TBM.

d) Immunodiagnostic approach

The CSF in patients with TBM contain breakdown products of tubercle bacilli i.e. mycobacterial antigens. Elevated immunoglobulins in CSF are antibodies intrathecally synthesised to *M tuberculosis.* Therefore the immunodiagnostic approaches in TBM are based to detect the presence of the mycobacterial antibodies or mycobacterial antigens in CSF specimens. Immunodiagnostic methods described for the laboratory diagnosis of TBM are

- Fluorescent Immunoassay
- Radio Immunoassay
- Enzyme Linked Immunoassay
- Immunoblot assay
- Dot Immunobinding Assay

e) Molecular biological approach

The CSF in patients with TBM contains genomic DNA of *M tuberculosis*. The mycobacterial DNA can be amplified with the help of primers. The different molecular biological method used to detect the mycobacterial DNA in the CSF include

- Polymerase chain reaction (PCR)
- Mycobacterium direct test (MDTB)
- Ligase chain reaction (LCR)

1.10 PROBLEMS IN LABORATORY DIAGNOSIS OF TBM

Cytological

The cytological features in CSF of patients with TBM often show marked variation.

a) The leukocyte count may be normal.

b) Lymphocytosis and pleocytosis can be seen in meningitis due to non-tuberculous aetiology such as partially treated pyogenic meningitis, fungal meningitis and granulomatous meningitis like sarcodosis. Cytological appearances are more or less similar in these diseases.

c) ATT chemotherapy administration prior to CSF studies can significantly alter the cytopathological picture.

d) Wandering tubercles are seldom seen in TBM.

Due to the above reasons, the cytological method cannot be relied upon, for the laboratory diagnosis of TBM.

Biochemical

Elevated proteins and reduced glucose concentrations in CSF can be observed in other neurological diseases like chronic meningitis, demyelinating disorders and viral meningeoencephalitis. Therefore, routine biochemical parameters in CSF are non-specific and seldom contribute to the laboratory diagnosis of TBM.

Bacteriological methods

Demonstration of *M tuberculosis* from CSF by bacteriological methods i.e. smear as well as culture still remains the "Gold standard" for the laboratory diagnosis of TBM. Inspite of several advances in bacteriological methods, successful demonstration of *M tuberculosis* in CSF is infrequent. (21) Those laboratories which employ conventional media such as Lowenstein Jensen media may require 8-10 weeks to culture *M tuberculosis* in CSF specimens. Short-term culture method although require less time, remain insensitive, particularly in CSF specimens (12). Demonstration of acid-fast bacilli in Ziehl-Neelsen stained smears is often infrequent and carry low sensitivity. Kennedy et al. 1979 (76) has reported that minimum 100 mycobacteria per milliliter of CSF are required for the successful demonstration of AFB in CSF. Most CSF in TBM does not contain sufficient number of tubercle bacilli. Thus, the bacteriological methods are often ineffective and not useful for the early laboratory diagnosis of TBM.

There are several reasons for the infrequent isolation of *M tuberculosis* in the CSF of patient with TBM a) CSFs from most patients with TBM are obtained from the lumbar route. The lumbar CSF contains low concentration of *M tuberculosis* bacilli than

ventricular and cisternal CSF (128) b) Secondly tubercle bacilli become embedded in the dense exudates in the base of the brain and these exudates form a barrier for *M tuberculosis* to circulate in the lumbar CSF c) Thirdly and perhaps more importantly, most of the patients with TBM would have received a course of ATT before they are referred to specialized centers for neurological diseases. Lumbar CSF from partially treated patients with TBM will seldom contain *M tuberculosis;* hence the culture is invariably negative. For these reasons, bacteriological methods are ineffective as far as early laboratory diagnosis of TBM is concerned.

Biochemical methods

The sophisticated biochemical methods such as estimation of tuberculostearic acid, 3,2 ketohexyl indoline assay, radiolabelled bromide partition test and adenosine deaminase activity employed in the laboratory diagnosis of TBM, hold definite promise for the laboratory diagnosis of TBM. However these are not feasible for routine application in laboratories of developing countries because of the need for sophisticated and technical expertise.

Immunodiagnostic approaches

The CSF in patients with TBM usually contain a) antibodies to *M tuberculosis*, b) circulating mycobacterial antigens. Any attempt to demonstrate either circulating mycobacterial antigen or antibody by an immunological technique will be useful as an adjunct in the diagnosis of TBM. Several types of mycobacterial antigens have been

isolated from the cultures of *M tuberculosis* and these have been used for the immunodiagnosis of TBM such as ELISA, western blot and Dot-Iba. Though detection of antibody to *M tuberculosis* in CSF carries diagnostic significance, it has several disadvantages. 1) Detection of antibodies cannot distinguish the active disease from the inactive stage. 2) Wherever there is disruption in blood-brain-barrier, serum antibodies to *M tuberculosis* will mix with the CSF, which will yield erroneous results. This phenomenon can also occur in patients with partially treated pyogenic meningitis and thus the detection of antibodies to mycobacterial antigen may give false positive results. 3) There are several cross-reactive antigens, which may react with the antibodies in CSF and thus false positive results can occur.

Demonstration of mycobacterial antigens in CSF of patients with TBM has immense diagnostic value. Several sophisticated immunoassays like Inhibition ELISA (6), Competitive ELISA (133), Sandwich ELISA (138) and Dot-Iba have been established in the past three decades for the laboratory diagnosis of TBM. However, the following problems are often encountered 1) requirement of specific antibodies, which will react only with *M tuberculosis* antigen and not with other microbial agents 2) requirement of the technical expertise 3) the immunodiagnostic method may yield false negative results in a patient who has already received ATT for more than 4 weeks. In a tertiary referral center like ours most the patients have already received ATT for more than 4 weeks. Hence immunoassay for antigen detection may yield false negative result.

Molecular biological methods

Although considerable data are now available on their use with respiratory specimens for the diagnosis of pulmonary tuberculosis, the precise role of PCR for the routine diagnosis of TBM is still not defined. The low sensitivity of PCR for the diagnosis of TBM could be due to following reasons a) Pausibacillary status of CSF in patients with TBM and particularly the lumbar CSF contains fewer tubercle bacilli than cisternal or ventricular CSF (128) b) The presence of host factors in CSF which may inhibit the PCR c) The methodology used for the isolation of DNA requires breaking the tough cell wall of *M tuberculosis*, and this in turn result in sub-optimal yield of amplifiable mycobacterial DNA and this may explain low PCR sensitivity. d) In a tertiary referral hospital like our institute, many patients with TBM are referred from peripheral hospitals and majority of these patients have already received partial treatment with anti tuberculosis chemotherapy (ATT). ATT treatment may also have an effect on the bacterial load, which in turn result in reduced yield of DNA. Bonignton et al (12) are of the opinion that the sensitivity of PCR can be enhanced by increasing the volume of CSF and also emphasised the importance of obtaining CSF before the commencement of ATT, e) Above all, requirement of clean laboratory area, sophisticated instrumentation, expensive chemicals and reagents and an unforgiving precaution in reagent and sample handling, PCR is regarded to be inappropriate for routine laboratory application in developing world.

1.11 OBJECTIVE OF THE STUDY

I. To establish a simple, rapid, reproducible, inexpensive immunodiagnostic assay for the laboratory diagnosis of TBM, which would suit the laboratories in developing countries.

II. To evaluate the utility of PCR in CSF of patients with TBM for the laboratory diagnosis of TBM.

III. To demonstrate mycobacterial antigens in the cytospin smears of CSF of patients with TBM using immunocytochemical methods.

IV. Demonstration of mycobacterial antigen in intracranial tuberculoma using the immunohistochemical techniques.

V. To evaluate the utility of PCR for tubercular aetiology in formalin fixed paraffin embedded tissues of intracranial tuberculoma.

REVIEW OF LITERATURE

CHAPTER-II

2.1 MYCOBACTERIAL ANTIGENS IN THE IMMUNODIAGNOSIS OF TUBERCULOUS MENINGITIS

M tuberculosis is a complex organism. Likewise the antigenic components of *M tuberculosis* are also complex. The antigenic components of *M tuberculosis* are derived from the cell wall and from the cytoplasm of the *M tuberculosis*. Distinct and varied immunoreactive properties have been demonstrated in the lipid, polysaccharide and protein components of *M tuberculosis*. It is also documented that some of the antigenic components of *M tuberculosis* possess immunosuppressive properties while others are capable of inducing macrophage activation, adjuvanticity as well as promote granuloma formation (57,58). Some of the antigens derived from mycobacteria are species specific while others are known to be shared among many species and thereby contribute to the antigenic cross-reactivity (36). In this review an attempt will be made to highlight the immunoreactive characteristics of only those mycobacterial antigens that are relevant for the immunodiagnosis of TBM.

It was not until the work of Janicki and his collaborators in 1971 (66,67,31,32), that a reference system for the identification and nomenclature of individual mycobacterial antigen became available. Janicki and associates (67) prepared an unheated CFA from the $H_{37}Rv$ strain of *M tuberculosis* and they raised a homologous polyvalent antiserum in goats. Subsequently additional reagents were prepared to provide a reference system for the cell-extracts and for the CFA of *M tuberculosis*. An IEP

method was applied to evaluate the individual mycobacterial antigens against the goat polyvalent antiserum to *M tuberculosis*. Precipitin arcs obtained in IEP were numbered sequentially. Eleven major mycobacterial antigens were recognised in the IEP. Janicki and co-workers (67) emphasised the importance of the uniform standardisation of this reference system. Daniel et al. (31) demonstrated different antigenic preparations of *M tuberculosis* demonstrated different patterns for IEP, eventhough identical laboratory conditions were provided in the culture method and immunisation schedule. Daniel et al however emphasised that this variation of patterns in IEP did not limit the usefulness of the reference system for mycobacterial antigens. Additional supplies of standard reagents including cell-sonicate antigens from the same strain of *M tuberculosis* as well as its homologous goat antiserum to *M tuberculosis* were prepared and made available for researchers who were actively involved in the field of mycobacterial immunology (29). The reference system was designated as United-States-Japan Reagents because they were formed under the sponsorship of the United States-Japan Co-operative Medical Sciences Programme through the National Institute of Allergy and Infectious diseases, USA.

Chaparas and Hedrick (22) used the reference antiserum to demonstrate the antigenic similarity between *M bovis* and BCG strain of *M tuberculosis* and they showed no significant antigenic variation between these two strains of mycobacteria. Subsequently Chaparas (23) studied culture filtrates from twelve species of mycobacteria with the reference antiserum and observed that none of the antigens detected by the reference antiserum are unique to *M tuberculosis*.

Robert Koch in 1890 first reported an antigenic preparation of tubercle bacilli, which he named as OT. It consisted of a concentrated sterile filtrate of autoclaved, heat-inactivated liquid cultures of *M tuberculosis*. Subsequently several investigators substituted synthetic media and improved the methods of preparation of the OT. Tuberculin skin testing, using such material was widely used in the clinical and epidemiological fields. However non-specific reactions with OT were soon encountered. Later it was recognised that non-specific reaction, could have resulted from the host environmental contact with other non-pathogenic strains of mycobacteria. This non-pathogenic mycobacteria contained cross-reacting antigens, which shared with the antigens of virulent mammalian tubercle bacilli.

Seibert et al in 1932 (144,143) first prepared a purified tuberculo-protein from the OT by using trichloroacetic acid precipitation method and they designated the preparation, as tuberculin PPD. Tuberculin PPD was subsequently prepared from the OT by repeated precipitation with ammonium sulphate at 50% saturation. Seibert et al (145) later used 8 weeks-old surface cultures of *M tuberculosis*. Filtrates were prepared from these cultures without prior heating and thus avoiding the heat denaturation that usually occurs during the preparation of OT and PPD. Subsequently Seibert et al. characterised four proteins as well as two polysaccharides, each of which was distinct in their physicochemical properties. Seibert et al designated these proteins as A, B, C, D and polysaccharides as I and II. Protein A was capable of eliciting tuberculin skin reactions in humans and was more potent than PPD (160). Protein A contained atleast two components, and possessed a molecular weight of 35,000-42,000 daltons (147). IEP

studies by Daniel and Affronti (30) have shown that protein A contained antigens 1, 2, 5, 6 and probably also 4. Protein B also was capable of eliciting tuberculin skin reactions in humans and was also more potent than PPD (160). Protein B was found to have a sedimentation constant of 2.0s and a molecular weight of approximately of 20,000 daltons (147). IEP showed protein B contained antigens 1,2,5,6,7 and found to be distinct from protein A by the virtue of containing much less antigen 6 and significantly higher quantities of antigen 7. Protein C was relatively inactive as a skin test antigen (160). On electrophoresis, protein C contained a rapidly moving component, which Seibert designated as protein D (147). Protein D was not isolated or studied further.

Polysaccharide I was further characterised by Birnbaum and Affronti (11) as a heteropolysaccharide containing arabinose, galactose and mannose. It was initially described as being non-reactive in the sensitised guinea pigs as well as in humans (105,147). Subsequently polysaccharide I was shown to elicit cellular hypersensitivity responses in-vivo as well as in-vitro. However in these instances its antigenicity has been attributed to the presence of the contaminating proteins (59,10). It did not react with the polyvalent goat antiserum to *M tuberculosis* (10). Immunoelectrophoretic studies revealed polysaccharide I contained predominantly antigen 2, with smaller amounts of antigen I and also an unidentified anodal component (30). Polysaccharide II was found to be a glucan with a molecular weight of 100,000 daltons (146,147). There was a variation in its concentration in different culture filtrate preparations. In IEP, polysaccharide II was shown to be antigen-3 (30). The predominant source of polysaccharides is the cell wall of *M tuberculosis* (36). Arabinogalactans, arabinomannans, mannans and glucans have been

isolated from the cell wall of mycobacteria. This polysaccharide did not elicit the delayed type skin reactions (3,4,5,110,111,112,113).

An important breakthrough in the characterisation of antigens was achieved initially by the production of monoclonal antibodies to mycobacterial antigens (24,48,77) and by the development of recombinant DNA systems for the expression of mycobacterial genes in *Escherichia coli* (168).

Several **semi-purified mycobacterial antigens** have been further purified by chemical or immunological methods and have been evaluated for the immunodiagnosis of tuberculous meningitis. These include Antigen 5, A 60 antigen and LAM antigens.

Antigen 5

Daniel and Anderson used an immunoabsorbent affinity chromatography method to isolate a dialysable antigen from the unheated culture filtrates of $H_{37}Ra$ strain of *M tuberculosis* (34,35). This antigen was found to be a protein and its amino acid composition suggested that, it is derived from the cytoplasm of the tubercle bacilli. It had a sedimentation constant of 2.0 s and a molecular weight of 35kDa. This was termed as antigen 5 by Daniel et al. as it identified with antigen 5 in the numeric number of the nomenclature of mycobacterial antigens. Daniel et al. also found that antigen 5 is present in 4 strains of *M tuberculosis* and 6 strains of *M bovis* and absent in 30 other strains of mycobacteria. As a diagnostic tool, Benjamin and Daniel (9) found that antigen 5 carried a higher specificity than other mycobacterial antigens for the diagnosis of PT and TBM and suggested that antigen 5 as an ideal antigen for application in immunoassays. Further

studies by Daniel et al. (36) indicated that antigen 5 is also composed by arabinomannan and arabinogalactan.

A60 antigen

A60 was isolated from the cytoplasm of *M bovis* and designated A60 by Cocito and colleagues (25,26). It is a high molecular weight complex (10^6-10^7 daltons) containing the moieties of free lipids, lipopolysaccharides and lipoproteins (51). A60 is heterogenous antigen that contained about 30 components including proteins of 65, 40, 38, 35, 19 and 14 kDa, LAM and a glycolipid (27) and these components account for the thermostable property of PPD. It has been suggested that LAM is the major component of antigen A60.

Lipoarabinomannan

LAM is a lipopolysaccharide, present in the cell wall of *M tuberculosis*. Hunter et al. (63) first isolated this arabinomannan from *M tuberculosis* and *M leprae* by anion exchange and gel filtration chromatography. In polyacrylamide gel electrophoresis, LAM yielded a broad diffused mass of 30-35 kDa. Subsequently the analysis of LAM showed that in addition to arabinose and mannose, it also contained glycerol, inositol, phosphate, lactate, succinate, palmitate and tuberculostearate. LAM exhibited a wide spectrum of immunoregulatory functions. LAM is one of the major antigens used for the immunodiagnosis of TBM.

Plasma membrane antigen

Krambovitis (83) prepared plasma membrane antigen from *M tuberculosis* $H_{37}Rv$ (NCTC 7416) on treatment with buffer containing SDS at 80°C for 1h. The plasma membrane antigen contained several soluble mycobacterial antigens.

Several distinct **protein antigens** have been identified using monoclonal antibodies or immunoblotting. The molecular weight of these antigen are 6, 10, 14, 17, 19, 20, 23, 24, 28, 30/31, 33, 35, 38, 45, 47, 58/60, 65, 70 and 85 kDa (48). Prominent among the above antigen in the immunodiagnosis of tuberculosis are 14, 19 and 38 kDa.

14 kDa

The 14 kDa is a prominent protein antigen designated as MPT40, is also referred as 16 kDa. Using monoclonal antibodies Lee and colleagues in 1992 (87) purified a major membrane protein of *M tuberculosis.* This was identical to 14 kDa antigen in its physicochemical properties. 14 kDa has been expressed in Lambda gt11 recombinant DNA clones (167). It has 4 epitopes as defined by MAbs. It is related to the alpha crystalline family of heat shock proteins (161,162,78). The results of studies using synthetic peptides derived from the amino acid sequence of MPT40 indicated the presence of T and B cell epitopes within the MPT40 protein (52). Antibodies of 14 kDa antigen have been regarded to be specific for the detection of smear negative cases of PT and TBM.

19 kDa

The 19 kDa is a lipoprotein antigen and is one of the immunodominant antigen of *M tuberculosis.* It has been purified from *M tuberculosis* by an affinity column chromatography by using MAb TB 23. The antigen has been found to possess diagnostic utility for the immunodiagnosis of tuberculous meningitis (20).

38 kDa

38 kDa is a lipoprotein and is one of the major constituents of *M tuberculosis* culture filtrates. Several studies using two-dimensional crossed immunoelectrophoresis suggested that the antigen is similar to *M tuberculosis* antigen 5 (35). 38 kDa antigen was obtained by immunoaffinity purification based on murine monoclonal antibodies (13), but currently by cloning the gene of this antigen which has offered the potential for its greater availability (1). This antigen is recognized by monoclonal antibodies TB71 and TB72. In addition to the original reference antibodies TB71 and TB72 (24) a number of other monoclonal antibodies to 38 kDa antigen has been raised i.e. HYT28 (141), HGT3 (71), HBT12 (93).

2.2 IMMUNOASSAYS FOR THE DIAGNOSIS OF TBM

2.2.1 Immunoassays for the detection of antibodies to *M tuberculosis* in cerebrospinal fluid

Earlier published studies for the detection of CSF antibodies to *M tuberculosis* in patients with TBM are shown in Table 2-1.

Table 2-1: **Detection of antibody to *M tuberculosis* in CSF of patients with TBM - Review**

Authors	Antigen	Assay
Kuo et al. (1969)	PPD	IHA
Munoz et al. 1978)	PPD	IHA
Kalish et al. (1983)	PPD	Indirect ELISA
Samuel et al.(1983)	Sonicated Ag	RIA
Hernandez et al.(1984)	BCG	Indirect ELISA
Chandramuki et al.(1985)	MTSE	RIA
Coovadia et al. (1986)	Mtb Ag	Indirect ELISA
Prabhakar et al. (1987)	Sonicated Ag, PPD, BCG	Indirect ELISA
Ashtekar et al. (1988)	BCG	Indirect ELISA
Watt et al. (1988)	BCG	Indirect ELISA
Van vooren et al. (1989)	P-32 Ag	Dot-Iba
Chandramuki et al. (1989)	LAM, 38 kDa, 19 kDa, 14 kDa	Indirect ELISA
Sindic et al. (1990)	A60 Ag, M tb cytoplasmic Ag	Immunoblot
Mathai et al. (1991)	Ag5	Indirect ELISA
Radhakrishnan et al. (1991)	Ag5	Dot-Iba
Park et al. (1993)	PPD, LAM	Indirect ELISA
Srivastava et al. (1994)	Sonicated AG	Indirect ELISA
Mathai et al. (1994)	CFA	Immunoblot
Patil et al. (1996)	MTSE	Immunoblot

Kuo et al. (84) standardised an indirect haemagglutination test (IHA) for the immnunodiagnosis of TBM. They used (a) Pasteur antigen polysaccharide antigen), (b) tuberculin PPD antigen to sensitise the red blood cells in the assay. The antibodies to *M tuberculosis* in the CSFs of patients with TBM and control groups were tested by the haemagglutination method. The assay gave positive results in 33 out of 34 patients with TBM and thus carried a sensitivity of 97.1%. The assay gave negative results in 44 patients with other CNS infections. AFB was demonstrated in CSF smear by the Ziehl-Neelsen method only in 55.9% and *M tuberculosis* was isolated by the culture in 70% of patients with TBM. Kuo also observed higher globulin content in the concentrated CSFs and therefore they emphasised the need to concentrate CSF specimens prior to, their application in the test. They emphasised that the concentration of the CSF specimens yielded more positive results and helped in establishing the diagnosis of TBM as well as increasing the sensitivity of the test.

Munoz et al. (116) introduced an indirect haemagglutination test (IHA), using PPD antigen for the diagnosis and prognosis of patients with meningoencephalitis. The test gave positive results in all the 22 patients with TBM i.e. the sensitivity of test were 100%. In their study *M tuberculosis* was isolated only in. 31.8% of patients with TBM. IHA test according to these authors was specific as no false positive results were recorded in any of the 53 patients in the control groups. Munoz et al. observed no correlation between the antibody titer and severity of the disease. They also emphasized, small volume of CSF after concentrating the CSF (modification to Kuo's haemagglutination) was sufficient for the test and that the CSF samples following ammonium sulphate

precipitation should be dialysed thoroughly prior to their application in the test as ammonium sulphate present in the CSF specimen can interfere with the haemagglutination reaction.

Kalish et al. (73) for the first time introduced an indirect ELISA method for the immnunodiagnosis of TBM. In their study, they found high titers of IgG antibodies to PPD antigen in three culture proven patients with TBM. CSFs from 33 patients with chronic meningitis (control group) gave negative results for IgG antibody to PPD antigen. In order to demonstrate that IgG antibodies are produced intrathecally in patients with TBM during the course of illness, Kalish et al. measured IgG and albumin concentration in the serum and CSF simultaneously in patients with TBM and calculated the CSF-IgG index. An elevated CSF-IgG index in patients with TBM suggests that the IgG antibodies are synthesised within the CNS during the course of the disease, rather than be present due to the passive transfer of serum IgG across the blood brain barrier. Serial CSF and serum specimens of their patients with TBM demonstrated greater amounts of IgG antibody to PPD in CSF than in the serum. This observation also suggests a intrathecal synthesis of antibody to *M tuberculosis* in patients with TBM. The antibody titers in CSF showed positive correlation with the clinical course of the illness as well as with the leukocyte cell-count, protein as well as glucose concentration in the CSFs. Kalish et al. also emphasised, ELISA method for measuring the IgG antibodies to PPD antigen should be evaluated as one of the means for the early diagnosis in patients with TBM.

Samuel et al. (140) applied a RIA for the detection of mycobacterial antigen and antibody in CSF specimens of patients with TBM. They studied four groups- controls,

pyogenic meningitis, TBM and pulmonary tuberculosis without meningitis. In their study, detection of mycobacterial antigen in CSF specimen was not found to be useful for the diagnosis of TBM. However antibody titers to *M tuberculosis* in CSFs of patients with TBM were statistically higher than the other three groups of patients. Samuel et al. also observed a decrease in the antibody titer between second week to eight months after the commencement of ATT. They attributed the decrease in the antibody titer due to the steroids administration because steroids are known to suppress the antibody production. They also studied the effects of dilution of antibody titer with the development of hydrocephalus in their patients with TBM.

Hernandez et al. (60) used BCG antigen as the solid phase absorbent in the ELISA. They evaluated the sensitivity of the ELISA in 20 bacteriologically proven cases of TBM and the specificity was assessed in 31 patients with acute pyogenic meningitis, 20 patients with viral meningitis and 10 patients with non-infectious disease of CNS. In their study, Hernandez et al used a disk coated with BCG antigen as the solid phase absorbent instead of the conventional microtiter plates and they consider that this has made the test more economical. The plate method required expensive equipment like ELISA readers for the interpretation of the results. In contrast, disk method required less expensive equipment. Hernandez et al. simultaneously measured both IgG and IgM antibody titers in ELISA and observed a sensitivity of 100%. A marked difference in the antibody titer between the CSF samples of TBM and non-TBM group was observed. They concluded that, on the basis of predictive value of the assay, the analysis of their data showed a sensitivity and specificity of 100% for the diagnosis of TBM.

Chandramuki et al. (19) standardised three different assays to detect antibodies in CSFs of their patients with TBM and pyogenic meningitis. (a) A solid-phase RIA in which the microtiter plates were coated with a soluble extract of mycobacterial antigen (MTSE). The microtiter plates were incubated overnight at 4°C with four serial doubling dilutions of CSF specimens. The plates were washed and incubated with ^{125}I labeled rabbit anti human immunoglobulin. The presence of radioactivity in the CSF specimen was expressed as the relative value. Antibody levels were expressed as the reciprocal dilutions of CSF that gave 50% binding. (b) An antibody competition test for the detection of MAb like- epitope-specific antibodies in CSF. ^{125}I labeled probes were prepared using the following MAbs -ML 34, TB-72, ML-30, TB-23, TB-68, TB-78 and TB-71. Competitive inhibition of binding of each of these MAbs to MTSE coated microtiter plates in the presence of antibodies in CSF samples were measured. The results were expressed as relative inhibition (%) of ^{125}I -MAb binding. (c) An immunoblot method to detect antimycobacterial antibodies in the CSF. The MTSE antigen was resolved by a discontinuous polyacrylamide gel electrophoresis. Subsequently the proteins were transferred to nitrocellulose papers (NCPs). The NCP were then incubated with CSF samples and they were that treated with ^{125}I-RaHG. The radioactive blots were developed by autoradiography. Analysis of the data showed that the assay carried 74% and 66% sensitivity in culture positive and culture negative patients with TBM. However the assay also showed the positive results in 37% of patients with pyogenic meningitis and 18% of patients with non-meningitic neurological diseases.

Coovadia et al. (28) evaluated three parameters- (a) Adenosine deaminase activity (ADA) activity in the CSF, (b) an indirect EL1SA to detect antibody to *M tuberculosis* antigen 5 and (c) a radioactive bromide partition test in patients with TBM. Most of the patients in their study were in the paediatric age groups. Three groups of patients were studied. Group A consisted of 38 children with TBM and this group was further subdivided into two subgroups. Group A-l consisted of 13 children with a strong clinical suspicion of TBM. These patients showed typical CSF-cytological and biochemical findings and they also had positive Mantoux test. Radiogram of the chest was suggestive of pulmonary tuberculosis. CSF was positive for AFB either by direct Z-N stain or by culture. These children were assessed within 72 h of admission or before the commencement of ATT. Group A2 comprised 25 children who are on ATT for varying periods. Group B was made up of 47 children presenting with clinical features that were suggestive of meningitis. This group included 16 patients with non-tuberculous bacterial meningitis (B2), 13 patients with aseptic meningitis (group B3) and 20 with normal CSF (group B). Group C included 14 children with pulmonary tuberculosis without any clinical evidences of meningitis. At CSF dilutions of more than or equal to 1:20, 1:40 and1: 80, the ELISA test showed sensitivities of 53% (8/15) 40% (6/15) and 27% (4/15). In the control groups B and C, a titer less than 1:20 was [90% (45/50)] specific for the absence of TBM whereas titer less than 1:40 and 1:80 were 94% (47/50) and 100% (50/50) specific for the absence of TBM. Peak concentration of CSF antibodies was recorded as early as one weak or as late as two and half months of the disease. Coovadia

et al. did not observe any correlation between antibody concentration and severity of the disease or the presence of neurological sequelae of the disease.

In an ELISA, Prabhakar and Oommen (125) used three mycobacterial antigens-commercial PPD, BCG and sonicate antigen of $H_{37}Rv$ *M tuberculosis* for the detection of antimycobacterial antibodies in the CSFs of patients with TBM. In a retrospective study, a total of 260 CSF samples were analysed. The CSF samples were classified into 6 groups on the basis on their clinical diagnosis. Group I was composed by patients with a clinical diagnosis of. TBM and these patients had either positive CSF culture for *M tuberculosis* or showed definite clinical response to ATT. The patients in this group were -regarded as being 'definite TBM' (n=10). Group II consisted of 'Probable' cases of TBM (n=15). Patients in this group had negative bacteriological culture for *M tuberculosis* and they also showed an equivocal clinical response .to ATT. Group III consisted of patients who underwent spinal anesthesia or lumbar puncture for myelographic studies (n=118). Group IV consisted of patients with CNS infections due to non-tuberculous aetiology *viz.*, pyogenic meningitis, viral meningo-encephalitis and cerebral cysticercosis (n=23). Group V comprised of patients with post-infectious demyelinating disorders (n=37). Group VI consisted of patients with altered blood brain-barrier- strokes, brain tumors, recurrent seizures and benign intracranial hypertension (n=37). This particular group was selected to study whether the transfer of antimycobacterial antibodies occurs across the blood brain barrier. The CSFs from all the patient groups were used at different dilutions and tested in the ELISA. Sonicate antigen of $H_{37}Rv$ *M tuberculosis* gave 70% sensitivity and 90% specificity in the ELISA. Lower sensitivity was observed with the BCG antigen

although specificity was found to be 90%. Studies with PPD as antigen indicated a weakly positive reaction in five 'definite' TBM patients and hence PPD antigen was not used further in their study. Based on these results Prabhakar and Oommen concluded, sonicate antigen of $H_{37}Ra$ strain of *M tuberculosis* is the most appropriate antigen and that there is a good correlation between ELISA results and the cytological picture in the CSFs of patients with TBM. The ELISA test in, their study differentiated TBM from non-TBM patients and that they emphasised the use of ELISA as an adjunct in the laboratory diagnosis of TBM.

Astekar et al. (2) used RIA for the detection of specific mycobacterial antigen and antimycobacterial antibody in CSF and serum of patients with TBM. They studied 84 TBM patients, categorised on the basis of (a) clinical features of TBM, (b) history of contact, (c) X-ray evidence of primary complex or progressive tuberculous disease, (d) CSF cytology and biochemical findings, (e) demonstration of *M tuberculosis* in smear and culture of CSF specimen. 30 control subjects having pyogenic meningitis, viral meningitis and patients with febrile illness was also studied. 75% of the patients with TBM were positive either for smear or culture of *M tuberculosis*. The results of their study revealed that mycobacterial antigen and CICs are significantly elevated in patients with TBM than in controls. They also observed a positive correlation between the isolation of *M tuberculosis* by culture and estimation of mycobacterial antigen in the CSF specimen. Ashtekar et al. concluded that the detection of mycobacterial antigen and antibodies are more specific and therefore RIA should be carried routinely in all the patients with TBM.

Watt et al. (164) applied an indirect ELISA to detect antimycobacterial antibody in 29 bacteriologically confirmed patients with TBM and 83 patients with non-tuberculous infectious diseases of CNS. They compared the sensitivity of the assay with the direct Ziehl-Neelsen staining method and also evaluated the specificity of the assay in the non-tuberculous subjects. ELISA was performed with BCG as the antigen. Watt et al. recorded positivity in only 7 out of 29 culture positive patients with TBM. However, the assay carried 98% specificity.

Van Vooren et al (159) established a dot-immunoblot assay to detect antimycobacterial antibody in CSFs, using a, purified BCG P-32 antigen. They studied, five patients with pulmonary tuberculosis with suspected meningeal involvement. The dot-immunoblotting procedure is much simpler than the conventional ELISA. Specific IgG class of antimycobacterial antibodies was found in all the five patients in the serum as well as in their CSFs. However, a higher ratio of anti-p32 immunoglobulins and total immunoglobulin was observed in the CSFs of patients with TBM than in serum while a reverse situation was observed in non-tuberculous patients. Van Vooren et al. concluded, specific antimycobacterial antibodies are synthesised intrathecally in patients with pulmonary tuberculosis with meningeal involvement.

Chandramuki et al. (20) detected specific antibody to five mycobacterial antigens by an indirect ELISA in the CSFs of patients with TBM. The mycobacterial antigens used were (a) soluble extract of $H_{37}Rv$ *M tuberculosis* (b) lipoarabinomannan (c) 14,19,38 kDa antigens from $H_{37}Rv$ *M tuberculosis* isolated by an affinity chromatography (d) a recombinant 65 kDa antigen. They studied 74 patients with TBM,

26 culture positive and 48 culture negative. The control subjects included 26 patients with purulent meningitis and 29 patients with other neurological diseases. Chandramuki et al. observed antibody response to 14 kDa antigen as well as lipoarabinomannan were more than with other antigens. The assay gave 100% specificity and 74% sensitivity with these two antigens. Chandramuki et al. emphasised, estimation of levels of antibody in CSFs to selected mycobacterial antigens should be considered as a valuable aid in the diagnosis of TBM.

Sindic et al. (150) studied the CSFs and sera from six bacteriologically proven and two clinically suspected cases of TBM for the presence of antimycobacterial antibodies against an unfractionated cytoplasmic antigen of $H_{37}Rv$ *M tuberculosis*. They used an irnmunoblot technique. This technique is based on agarose gel isoelectric focusing of paired CSF and serum of patients with TBM as well as transfer of the specific IgG antibodies against mycobacterial antigen loaded to nitrocellulose sheets. An intrathecal synthesis of antimycobacterial oligoclonal IgG antibodies often superimposed on the diffuse polyclonal production and this was shown in all patients with TBM, but not in patients with tension headache or other neurological disorders. Similar results were obtained with another purified mycobacterial antigen (A60) prepared from *M bovis*. This antigen was used for the coating the NCP sheets instead of whole mycobacterial homogenate. In their study, the number of antimycobacterial oligoclonal IgG bands increased with the time and persisted for years even in clinically cured patients. The authors concluded the demonstration of such antimycobacterial antibodies in CSF could

be a useful adjunct for the diagnosis of TBM especially in patients with negative CSF culture for *M tuberculosis.*

Mathai et al. (103) used ELISA for the detection of circulating antimycobacterial antibody against antigen 5 for the diagnosis of TBM. They used CSF from 40 patients with clinical diagnosis of TBM and 42 patients with non-tuberculous neurological diseases. The sensitivity and of the assay in 1:40, 1:80, 1:160 was 94%, 75%, 34%. While the specificity was 73%, 100%, 100% respectively. At 1:80 dilution 100% specificity of the assay was achieved but it became less sensitive. Their study concluded that IgG antibody detection against antigen 5 by non-competitive ELISA has potential application in the laboratory diagnosis of TBM.

Radhakrishnan et al. (129) used a simple dot immunobinding assay (Dot-Iba) as an alternative method for the laboratory diagnosis of TBM. Based on clinical features, CSF cytological and biochemical parameters as well as response to ATT, 75 patients were categorised as TBM patients. Of the 75 patients, 15 patients were culture positive while 60 patients were culture negative. Antibody to *M tuberculosis* Antigen 5 was detected using Dot-Iba. Using Dot-Iba, antimycobacterial antibody was positive in all the 15 culture positive and 36 out of 60 culture negative patients with TBM. A cut-off point was determined 1:16 dilution as is gave the best discrimination between tuberculous and non-tuberculous groups. In control groups none of the samples (n=75) gave positive results in 1:16 dilution. Thus the sensitivity of Dot-Iba in culture positive and culture negative samples was 100% and 60% respectively, while the specificity was 100%. They

concluded Dot-Iba that as a simple, rapid, specific test as an alternative to standard bacteriological method for the laboratory diagnosis of TBM.

Park et al. (122) devised an ELISA method to detect IgG antibodies in CSF against, two mycobacterial antigens i.e. PPD and LAM for the laboratory diagnosis of TBM. Patients in their study group included TBM (n=27), aseptic meningitis patients (n=29), and patients with non-inflammatory neurological illness as controls (n=49). TBM group included both definite (n=11) and probable (n=16) patients, categorized based on positive CSF smears from acid-fast bacilli, culture for *M tuberculosis,* clinical CSF findings, and compatible radiological features of pulmonary tuberculosis. In their study they used PPD and LAM antigens. CSF IgG antibodies to LAM antigen were significantly elevated in 23 CSF out of 27 (85.2%) patients with TBM. While antibodies to PPD was present in 16 (59.2%) patients. Two out of 29 patients with aseptic meningitis showed increased IgG antibodies to LAM and PPD antigen. The sensitivity and specificity of IgG antibodies for the diagnosis of TBM were 85.2% and 95.9% for LAM and 52.9% and 93.9% for PPD. Park et al. further measured IgG antibody in CSF and sera in these patients to investigate the possibility of passive transfer of IgG from serum to the CSF through blood brain barrier. CSF-IgG index were significantly elevated in TBM and was more frequently positive in the CSF than in the sera, suggesting local intrathecal synthesis of TBM.

Mathai et al. (104) used immunoblot method as a confirmative method for the laboratory diagnosis of TBM. They selected 30 patients with clinical diagnosis of TBM as well as an equal number of patients with non-tuberculous meningitis as controls.

Immunoblot method was used for the detection of specific antibody to mycobacterial antigens. They used CFA and *M tuberculosis* Antigen 5 as the antigen in the assay. Both the antigens were subjected to SDS-PAGE and transferred to NCM. NCM strips were cut and stored at 4°C. NCM discs were treated with CSF from TBM and control groups and immunostaining was carried by the conventional method. Accordingly CSF samples from most of the patients in the control group either did not gave demonstrable band but in some give positive immunoassay at 27, 30 and 43 kDa antigen The most striking observation was the presence of antibody to 35 kDa antigen of CFA. In all the 5 culture positive as well as 25 CSF samples from clinically probable but culture negative cases of TBM showed specific immunostaining at 35 kDa. The immunoblot method gave a sensitivity of 86% and a specificity of 100%. Their study concluded that by means of immunoblot method they were able to demonstrate the presence of antimycobacterial antibody in the CSF of patients with TBM, which specifically reacted with the 35 kDa of CFA

Patil et al. (123) applied a western blot and ELISA method to detect antibody response to *M tuberculosis* in the CSF of TBM patients. A total of 124 CSF samples were analysed by Western blot method. These included CSF samples from patients with clinically diagnosed TBM, showing antibody response to *M tuberculosis* antigens by ELISA (n=30). The control CSF included samples from patients with pyogenic meningitis (n=10), cryptococcal meningitis (n=6), neurocysticercosis (n=28), neurosyphilis (n=8), viral meningoencephalitis (n=8), carcinomatous meningitis (n=8),

iatrogeninc meningitis (n=6) and CSF of patients who underwent spinal anesthesia (n=20). SDS-PAGE gel electrophoresis of *M tuberculosis* saline extract (MTSE) antigen was carried out at a constant voltage in 1% homogenous gel in discontinuous buffer system. Gel containing the mycobacterial antigens was transferred on NCM. NCM containing MTSE antigen was cut into strips and incubated with CSF samples (1:10) of TBM and control groups. Immunostaining was carried by conventional method. Using an ELISA Patil et al. recorded the presence of anti-LAM antibodies in 70.6% and anti-38 kDa antibodies in 41 % patients with clinical diagnosis of TBM. Patil et al found that 30-40 kDa and 14kDa antigens of *M tuberculosis* are immunodominant in Western blot method. Using monoclonal antibodies (ML34 and TB-68) they established that this 30-40 kDa component to be lipoarabinomannan and 14 kDa to be a protein component with diagnostic specificity for detecting antibody response in TBM patients. They concluded that immunoblotting test to detect immune reactivity to 30-40 kDa and 14 kDa antigens would be an adjunct that can be used for the diagnosis of TBM.

Srivastava et al. (153) used sonicate antigen of *M tuberculosis* in ELISA. They studied 68 patients with clinical diagnosis of TBM to detect antimycobacterial antibodies in CSF and serum. They found 90% (18/20) of proven cases of TBM, antibodies were also.. In patients with clinically suspected TBM antibodies in CSF were present in 87.5%. In control group antibodies were present absent in the CSF samples. The results of their study indicated that ELISA test using sonicated $H_{37}Ra$ *M tuberculosis* antigen is a sensitive and specific test for diagnosis of TBM.

2.2.2 Immunoassays for the detection of mycobacterial antigen in CSF

Earlier published studies for the detection of mycobacterial antigens in the CSF are summarized in Table: 2-2.

Table 2-2: **Detection of mycobacterial antigens in CSF of patients with TBM- Review**

Authors	Antibody used	Assay
Sada et al. (1983)	Anti BCG	Sandwich ELISA
Bal et al. (1983)	Ab to sonicated Ag	Inhibition ELISA
Krambovitis et al. (1984)	Ab to Plasma membAg	Latex agglutination
Chandramuki et al. (1984)	M Ab to MY4 Ag	Reverse passive haemagglutination
Kadival et al. (1986)	Anti BCG	Sandwich ELISA
Donald et al. (1987)	Anti BCG	Sandwich ELISA
Kadival et al. (1987)	Anti Mtb	RIA
Watt et al. (1988)	Anti BCG	Sandwich ELISA
Ramakisson et al.(1988)	Anti BCG	Competition ELISA
Wu et al. (1989)	Anti BCG	Sandwich ELISA
Dhand et al. (1989)	Anti BCG	Sandwich ELISA
Mathai et al. (1991)	Ab to Antigen 5	Inhibition ELISA
Radhakrishnan et al. (1991)	Ab to Ag5	Dot-Iba
Mastroianni et al. (1991)	Anti BCG	Dot-Iba

Sada et al. (138) standardised a Sandwich EL1SA for the detection of mycobacterial antigen in CSF of TBM patients. They studied 16 patients with TBM and 12 patients with non-tuberculous infectious diseases of CNS and 11 patients without any neurological diseases. The wells in microtiter plates were coated with rabbit anti-BCG

antibody (1:800). Following 12h incubation, the CSF samples from TBM and non-TBM groups were added and incubated for 2 h. The amount of antigen present in the CSF was measured by the addition of anti-rabbit IgG-alkaline phosphatase conjugate and p-nitrophenyl phosphate was used as a substrate. The microtiter plates were read at 405nm. The assay showed a sensitivity of 81.25% and a specificity of 95% for tuberculous aetiology. False positive result was obtained in patients with Cryptococcal meningitis. This false positive reaction may be due to the cross reactivity of the rabbit anti-BCG antibody with the polysaccharide capsular antigen of *Cryptococcus neoformans*. The authors concluded, serial determination of antigen concentration in the CSFs of patients with TBM during treatment might be useful in the follow-up of the patients as well as assessing the progress of the disease.

Bal et al. (6) estimated mycobacterial antigen in CSF specimens using an inhibition ELISA. They studied 41 CSFs from patients, of which only 9 were clinically diagnosed as TBM and the remaining 32 CSFs were proved to be of non-tuberculous aetiology. In their assay, irradiated cells of $H_{37}Rv$ *M tuberculosis* was used as the solid-phase absorbent. A polyvalent antibody to $H_{37}Rv$ *M tuberculosis* was raised in rabbits. In the standardisation of inhibition ELISA, the globulin fraction of rabbit serum was conjugated with an alkaline phosphatase. The antibody-enzyme conjugate and the soluble fraction of mycobacterial antigen were incubated at 37°C for 1 h. This antigen-antibody complex was added to a solid phase and incubated for 12 h at 4°C. The enzyme activity of the conjugate adherent to solid phase was estimated by the addition of p-nitrophenyl

phosphate. The difference between the absorbance in the test and control sample was plotted against the logarithmic value of the antigen concentration. A linear relation was obtained. The CSF samples from TBM and non-TBM groups were similarly tested. The antigen concentration of CSFs was calculated directly from the standard graph. Any reduction of absorbance of 0.15 or more was considered as significant. Of the 9 patients who were clinically diagnosed as TBM, 7 were proved to be having definite tuberculous aetiology. Besides the ELISA, the CSF specimens were also subjected to biochemical, cytological and bacteriological investigations. All the patients were followed-up either to confirm or to exclude the initial diagnosis of TBM.

Krambovitis et al. (82) described a simple latex particle agglutination test for the rapid detection of mycobacterial antigen in the CSFs of 18 children with TBM and 134 controls with other neurological disorders. 15 out of 18 children with TBM demonstrated AFB in the CSF smears. The test was carried out by the sensitisation of latex particles with a purified rabbit immunoglobulin against the plasma membrane antigen of *M tuberculosis*. The control latex reagent was similarly prepared with the normal rabbit immunoglobulin. The CSFs were heat-inactivated at 56°C to remove the non-specific proteins. 40 μl of CSF was placed around two black circles in a glass test slide. 20 μl of latex reagent was added to the first circle and 20 μl of control latex reagent to the second circle. A positive result was indicated by the development of agglutination within 3 minutes. Those CSFs samples that did not contain any mycobacterial antigen showed no agglutination. The latex agglutination test was positive in 17 out of 18 CSFs and was negative in 130 out of 134 CSFs from the control group. A patient with *Haemophilus*

influenzae meningitis gave a false positive result. The authors concluded, latex particle agglutination test has potential application in the laboratory diagnosis of TBM. The test is inexpensive and does not require elaborate instrumentation.

Chandramuki et al. (19) applied a reverse passive haemagglutination test for the detection of soluble non-protein mycobacterial antigen using an IgM murine MAb The murine IgM-MAb was prepared by immunising BALB/C mice with the MY4 antigen. The globulin fraction in the ascitic fluid of the mouse contained the monoclonal IgM antibody against MY4 antigen. A reverse passive haemagglutination test was performed by coupling IgM-MAbs to chymotrypsin treated sheep-red-blood-cells (SRBC). (0.033% chromic chloride was added by drops to a mixture of 25μl of ML 34 globulin (400ng/ml). This suspension was incubated on a mixer for 1 h at 20°C. The cells were washed and made up to 1% suspension in PBS. Serial doubling dilutions of CSFs were made in the 'U' shaped microtitration plates. ML 34-coated red-blood-cells were added and the haemagglutination reaction was read after the incubation for 1 h at 20°C. Controls included neat CSF samples with uncoated SRBC and coated SRBC. The ML-34 antibody coated SRBC agglutinated by an MTSE was used as a positive control. Haemagglutination occurred in presence of 1.0 μg / ml MTSE. The test was applied to analyse the CSFs of 89 patients with. TBM from India, 127 control subjects from UK and India. The mycobacterial antigen was demonstrated in 88% culture positive and 73% culture negative patients of TBM. However the test also detected false positive results in 21% of Indian patients with pyogenic meningitis.

Kadival et al. (70) standardised a double antibody sandwich ELISA to detect mycobacterial antigen in the CSFs of treated and untreated patients with TBM. They used rabbit anti-BCG antibody as the primary antibody and anti rabbit IgG-biotin conjugate as the secondary antibody. Avidin-alkaline phosphatase and p-nitrophenyl phosphate were used as enzyme and substrate respectively. Kadival et al. studied a total of 90 patients. Group I consisted of 38 CSF samples from patients with TBM that was further subdivided into treated (n=14) and untreated groups (n=24). Only 4 of these 38 patients with TBM had shown the presence of AFB in their CSF specimens. The diagnosis of TBM in the rest of 34 patients was based on the clinical features and biochemical abnormalities in the CSFs. Group II consisted of 22 control samples from patients with febrile convulsions, epilepsy, viral encephalitis and tetany. None of the patients in Group II had evidence of pulmonary tuberculosis. Group III consisted of 30 CSF samples from patients with symptomatic aseptic, non-mycobacterial meningitis. The CSF showed elevated proteins in most of these patients with culture proven bacterial meningitis. The sandwich ELISA was performed in the microtiter plates. The wells were coated with anti mycobacterial antibody for overnight at 28°C. After the three washes, the micotiter plates were incubated with the sonicate antigen of *M tuberculosis*. To study the cross-reactivity with other mycobacterial species, sonicate antigens of *M intracellulare, M avium, M kansasii, M vaccae, M fortuitum* were used. PBS with 1% BSA was used as the washing buffer in between the phases of incubation. Anti-BCG rabbit serum was added and incubated for 2 h. This was followed by anti rabbit IgG-alkaline phosphatase conjugate. The plates were incubated for 2 h at 20°C. The absorbances were read after the addition

of p-nitrophenyl phosphate. The biotin-avidin system was precisely used in this study to improve the sensitivity of the assay. With the avidin-biotin system, the assay detected the concentration of mycobacterial antigen as low as 3ng/ml while the sandwich ELISA method detected only upto 15ng/ml or more. The specificity of both types of ELISAs was evaluated with sonicate preparation from other mycobacteria i.e. *M intracellulare, M avium, M kansasii M fortuitum and M tuberculosis*. Sandwich ELISA showed a cross-reactivity of 5% with *M kansasii*. Only 2% cross- reactivity with other mycobacteria was observed in the ELISA with avidin-.biotin system. The authors emphasised although the avidin-biotin system was five times more sensitive than the sandwich ELISA but it was less specific. Therefore sandwich ELISA was preferred for the detection of mycobacterial antigen in the CSF samples. The results in their study showed in 75% of the 24 untreated subjects with TBM, the antigen concentration ranged between 0.3 to 10ng/ml. Among the 14 treated patients, only 5 patients showed the presence of antigen in the CSF. Among the patients with' aseptic meningitis, non-tuberculous and non-bacterial meningitis, the antigen concentration in CSFs was present only in insignificant concentration and the ELISA gave no false positive results in the non-tuberculous subjects. The authors have concluded that a good correlation was obtained between the demonstration of mycobacterial antigen in the CSFs and active stage of the disease. They recommended the application of ELISA technique for the diagnosis of patients with a clinical suspicion of TBM.

Donald et al. (43) investigated 53 CSFs from patients with meningitis and detected the presence of mycobacterial antigen in CSF by a sandwich ELISA method.

Prior to the application, the CSFs were heat-inactivated at 56°C for 1 h to eliminate non-specific reactions. 22 out of 53 patients who presented with the clinical features of TBM, positive culture for *M tuberculosis* was obtained only in 9 patients. In 31 other patients with meningitis, the aetiology was due to the following organisms *Haemophilus influenzae* (n=9) *Streptococcus pneumoniae* (n=8) *Neisseria meningitidis* (n=4) *Cryptococcus neoformans* (n=2) *Staphylococcus aureus* (n=l) *and Klebsiella pneumoniae* (n=l), Mumps virus (n=2) Coxsackie A virus (n=l). The CSFs were obtained either before the start of the therapy or within a week after the therapy in patients with TBM while in non-tuberculous subjects the CSF was studied only with the initial CSF specimens from which the organisms were grown. The ELISA was standardised in the microtiter plates with varying concentration of BCG antigen. The plates were coated with rabbit anti-BCG antigen. The amount of antigen was determined using anti-BCG rabbit IgG-peroxidase as the enzyme conjugate and O-phenylenediamine as the substrate. The absorbances were read at 494 nm. The assay gave 100% sensitivity and 81% specificity for tuberculous aetiology in their patients with TBM.

Kadival et al. (72) applied a biotin-avidin RIA for the estimation of mycobacterial antigen in the CSFs of patients with TBM. The assay involved sandwiching mycobacterial antigen between- the two antibodies to *M tuberculosis* raised in burros and rabbits. The reaction is amplified by the use of anti rabbit IgG-biotin conjugate and ^{125}I labeled-avidin. The RIA detected antigen concentration up to 20ng/ml and showed less than 5% cross reactivity with six other strains of mycobacteria. The authors investigated 17 treated and 19 untreated patients with TBM, 26 patients with non-bacterial meningitis

and 30 patients with bacterial meningitis. The results showed the antigen concentrations in 15 out of 19 untreated patients in the TBM ranged between 20-10,000 ng/ml. However in the treated group only 2 out of 17 CSFs were positive for the presence of antigen. The authors concluded that RIA promises to be a rapid adjunct for the early diagnosis of TBM.

Watt et al. (164) evaluated the use of sandwich ELISA to detect mycobacterial antigen in the CSFs of 29 culture proven patients with TBM and 83 patients due to non-tuberculous CNS infections. The ELISA was performed with anti-BCG antibody as the primary antibody. The CSFs from tuberculous and non-tuberculous subjects were added in their respective wells. BCG protein standards were used as the control. Following this, anti rabbit IgG-HRP was added as the enzyme conjugate and o-phenylenediamine was added as the substrate. Watt et al. compared the sensitivity of the assay with the direct demonstration of AFB. Specificity of the assay was critically evaluated in the non-tuberculous subjects. The authors have observed that the sandwich ELISA detected mycobacterial antigen in 11 out of 28 patients with TBM while AFB was present in only 2 out of the 28 patients. Thus the ELISA was more sensitive than the direct smear examination in patients with TBM. The assay showed a specificity of 98%. False positive results occurred in 4 patients with bacterial meningitis. The authors also estimated predictive values for two extremes of prevalence. They observed a positive predictive value of 93% in their high prevalence setting, which means that positive ELISA is very helpful whereas a negative test is much less useful. They have also emphasised that in area of low prevalence a negative test is very useful in excluding the diagnosis of TBM.

Ramkisson et al. (133) described a competition ELISA for the detection. of mycobacterial antigen in the tuberculous exudates. They studied 59 CSFs, 25 ascitic and pleural fluids of patients with tuberculosis. The technical procedure of the ELISA consisted of incubation of equal volumes of CSF specimen and the rabbit IgG anti-BCG antibody in unsensitised wells of a flat-bottomed polystyrene microtiter plate. Following overnight incubation, 100 μl of CSF-rabbit anti-BCG complex was transferred onto each well in a microtiter plate. The wells were presensitised with 1 pg of BCG antigen. Following the incubation, goat anti rabbit IgG-alkaline phosphatase conjugate was added into each well. p-nitrophenyl phosphate was used as the substrate. The absorbance was recorded. The results showed significant differences between the absorbance in CSFs of patients with TBM and control groups. The authors suggested that the competition ELISA seems to possess a clinical application for the diagnosis of TBM.

Wu et al. (166) used commercial antibody against *M bovis* in a sandwich ELISA and detected mycobacterial antigen in the CSFs of five patients with TBM. The ELISA was capable of detecting antigen as low as 4ng/ml of antigen. Absorbance for five patients with confirmed TBM ranged between 0.150-0.600 (0.27±0.19). For 134 patients with non-meningitic neurological diseases and 6 treated patients with probable TBM the absorbances were 0.032±0.009 and 0.029±0.10 respectively. Specificity of the ELISA was evaluated by the negative results (0.028±0.066) obtained with the bacterial and cryptococcal antigens. The authors emphasised that the ELISA will be useful for monitoring the effectiveness of anti-tuberculosis treatment in patients with TBM. The authors also observed that the chemotherapy may be responsible for the rapid decline of

antigen levels .in CSFs of their patients with TBM. The authors concluded that the detection of antigen is more useful than the detection of antibody as the detection of antibody will not differentiate patients with active TBM from patients with an inactive TBM.

Dhand et al (42) developed a sandwich ELISA for the detection of mycobacterial antigen in the CSFs of 20 patients with TBM, 9 patients with pyogenic meningitis and 15 others with a normal CSF. Double antibody sandwich method was employed in their study. Anti-BCG antibody was raised in rabbits by immunising the rabbit with 100 μg *M bovis* mixed with Freund's adjuvant. The second antibody was prepared from pooled immune sera from tuberculous subjects. The immunoglobulin was separated from the immune sera and was further purified by an affinity chromatography. The results showed 100% sensitivity in 7 culture-positive patients with TBM. Among the 13 culture negative patients, the assay was positive in 6 patients. The assay was negative in all the 9 patients with pyogenic meningitis but false positive results were seen in one case each of viral meningitis, neurocysticercosis, and enteric encephalopathy.

Mathai et al. (102) used Inhibition ELISA to detect mycobacterial antigen in CSF. They used CSF specimens obtained from 50 patients with a clinical diagnosis of TBM and 50 CSF specimens from patients with non- tuberculous neurological diseases as controls. A cell-free culture filtrate of $H_{37}Ra$ *M tuberculosis* was used as antigen in the study and the antibody used was rabbit IgG to culture filtrate antigen. Prior to patient sampling, the assay was standardised using different concentration of *M tuberculosis* Antigen 5 (1-1000ng/ml). The standardisation assay showed a detection limit of 5ng/ml.

The assay gave positive results in all the 10 culture confirmed cases of TBM and 28 out of 40 patients with probable TBM gave positive results. In these patients concentration of CFA concentration ranged from 14.5-290ng /ml, while in control CSF the mean concentration was 1.45± 3.9ng/ml. The sensitivity of the assay was 100% and 70% respectively, for culture positive and culture negative patients with TBM. The assay gave a specificity of 100%. Their study concluded that the detection of mycobacterial antigens in CSF is more sensitive and specific for the diagnosis of TBM.

Radhakrishnan et al. (130) used an inhibition ELISA to quantitate *M tuberculosis* antigen 5 in CSF specimen of 40 patients, with a clinical diagnosis of TBM. Out of the 40 patients, CSF from10 patients were culture positive (confirmed patients) and in 30 patients culture were negative (probable patients). The control group included CSFs from 40 patients with non-tuberculous neurological diseases. The antigen used was *M tuberculosis* Antigen 5 isolated from cell free culture filtrates of *M tuberculosis* strain $H_{37}Ra$ by immunoabsorbent affinity chromatography. The antibody, used was rabbit IgG to *M tuberculosis* Antigen 5. Prior to patient sampling, the assay was standardised using different concentration of *M tuberculosis* Antigen 5 (1-500ng/ml). The standardisation assay showed a detection limit of 5ng/ml. The assay gave positive results in all the 10 culture confirmed cases of TBM and 21 out of 30 patients with probable TBM gave positive results. In these patients, concentration of Antigen 5 concentration ranged from 9 to 82 ng /ml, while in control CSF the mean concentration of the antigen was 1.45ng/ml. They also studied correlation between clinical status and antigen concentration in serial CSF samples from eight patients with TBM during chemotherapy. Antigen concentration

showed a gradual decrease between 2-4 weeks after the commencement of therapy. Of the 8 patients studied, 5 patients showed good clinical recovery at the time of discharge from hospital. Thus they concluded that inhibition ELISA as specific method for the detection of mycobacterial antigens for the diagnosis of TBM. This method can also be used to monitor therapeutic response to ATT in patients with TBM.

Radhakrishnan et al. (129) also used Dot-Iba for the detection of mycobacterial antigen in CSF of patients with TBM and compared the sensitivity and specificity with that of inhibition ELISA. Study population included CSFs from 75 patients with clinically diagnosed as TBM and CSFs from 75 control patients with non-tuberculous diseases. 15 out of 75 patients with TBM were culture positive results, while the remaining 60 CSF samples were negative for *M tuberculosis* culture. The antibody, used was rabbit IgG to *M tuberculosis* Antigen 5. In the 15 culture positive patients with TBM, the Dot-Iba detected antigen only in 6 patients while in 60 culture negative patients with TBM Dot-Iba was positive in 30 patients. The Antigen 5 concentration in CSFs from the control group ranged between 1.05-3.1ng/ml. The overall sensitivity and specificity of Dot-Iba was 80% and 100%. They emphasised that the Dot-Iba have several advantage (a) the results can be interpreted visually, (b) there is no need for any sophisticated instruments. This observation is relevant in developing countries where the incidence and mortality rates of TBM are still high and the laboratory resources are limited.

Mastioianni et al. (101) standardised a dot-blot enzyme immunoassay for the detection of mycobacterial antigen in the CSFs of patients with TBM. A total of 38 CSF specimens were collected for the assay. 11 patients were diagnosed as having TBM. In 8

patients, TBM was proved bacteriologically by culturing the bacilli from the CSF. In 3 patients, the diagnosis was made on the basis of, clinical features together with the characteristic changes in the CSF (lymphocytosis, increased amount of protein and a reduced concentration of glucose) as well as response to antituberculosis treatment. 27 CSFs from patients with bacterial, fungal and viral meningitis were also examined. The dot-blot assay was performed in the NCP sheets. The CSFs were spotted onto the NCP. Following this, rabbit anti BCG-HRP conjugate was added and incubated for 2h. After washing, the substrate (0.3% 4-chloro-naphthol in anhydrous methanol plus 0.15 M PBS) was added. Positive reaction appeared as a well-defined blue dot against the NCP in a white background. In order to estimate the sensitivity of the dot-blot assay, sonicate antigen prepared between *M tuberculosis* was tested in concentrations ranging between 5 and 500ng/ml. The amount of antigen in CSFs detected by the dot-blot technique was 100 ng/ml and above. Mycobacterial antigen was demonstrated in all the CSF (11/11) from patients with TBM. 2 out of 25 CSF samples from patients with non-tuberculous meningitis also gave a positive reaction. Serial CSFs in patients with TBM following therapy showed a progressive decline in the concentration of antigen. Mastioianni et al. concluded that dot-blot assay is simple, sensitive and rapid for the early diagnosis of TBM. It can, be easily performed in clinical laboratories without the use of expensive apparatus and best suited to developing countries where the prevalence of TBM is still high.

2.3 POLYMERASE CHAIN REACTION FOR THE DIAGNOSIS OF TUBERCULOUS MENINGITIS

Advent of molecular biological techniques such as Polymerase chain reaction (PCR) has opened a new avenue for the laboratory diagnosis of tuberculosis. In paucibacillary form of tuberculosis, such as tuberculosis of the central nervous system, the concept of amplifying specific genomic mycobacterial DNA to detectable level appears attractive (7). The published reports of the PCR studies for the laboratory diagnosis of TBM are shown in Table 2-3.

Table 2-3: **PCR for the diagnosis of TBM- A review**

Authors	Target Sequence
Kaneko et al. (1990)	MPB 64
Sankar et al. (1991)	MPB 64
Donald et al. (1993)	IS6110
Lee et al. (1994)	MPB 64
Folgueria et al. (1994)	IS6110
Liu et al. (1994)	MPB 64
Lin et al. (1994)	MPB 64
Lin et al. (1995)	MPB 64
Kox et al. (1995)	IS6110
Nguyen et al. (1996)	IS986
Miorner et al. (1995)	IS6110
Seth et al. (1996)	MPB 64
Melzer et al. (1999)	IS6110
Caws et al. (2000)	IS6110
Narayanan et al. (2001)	IS6110 & TRC_4

The use of primers derived from the insertional sequence IS6110 and gene sequence encoding for MPB64 protein of *M tuberculosis*, have been used in most of the published studies. However the sensitivity of PCR for tuberculosis in CSF have showed great variation ranging between 33-99.5 % in these published studies.

Brisson and Noel in 1989 (14) were first to introduce PCR technique for the rapid diagnosis of tuberculosis. They used primers, which amplified a 383 base pair DNA fragment that encoded the 65kDa mycobacterial antigen. They collected 35 clinical specimens (sputum, gastric aspirate, biopsy sample) from 34 patients in whom tuberculosis was clinically suspected. PCR yielded positive results in 15 specimens and in the remaining 20 specimens, the PCR yielded negative results. They emphasised that the new amplification method possess several advantages over bacteriological method for the laboratory diagnosis of TBM.

Kaneko et al. (74) used PCR for the rapid diagnosis of TBM. They studied six patients who were clinically diagnosed as tuberculous meningitis. The controls included in these study were 10 patients with bacterial and viral meningitis; 10 CSFs from normal subjects. Mycobacterial DNA in CSF samples was extracted by treating the CSF samples with SDS and proteinase K, followed by phenol chloroform extraction and ethanol precipitation. Target sequence was a region in the gene sequence encoding for MPB64 protein of *M tuberculosis*. The PCR products were analysed on ethidium bromide stained agarose gel electrophoresis, followed by Southern blot hybridisation. Positive PCR were obtained in 5 out of 6 CSF specimens of TBM. PCR gave negative results in the control

group. The PCR assay gave an overall sensitivity and specificity of 83.3% and 100% respectively for the laboratory diagnosis of TBM.

Shankar et al (149) in their study of TBM compared PCR with conventional bacteriological method and also with an ELISA for antibodies to *M tuberculosis*. Based on the clinical features, CSF findings and results of bacteriological methods, they classified 34 TBM patients as highly probable (n=20), probable (n=7) and possible (n=7) cases. Mycobacterial DNA was extracted from the centrifuged deposits of CSF, using the conventional method. A 240 bp region in the gene coding for MPB 64 protein was selected for amplification of mycobacterial DNA. In ELISA the mycobacterial antigen used for the detection of antimycobacterial antibodies in CSF, was *M tuberculosis* saline extract of (MTSE). Of the 34 CSF samples none of them were smear positive, while culture gave a sensitivity of 11.7% (4/34). ELISA gave a sensitivity of 44% (15/ 34). The sensitivity of PCR was 75%(15/20) in highly probable cases of TBM, 57%(4/7) in probable cases and 43%(3/7) in possible cases of TBM. The overall sensitivity of PCR was 65% (22/34). Six control subjects initially tested were PCR positive but the DNA preparations from their stored CSF samples were all PCR negative, suggesting that the false positive results in the control were due to cross contamination during the PCR assay. In view of this, Shanker et al. emphasised the potential application of PCR for the laboratory diagnosis of TBM.

Donald et al (45) used PCR for the rapid diagnosis of TBM in patients in the paediatric age group. The assay detected a 123bp region from the *M tuberculosis* complex specific IS6110. Of the 43 CSF specimens from 20 children, 27 (63%) yielded

positive results, 9 (21%) equivocal results and in 7 (16%) cases PCR were negative. Positive results were recorded during the third and fourth weeks after the commencement of therapy. According to Donald et al. the reason for the equivocal results in 9 CSF samples may be due to technical error in sample preparation, procedure inadequate amount of specific template in the sample.

Lee et al (88) evaluated PCR for the rapid diagnosis of tuberculous meningitis. They collected 19 CSF clinical samples from 6 TBM patients and 13 CSF samples from disease controls. Of the 6 TBM patients, 4 were culture positive and two were diagnosed as TBM based on positive response to ATT. A 240 bp region from a gene coding for MPB 64 protein, specific for *M tuberculosis* complex was selected for amplification. PCR detected mycobacterial DNA in 5 of the 6 CSF samples from TBM and one CSF became positive only when PCR was followed by Southern blot hybridization. Thus PCR gave positive results in all the 6 TBM patients. 12 of the 13 disease controls gave negative results in the PCR. Lee et al. emphasised that the sensitivity of the PCR technique is dependent on the number of amplification cycles and they found 40 cycles to be optimum.

Folgueria et al (53) used PCR for the detection of *M tuberculosis* DNA in 11 CSF samples from 10 HIV seropositive patients in whom TBM was suspected. The PCR assay detected 123 bp region from the *M tuberculosis* complex specific insertion sequence IS6110. The PCR products were analysed on Agarose gel and followed by Southern blot hybridisation using an endlabelled probe. The culture for *M tuberculosis* was positive in 5 out of eleven CSFs and in one patient auramine-rhodamine stain was positive for *M*

tuberculosis. PCR was positive in all those patients in whom smear or culture were negative. None of the non-tuberculous patients specimen were positive by PCR. Their study emphasised sensitivity of PCR for the rapid laboratory diagnosis of TBM in HIV patients.

Liu et al (92) developed a simplified protocol based on nested PCR for early laboratory diagnosis of TBM. Nested PCR applied two sets of primers i.e., an outer primer capable of specifically amplifying the gene sequence encoding the MPB64 protein of *M tuberculosis,* while the inner primers amplified 20 base oligonucleotides corresponding nucleotide 381-500 and 680-661 of the gene sequence encoding the MPB64 protein. For verification, the PCR products were subjected to digestion with restriction enzymes Sma I, which yielded two distinct bands of 128bp and 72bp. Using this protocol they detected *M tuberculosis* genomic DNA within 24 h in the CSF of the 19/21 TBM patients. Culture method yielded positive results only in 6 (28.5%) patients. The PCR gave negative results in all 79 patients with non-tuberculous meningitis subjects. According to Lee et al. nested amplification is more sensitive for the detection of *M tuberculosis*. This may be due to the low copy number of the DNA in CSF samples of the TBM patients. Compared with the conventional single-step PCR, nested amplification can enhance the sensitivity approximately to 1000-fold. In addition, the use of restriction enzyme *Sma* I to digest the PCR products appear to be much simpler and faster than hybridisation with a radiolabelled probe. Thus they concluded that nested amplification protocol is more useful for the rapid diagnosis of TBM.

Lin et al (90) used primers that amplified 240bp region within the gene fragment within the gene, which encoded for specific MPB64 protein of *M tuberculosis* complex for the rapid diagnosis of TBM. They studied 47 CSF samples from 45 patients i.e. twenty CSF samples from patients with clinically suspected TBM and another 27 CSF samples from clinically not suspected TBM. Mycobacterial DNA was detected in 14 CSF from clinically suspected group. None of the CSF samples were positive by direct smear, while 4 samples gave positive culture results Sensitivity and specificity of PCR was 70% (14/20) and 100%, while that of culture method showed 15% and 100%. In their study, one highly probable TBM patient had repeated CSF analysis and PCR was positive on the third CSF sample. They regard the negative PCR results of earlier CSF samples may be due to inadequate number of tubercle bacilli and that the number of tubercle bacilli increase with the course of the illness. According to them repeated CSF studies should be done in clinically suspected TBM patients.

Lin et al (89) studied the application of PCR to monitor *M tuberculosis* DNA in the CSF of patients with TBM after ATT. Their assay amplified a 240bp sequence encoding for specific MPB64 protein. PCR was used to detect *M tuberculosis* DNA in 29 CSF specimens collected from 7 patients with TBM after the commencement of ATT. Ten of the 13 CSF specimens were positive for PCR within the initial treatment for three weeks. By contrast only one of the remaining 16 CSF specimens was positive who received treatment for more than three weeks. They concluded that PCR can not only be successfully used for the diagnosis of patients with TBM, but also to monitor *M tuberculosis* DNA in the CSF following ATT in patients with TBM.

Kox et al (81) evaluated an improved PCR technique for the rapid and specific identification of *M tuberculosis* in CSF. The target for amplification was a nucleotide sequence located within IS6110. As a modification to the PCR protocol they used a small amount of DNA from *M smegmatis* strain 1008, containing modified IS6110 to PCR reaction mix as an internal control for inhibitors of PCR. The study samples included CSF samples from 42 patients diagnosed on the basis of clinical symptoms and signs, radiological and CSF findings. They compared the results of PCR assay with that of culture and acid-fast staining methods. The sensitivity of PCR, culture and acid fast smear was 48%, 39%, 9% and the specificity was 100%, 100%, 95% respectively. Their study also emphasised the importance of sample volume for PCR. They were able to detect *M tuberculosis* in CSF from a patient who had received antituberculous treatment for 6 weeks as the PCR assay in this patient was done with a CSF sample volume of 5ml. According to them, for PCR, the ideal minimum volume of CSF is 2 ml, to detect *M tuberculosis*.

Nguyen et al. (120) examined the diagnostic utility of PCR in CSF of patients with tuberculous meningitis. The primers used in their assay detected a sequence within IS6110 specific for the members of the *M tuberculosis* complex. *M smegmatis* 1008 DNA containing modified IS6110 was used for detection of inhibitors of the PCR. Prior to CSF sampling the stability of *M tuberculosis* DNA was tested by adding 10 fg of DNA to each of three samples stored at three different temperatures. The samples were stored for 8 weeks at room temperature, 37°C and −20°C or even if at −20°C were thawed and frozen 10 times, no damage in DNA was found. Their study included 136 patients initially

suspected of having TBM. They classified the TBM patients into confirmed TBM, n=39 (based on positive Ziehl Neelsen staining, culture or PCR results for *M* tuberculosis) and probable TBM, n=60 (based on clinical features, response to ATT treatment or evidence of extraneural tuberculosis). For small CSF volumes, DNA was extracted using guanidium thiocyanate method. In 99 patients with a diagnosis of confirmed and probable TBM the sensitivity of PCR, culture and microscopy was 32%, 17% and 1% respectively. Among the 39 patients with confirmed TBM, PCR was positive in 32 patients (82%), 17 (44%) had positive culture results, while only one case was proven positive by microscopy. There were no false-positive PCR results. According to them, the explanation for the low sensitivity of PCR was due to smaller volume of CSF (mean volume 400 μl) available for the assay. They recommend that an adequate volume of CSF (>2 ml) essential for the PCR assay in TBM patients.

Miorner et al (109) compared three immunoassays, an immune complex assay and PCR for the diagnosis of TBM. Clinical samples included 33 CSF samples from 33 patients, with a clinical diagnosis of TBM and 34 CSF samples from 34 patients with various other diseases of the central nervous system. They used mycobacterial antigens MTSE (10 mg/l), LAM (0.1 mg/l) and 38 kDa (1mg/l) in the ELISA for the detection of antibodies to mycobacterial antigens and applied a sandwich ELISA method for the detection of mycobacterial immune complexes in CSF specimens. PCR amplified a 263 bp fragment in IS6110 gene specific for *M tuberculosis* complex and recorded positive results in 54% with TBM. They also observed when the results of PCR combined with

immune-complex assay, yielded a sensitivity of 100% in culture positive and 74% in culture negative samples from patients with TBM.

Seth et al. (148) applied a double blind study to evaluate the efficacy of PCR for the rapid diagnosis of TBM. The assay amplified a specific sequence in gene MPB64. CSF samples from 40 patients with TBM and from 49 patients with other neurological disorders were studied. Based on the clinical characteristics the patients were categorised into highly probable (n=4), probable (n=20) and possible TBM (n=16), PCR was evaluated for each category of patients. PCR was positive in 2/4, 19/20, 13/16 patients with highly probable, probable and possible TBM respectively. According to them the reason for the two negative results among the highly probable group was because these two patients received ATT for more than 2 months prior to the assay. None of the CSF samples were positive by conventional bacteriological methods. In their assay 3/49 CSF samples from non-TBM patients were also found positive by PCR. Thus the sensitivity of PCR was 85% and the specificity was 93.8%.

Melzer et al (106), in a case study reported false negative PCR results in CSFs from culture proven patients with TBM. They emphasised the danger of overreliance on CSF-PCR in patients with suspected TBM. According to Gascoyne-binki et al. (56), the low sensitivity of PCR for TBM may be (a) due to the presence of inhibitors of PCR, (b) poor lysis of mycobacteria and uneven distribution of mycobacteria in the clinical specimens.

In a prospective, clinical, microbiological and molecular analysis of a national molecular diagnostic service for TBM, Caws et al. (18) used an in-house IS6110 targeted

PCR for molecular "Fastrack" diagnosis. Among the culture confirmed TBM cases, the sensitivity of PCR was 75% (3/4) and the specificity was 94%. The overall sensitivity of PCR in suspected cases of TBM was 34.7% (8/23). Accordingly they concluded PCR can provide and acceptable sensitivity in comparison to culture method for the diagnosis of TBM.

Narayanan et al. (117) evaluated a new set of primer TRC_4 developed in their laboratory and used them in PCR for the laboratory diagnosis of TBM and compared with the PCR results using IS6110 primer. The level of concordance between the results of IS6110 and TRC_4 in patients with clinically confirmed TBM was 80% and 86% respectively. They concluded that the sensitivity of PCR could be increased using both IS6110 and TRC_4.

Amplification studies differ in the clinical specimen for the study, amplification techniques including, lysing methods, target nucleic acid, primers and procedure used to detect amplified products etc. In order to overcome these difficulties two PCR commercial kits are available and they are discussed below.

A) PCR Amplicor MTB (Roche Diagnostic Systems, Somerville, N. J.)

COBAS Amplicor PCR (Roche Diagnostic Systems, Somerville, N. J.)

B) Transcription mediated amplification (Amplified *M tuberculosis* Direct Test (AMTD): Gen-Probe, San Diego, California)

These commercial kits are in use for the rapid diagnosis of tuberculous meningitis. Its utility is evaluated by the studies conducted by Pfyffer et al. 1996 (124), Ehlers et al. 1996 (46), Bonigton et al, 1998 (12), Lang et al. 1998 (86).

Table 2-4: **Molecular diagnostic kits used in the diagnosis of TBM**

Authors	Commercial kits used
Pfyffer et al. (1996) Ehlers et al. (1996) Lang et al. (1998)	Amplified *M tuberculosis* Direct Test (MTD) (Gen-Probe)
Bonington et al (1998) Reischl et al. (1998)	PCR Amplicor MTB and COBAS Amplicor PCR (Roche Diagnostic Systems)

Pfyffer et al. (124) conducted their study to develop a more sensitive and rapid for the diagnosis in a clinical situation in which only a few bacilli are present. They evaluated the diagnostic performance of AMTD with other Non-respiratory and respiratory specimens. Prior to CSF analysis MTD was standardised with artificially spiked dilution series of CSF using *M tuberculosis* bacilli. The standardisation assay detected as high as 100 cells/ ml to as low as 5 cells / ml. The overall sensitivity and specificity in their studies were 93.1% and 97.7% respectively for CSF.

Ehlers et al. (46) also used AMTD for the rapid diagnosis of TBM. Of the 51 CSF specimens examined, one was shown to contain acid-fast bacilli by auramine staining and five were positive by AMTD, one sample from control group gave false positive test result. The sensitivity and specificity of AMTD for the diagnosis of TBM was 66.7% and 97.8% respectively.

Lang et al. (86) studied 84 CSFs from paediatric patients with TBM and they used Gene Probe MTD. All the CSF was negative for acid-fast bacilli in Ziehl-Neelsen stained

smear. *M tuberculosis* was cultured in 5 patients. The sensitivity of MTD test was 33% and a specificity of 100% for the detection of *M tuberculosis* in the CSF samples. They consider MTD test as more sensitive than culture method.

Bonington et al. (12) performed a prospective study to assess the Roche Amplicor *M tuberculosis* PCR test (TB AMPLICOR) for the diagnosis of TBM and compared the results with Ziehl-Neelsen stained smears, radiometric culture for *M tuberculosis,* clinical and CSF findings. The sensitivity and specificity of Ziehl-Neelsen staining and radiometric culture was 20% and 7.5% respectively. The overall sensitivity of TB Amplicor was 25%. CSF sample were assayed after 9 days commencement of ATT, the sensitivity was increased to 28.6% and 60% for the detection of mycobacterial DNA in definite and probable cases of TBM. They concluded a) TB Amplicor is more sensitive than the combination of Ziehl Neelsen staining and radiometric culture for *M tuberculosis* b) PCR can be used as a rapid and specific diagnostic test for TBM. However, the sensitivity is often restricted by low concentration of mycobacteria within the CSF of patients with TBM. c) In order to increase the sensitivity, CSF should be collected before the commencement of ATT treatment. c) Sensitivity can further enhanced by increasing the volume of CSF used to 1 or 2ml.

Reischl et al (135) evaluated the COBAS Amplicor PCR system (Roche Diagnostic) for the routine detection of *M tuberculosis* complex in clinical samples. The concept behind the COBAS Amplicor PCR system is that, amplification and detection steps were fully automated. The sensitivity and specificity of COBAS Amplicor *M tuberculosis* assay were determined to be 82.3% and 98% respectively.

To conclude, PCR has an accepted role in the detection of *M tuberculosis* in clinical specimens of pulmonary tuberculosis, such as sputum but is not yet fully evaluated for the diagnosis of TBM. The sensitivity of PCR on CSF samples seems to be only a moderate improvement than that of culture. The specificity is comparable, but scrupulous laboratory techniques are needed to avoid DNA contamination. Large volumes of CSF are essential for the yield of positive PCR results. For these reasons the PCR is acknowledged to be inappropriate for its application in the developing world and current studies suggest that PCR does not solve the diagnostic challenge in TBM.

2.4 IMMUNOHISTOCHEMICAL AND MOLECULAR BIOLOGICAL TECHNIQUES FOR THE DIAGNOSIS OF TUBERCULOMA - A REVIEW

2.4.1 Immunohistochemical techniques for the demonstration of mycobacterial antigens in the formalin fixed paraffin embedded sections of intracranial tuberculoma

There are only 3 major published studies, which highlight the application of immunohistochemical (IHC) method for the demonstration of mycobacterial antigens in tuberculous lesion.

Humphery et al. (62) applied an IHC method to demonstrate mycobacterial antigens in the formalin fixed paraffin tissue sections of caseating pulmonary tuberculoma. They used rabbit immune serum to culture filtrate antigen of *M tuberculosis* as the primary antibody. The immune rabbit antibody to *M tuberculosis* was further

purified by bioaffinity immunoabsorbent chromatography. The formalin fixed paraffin sections of tuberculous lesions were then treated with primary antibody rabbit immune sera to *M tuberculosis* 4-5h at 4°C. This was followed by incubation with antirabbit IgG-biotin and rabbit–antiperoxidase complex. Subsequently the paraffin sections were incubated with a substrate i.e. DAB. In order to evaluate the specificity of the immunostaining, sections were incubated with pre immune rabbit serum instead of immune rabbit serum to *M tuberculosis*. This substitution gave negative results in all the specimens of tuberculosis. Distribution of mycobacterial antigen in the tuberculous lesions was demonstrated by the presence of brownish-pink granular particle within the cytoplasm of Langhan's giant cells and macrophages. These were not present in cases of fungal granuloma. In the caseous zones, clusters of mycobacterial antigen staining masses were also present and they were clearly demarcated from the adjacent amorphous caseous necrosis. The authors concluded that the detection of mycobacterial antigens is useful for establishing mycobacterial aetiology in cases of caseating pulmonary granuloma.

Barbolini et al. (8) emphasised the utility of IHC method for the detection of mycobacterial antigens in tuberculous granuloma. They used four monoclonal antibodies MAb 60.25, MAb 61.3, MAb 105.10 and MAb 2.16, which were directed to different proteins of *M tuberculosis.* An indirect avidin-biotin complex peroxidase-antiperoxidase method was used to detect mycobacterial antigens in the tuberculous granuloma. Mycobacterial antigens were demonstrated in all cases of tuberculosis in the form of rods

and fragments within the cytoplasm of macrophages in granuloma. They have concluded that immunohistochemical detection of mycobacterial antigens appears to be useful in establishing mycobacterial aetiology of caseating granuloma.

Radhakrishnan et al. (131) in their study evaluated the diagnostic utility of immunohistochemical techniques for the demonstration of mycobacterial antigens in intracranial tuberculoma and they compared its efficacy with those of conventional Z-N staining method in the formalin fixed paraffin sections. Ten surgical specimens of intracranial tuberculoma were selected for the study. The specificity of immunohistochemical technique was assessed in non-tuberculous granulomatous lesions. Primary antibody used in that study was polyvalent rabbit antibody to CFA $H_{37}Ra$ of *M tuberculosis*. Immunohistochemical studies for the demonstration of mycobacterial antigens were performed by the peroxidase-antiperoxidase method. All the ten specimens of intracranial tuberculoma were negative for acid-fast bacilli, while they were positive for the presence of mycobacterial antigens by the IHC method. They observed two types of distribution i.e. mycobacterial antigens in non-caseating zone, which were characteristically located within the cytoplasm of macrophages, giant cells and appeared as diffusely stained granular brownish-pink material. The second type distribution was observed in the caseous zones in which clusters of mycobacterial antigen stained masses were present and this was well demarcated from the amorphous necrotic debris. In both caseous and non-caseous zone, characteristic size and shape of acid-fast bacilli was not identified. According to them, this could be due to the phagocytic activity within the granuloma, resulting in distortion and fragmentation of bacilli. In none of the non-

tuberculous granulomatous lesions, characteristic intracytoplasmic localisation of mycobacterial antigens was present. Their study indicated that immunohistochemical technique is far more sensitive than conventional Ziehl-Neelsen method and is specific to distinguish cases of tuberculoma from non-tuberculoma aetiology in a caseating granulomatous lesion. Secondly this method is simple for its application in any routine laboratory.

2.4.2 PCR for the detection of mycobacterial DNA in formalin fixed paraffin embedded tissues of intracranial tuberculoma

PCR has been regarded as a useful tool for the laboratory diagnosis of tuberculosis. PCR has been performed with clinical specimens such as sputum, CSF, pleural fluid, ascitic fluid. This has widened its utility in formaline fixed paraffin embedded tissues. Several studies have been conducted to evaluate the utility of PCR for the detection of genomic DNA of *M tuberculosis* in formaline-fixed paraffin embedded tissues. Most of the studies have applied PCR in tuberculous lesions of lungs (99), lymphnode (118). So far only three reports emphasised the utility of PCR for the diagnosis of intracranial tuberculoma.

Isenmann et al. (65) were the first to apply PCR for the diagnosis of intracranial tuberculoma. They detected mycobacterial DNA in formalin fixed paraffin embedded sections of intracranial tuberculoma. Neuroimaging procedure such as CT and MRI scans in their patient was suggestive of meningioma. Histopathological examination of surgical specimen revealed granulomatous lesion. The diagnosis of tuberculoma was confirmed by isolation of *M tuberculosis* in culture. The patient recovered after surgery and

chemotherapy. They further confirmed the aetiology of tuberculous by PCR and detected mycobacterial DNA in the formalin fixed paraffin embedded tissue. They used two primers to amplify the target DNA sequences i.e. IS6110 and M65 specific for 65 kDa mycobacterial antigen. Thus their study highlighted that tuberculoma can mimick with the other space occupying lesions of CNS and detection of mycobacterial DNA using PCR may be a useful tool in the diagnosis of intracranial tuberculoma.

Monno et al. (115), emphasised the resurgence of tuberculosis is related to emergence of HIV epidemic. CNS involvement is five times higher in HIV patients; PCR can be used as a tool for the diagnosis intracranial tuberculoma by detecting mycobacterial DNA in CSF patients with intracranial brain lesion in whom the CSF examination did not grew *M tuberculosis* in culture. Their study included HIV positive patients with intracranial brain lesion without meningeal involvement (n=5), patients with extra-pulmonary tuberculosis without any brain involvement (n=6). Control subjects included intracranial brain lesion due to non-Hodgkins lymphoma (n= 3) and non-tuberculous infectious lesions such as cryptococcus (n=2), cytomegalovirus (n=2), cerebral toxoplasmosis (n=15). PCR was performed on CSF samples in all these patients. They used a primer, which amplified a 123 bp region within IS 6110 sequence of *M tuberculosis. M tuberculosis* DNA was present in CSF of patients with intracranial tuberculous lesions. While all the other CSF samples including those from control group gave negative results.

Singh et al. (151) reported a case of en-plaque tuberculoma diagnosed by MRI findings. This was either confirmed by a PCR assay, which was based on the

amplification 513bp of devR gene sequences, which encodes for regulatory protein of *M tuberculosis.* En-plaque tuberculoma is an extremely rare manifestation of CNS tuberculosis and demonstration of *M tuberculosis* by PCR is crucial in confirming the diagnosis. Their study concluded that PCR could be used as a routine diagnostic tool and holds promise for the rapid detection of *M tuberculosis* in paucibacillary clinical specimens such as CSF.

MATERIALS AND METHODS

CHAPTER-III

3.1 STUDY POPULATION

3.1.1 Group I- Patients with TBM

CSF samples were collected from 40 in-patients admitted to Neurology services of this institution with a clinical diagnosis of TBM. This hospital is a major tertiary referral center in Kerala State for neurological diseases and all the patients included in this study were referred from several outlying hospitals in and around Kerala and Tamil Nadu States.

A diagnosis of TBM in these patients was considered on the basis of relevant clinical manifestations of the disease i.e. history of fever, headache, vomiting, presence of neck stiffness, Kernig's sign and supported by compatible cytological and biochemical parameters in CSF namely pleocytosis, lymphocytosis, elevated protein (> 40mg %), reduced sugar (< 40mg % or less than 40% of the corresponding blood sugar level) as well as optimum clinical response following a course of ATT (3-4 weeks). A proforma was prepared for every patient to record the relevant clinical data as well as laboratory investigations (Appendix - D). Patients with a clinical diagnosis of TBM were further sub divided into a) "Confirmed" TBM, b) "Probable" TBM. "Confirmed" cases of TBM are patients in whom *M tuberculosis* was demonstrated either in the Z-N stained smears of CSF or by the culture. "Probable" cases of TBM are the patients in whom the clinical and CSF cytological and biochemical features were suggestive of TBM and these patients showed optimum clinical response following a course of ATT. However in none of the

patients with "probable" TBM, *M tuberculosis* was isolated in their CSFs on repeated cultures of CSF.

3.1.2 Group II- Patients with Non-TBM control (Disease control)

Disease control- CSFs were collected from 38 in-patients with a clinical diagnosis of bacterial meningitis (n=15), partially treated pyogenic meningitis (n=10), fungal meningitis (n=5) and viral encephalitis (n=8). The clinical features and CSF cytological parameters in patients with partially treated meningitis closely resembled with that of TBM and a distinction between these two diseases could not be made with any degree of certainty.

Non-disease control- CSF samples from 2 non-disease controls were also collected. Non-disease control included two patients who had received spinal anesthesia for neurosurgical procedure. During lumbar puncture, CSF samples were collected.

3.2 SPECIMEN COLLECTION

3.2.1 Collection of CSF

At the time of admission in the hospital 2-3 ml of lumbar CSFs were collected under aseptic conditions, in two separate sterile containers from all the patients with TBM and control groups. The CSF specimen in the first container was centrifuged and the smear was examined for acid fast bacilli under Z-N preparation and the reminder of the deposits were inoculated onto L-J medium for culturing *M tuberculosis.* The CSF in the second container was used for immunocytochemical, Dot-Iba and PCR studies.

Owing to the retrospective nature of the study, the CSF samples were stored at - 20°C until use. The CSF studies in 16 patients were repeated on two occasions.

3.2.2 Collection of tissues from neurosurgical material

Surgical specimens from ten cases of intracranial tuberculoma as well as non-tuberculous (fungal) granuloma were collected. These were used for the immunohistochemical demonstration of mycobacterial antigens as well as PCR for the detection of mycobacterial DNA in the lesions.

3.3 PREPARATION OF MYCOBACTERIAL ANTIGENS

Three mycobacterial antigens were prepared from culture filtrates of *M tuberculosis*. The methodologies involved in the preparation of each of the antigens are described below.

3.3.1 Preparation of culture filtrate antigen

Culture filtrate antigen (CFA) was prepared from cell-free culture filtrates of $H_{37}Ra$ strains of *M tuberculosis* (29). This strain was obtained from Tuberculosis Research Center (TRC) Chennai. Pellicle cultures from 10-14 days old "seed" cultures were grown on Sauton's medium for 8-10 weeks. At optimum growth, the cultures were initially clarified by filtration through Whatmann paper I and then Whatmann IV and finally through 0.45 μm millipore membrane. The cell-free culture filtrate was dialysed repeatedly for 48-72h against distilled water. Following that the CFA was concentrated to ten-fold using an ultrafiltration unit (Amicon –GMBH; Witten, Germany). The protein content of the dialysed CFA was estimated by Lowry's method (94). The CFA was reconstituted to 5mg/ml, dispensed in aliquots and stored at -20° C. Sodium merthiolate was added (1:10,000) as a preservative.

3.3.2 Isolation of specific mycobacterial antigen using immunoabsorbent affinity chromatography

10-15ml of cisternal CSF from a culture-proven patient with TBM was collected at autopsy. IgG content in these CSF was estimated, using single radial immunodiffusion

method. The IgG component in the CSF was collected, by passing the CSF through Protein A- Sepharose 4B column. The elute was repeatedly dialysed against 0.15 M PBS (pH 7.4) and concentrated to ten fold using an ultrafiltration unit. The protein content of the elute was estimated, reconstituted to 3 mg/ml and stored in aliquots at -20° C. The CSF-IgG to CFA was estimated using an indirect ELISA and this gave an end point antibody titer of 1:64000.

Immunoabsorbents were prepared (33) with activated Cyanogen Bromide-Sepharose 4B (Sigma Chemicals, St. Louis Mi USA). One gram of Cyanogen Bromide – Sepharose 4B was allowed to swell to 3.5ml in double distilled water and washed with large volumes (20 times original gel volume) of cold 0.1M Sodium bicarbonate buffer (pH 9). It was then suspended as a slurry of 50% (w/v) by adding 0.1M Sodium bicarbonate buffer (pH 9). Human CSF-IgG (3mg/ml) to *M* tuberculosis was dialysed against 0.1M Sodium bicarbonate buffer containing 0.5 M Sodium chloride (pH 9). CSF-IgG to *M* tuberculosis was added in equal volume to activated Cynogen Bromide-Sepharose 4B and the immunoabsorbents were incubated for 16h at 4°C. Excess IgG in the immunoabsorbent slurry was washed with large volumes (60 ml) of 0.1M Sodium borate (pH 9) alternating with 0.1 M Sodium acetate (pH 5) for five cycles starting and finishing with 0.1 M Sodium borate buffer. The immunoabsorbents were finally suspended in 0.15 M PBS, poured into chromatography column (1cm in diameter) and equilibrated in the column with 0.15 M PBS. The column was washed alternatively three times with 0.15 M PBS and 4 M Urea in 0.15 M Sodium bicarbonate buffer in order to

minimise 'leaching out' of gamma globulin from the immunoabsorbent column. CFA of $H_{37}Ra$ *M tuberculosis* (5mg/ml) was passed through the immunoabsorbent column. The column was run with 0.15 M PBS. Every 10 min, a one ml fraction was collected until a blank reading at 280 nm was obtained. The specific mycobacterial antigen that bound to the immunoabsorbent column was eluted with 4M Urea in 0.15 M Sodium carbonate buffer and the absorbance of fraction at 280 nm was recorded with a spectrophotometer (Shimadzu; Japan). Fractions with absorbance of >0.05 were pooled, dialysed against 0.15 M PBS and concentrated using an ultrafiltration unit. The protein content was estimated by Lowry 's method, dispensed in sterile aliquots (200 µg/ml) and stored at –20°C.

3.3.3 Preparation of tuberculin PPD

PPD antigen was prepared (95) from $H_{37}Ra$ *M tuberculosis.* Pellicle cultures from 10-14 day old "seed culture" were grown on Soutan's medium for 8-10 weeks. At the optimum growth, the cultures were filtered through Whatmann filter paper IV and 0.45 µm pore-sized millipore membrane. The culture filtrate was autoclaved at 120°C for 30 min and ammonium sulphate was added (297 g / liter of culture filtrate) to bring the saturation at 50 per cent. The precipitate was recovered by repeated centrifugation and reconstituted to 10ml in PBS. The reconstituted material was dialysed against PBS for 48h at 4°C. The protein content was measured, dispensed in aliquots and stored at -20°C.

3.4 CHARACTERISATION OF MYCOBACTERIAL ANTIGENS

3.4.1 SDS-PAGE

The CFA and affinity column purified antigen isolated from *M tuberculosis* were further characterised using Sodium dodecyl sulphate- polyacrylamide gel electrophoresis [SDS-PAGE (85)].

The electrophoresis was performed using a "Mighty small" electrophoresis unit (Pharmacia, Biotech Asia Pacific Ltd; Hong Kong). The dimensions of the gel slabs were 0.75x85x80 mm. The stacking gel contained 4% Acrylamide and 0.1% SDS (Sigma chemicals) in 0.15 M Tris-hydrochloride buffer (pH 6.8). The separation gel contained 12% Acrylamide and 0.1% SDS in 0.375 M Tris hydrochloride buffer (pH 8.8). The cross-linker was 2.6% N, N, Methylene bis acrylamide. 50 µg of CFA and 80 µg of affinity purified mycobacterial antigen were diluted 1:1 in the sample buffer [2% SDS, 5% mercaptoethanol, 10% glycerol and 0.02% bromophenol-blue in 0.06M and tris-hydrochloride buffer (pH 6.8)]. Both the mycobacterial antigens were incubated at 100°C for 5 min before they were applied to the gels. The electrophoretic tank contained 0.025M Tris 0.192M Glycine and 0.1% SDS (pH 8.3). The standards used as molecular weight markers were a) 94 kDa (Phosphorylase b), 67 kDa ((Bovine serum albumin), c) 30 kDa (Carbonic anhydrase), d) 20 kDa (Trypsin inhibitor), e) 14 kDa (∝-Lactoglobulin). Electrophoresis was carried out at a constant voltage of 100V for 2 h connected to an electrophoretic power pack (Pharmacia Biotech Asia Pacific Ltd.).

3.4.2 Staining with Coomassie-brilliant blue R-250.

After electrophoresis, the gels were stained with Phast Gel Blue R (Coomassie R 350 stain/ Pharmacia Biotech) for the demonstration of various protein components (55). The gel was fixed in 40% methanol and 10% Trichloroacetic acid for 1h before it was immersed in the staining solution. The gel was then stained in 0.1% Coomassie brilliant blue dissolved in 40% methanol and 10% acetic acid for 30 min. It was then destained with a solution containing equal volumes of 40% methanol and 10% acetic acid. The destaining solution was changed several times, until the background became clear against which the protein band appeared more prominent.

3.5 IMMUNISATION SCHEDULE FOR RAISING ANTIBODIES AGAINST MYCOBACTERIAL ANTIGENS

3.5.1 Raising polyvalent antiserum to CFA. (31)

Two adult male rabbits served as a source of antibodies against *M tuberculosis*. During primary immunization, each rabbit received 2 ml of water in oil emulsion that contained 2mg of CFA, 2mg of dried autoclaved bacillary sediment of *M tuberculosis*, mixed thoroughly with incomplete Freund's adjuvant (Sigma chemicals). The inoculum was injected in divided doses into two gluteal intramuscular sites and into 2 flank subcutaneous sites. The immunization schedule was repeated 14th, 21st, 28th, 35th day following primary immunization. The antibody titre was sequentially assessed during the immunisation schedule by immunoelectrophoresis and ELISA. A persistent and high

antibody titre could be demonstrated following fifth immunization schedule. 10 ml blood was collected from ear vein of rabbit and serum was separated, dispensed in aliquots and stored at –20°C.

3.5.2 Raising antibody to affinity column purified mycobacterial antigen (33)

Two adult male rabbits served as the source of antibody. For the primary immunisation, each rabbit was immunized with 3ml of water in oil emulsion that contained 1ml of affinity purified antigen (100µg), 1ml of incomplete Freund's Adjuvant and 1ml of *Bordetella pertusis* vaccine. The inoculum was injected subcutaneously at multiple sites. The secondary immunisation was repeated on the 14th day following the primary immunisation. Booster doses were given at weekly interval. High antibody titer was demonstrated on IEP following the 3rd booster dose. 10 ml venous blood was collected from ear vein, serum was separated and stored at –20°C.

3.6 EVALUATION OF ANTIBODIES RAISED IN RABBITS AGAINST CULTURE FILTRATE AND AFFINITY COLUMN PURIFIED ANTIGENS.

3.6.1 `Evaluation of antibodies to *M tuberculosis* by Immunoelectrophoresis. (IEP) (67)

IEP was performed on LKB horizontal electrophoretic apparatus. The buffer used for the electrophoresis contained 1.38g of barbital, 8.76g of Sodium barbital, 0.38g of Calcium lactate dissolved in 1000ml of distilled water (pH 8.6). The buffer used for the

preparation of agar contained 0.55g of barbital, 3.5g Sodium barbital, 0.31g of Calcium lactate and 10g of agarose in 1000ml of distilled water (pH 8.6). 15ml of agar buffer solution was spread uniformly on a (94x84 mm) clean glass slide. Wells and troughs were made in the agar with the help of LKB template. The diameter of each well was 4mm and the trough measured 2.5x57 mm. The space between the well and trough was 4mm. 10μl each of CFA and affinity chromatography purified mycobacterial antigen were placed to the respective wells and the antigens were separated electrophoretically for 90 min using a constant current of 5mA per slide. After electrophoresis, 150μl each of rabbit polyvalent antiserum against CFA and rabbit antiserum against affinity purified antigen were added in the respective troughs. Following IEP, the slides were incubated in a humid chamber (4°C) and examined daily for a period of 2-5 days.

3.6.2 Evaluation of antibodies to *M tuberculosis* by an indirect ELISA (49)

CFA and affinity column purified antigens were serially diluted through the range 5-80 μg/ml in 0.1M Carbonate bicarbonate buffer (pH 9.6). 100 μl of either antigen were added in the respective wells of a polyvinyl-chloride microtiter ELISA plate (Dynatech Laboratories, Alexandria, va; USA) and incubated at room temperature for 2 h. The plates were washed with 0.15 M PBS. The unreacted sites in the wells of the microtiter plates were blocked with 1% BSA (Sigma) in 0.15 M PBS (pH 7.4) for 1h. Rabbit antibodies to CFA and affinity purified antigen were serially diluted (1:625 - 1: 781,250) in 1% BSA / PBS. 100 μl of either of the rabbit antibodies were added to the respective

wells and the plates were incubated at 4°C for 12 h. The plates were washed for 5 times with PBS-T and 100μl of (1:10,000) anti rabbit –IgG-biotin conjugate (Sigma chemical) in 0.15 MPBS was added to each well and plates were incubated for 2 h at room temperature. The microtiter plates were washed several times with 0.15M PBS-T and 100 μl of (1:1600) Extr-avidin alkaline phosphatase (Sigma chemicals) was added to each well. The enzyme reaction was induced by the addition of 100 μl of substrate p-nitrophenyl phosphate 1mg/ml in 10% Diethanolamine buffer (pH 9.7). At 30 min., 25 μl of 3N NaOH was added to stop the reaction. The absorbance in all the wells was read at 405 nm, using an ELISA reader (Bio Rad Microtiter plate, Model 550; USA).

3.7 DOT-IMMUNOBINDING ASSAY (Dot-Iba) (129, 134)

Prior to application of Dot-Iba in CSF specimen, it was essential to standardize the Dot-Iba. The standardisation procedure of Dot-Iba is described below.

3.7.1 Standardisation of Dot-Iba

- 1 cm (diameter) circular Nitrocellulose membrane (NCM) was cut.
- NCM discs were placed in the well flat bottom 48 well delta multidishes (NUNC, Denmark).
- Different concentrations (5-500ng/ml) of affinity purified mycobacterial antigen isolated from culture filtrate of *M tuberculosis* were spotted onto NCM discs, incubated overnight at 4°C.

- NCM discs were incubated at 37°C for 2h and were washed repeatedly with 0.15M PBS-T.
- The unbound site in the NCM was quenched with 3% bovine serum albumin in 0.15M PBS for 1 h.
- The NCM discs were incubated with immune rabbit IgG to affinity purified mycobacterial antigen at room temperature for 1h and washed with 0.15MPBS/T.
- Subsequently the NCM was incubated with anti rabbit-IgG biotin and Extr-avidin alkaline phosphatase for 1 h respectively and washed 3 times with PBS/T.
- Finally NCM discs were immersed in substrate containing o-dianisidine tetratotised, (0.25 mg/ml), β-naphthyl acid phosphate (0.25 mg/ml) and magnesium sulphate (1.2 mg/ml), in 0.06 M sodium borate buffer (pH 9.7) for 10 minutes.
- The NCM discs were washed in PBS/T and fixed in a solution containing methanol: acetic acid: distilled water in 5:1:5 proportion.
- A positive reaction was indicated by the development of a purple-to-purple - pink colour in the center of NCM discs.

3.7.2 Detection of mycobacterial antigen in CSF by Dot-Iba

- CSF samples from TBM and control groups were inactivated by incubating at 56°C for 1h to remove non-specific substances. The NCM disc was incubated with 10 µl CSF (1:200-1:400 dilution), from TBM and non-TBM overnight at 4°C.
- The NCM discs were incubated at 37°C for 2h and were washed repeatedly with 0.15 M PBS-T.
- The unbound site was quenched with 3% bovine serum albumin in phosphate buffered saline (BSA / PBS-T).
- NCM discs were incubated immune rabbit serum to affinity purified mycobacterial antigen at room temperature for 1h and washed three times with 0.15 M PBS/T.
- NCM discs incubated with anti rabbit-IgG biotin conjugate and Extr-avidin alkaline phosphatase for 1 h respectively and washed repeatedly with PBS/T.
- NCM immersed in substrate containing o-dianisidine tetratotised (0.25 mg/ml), β-naphthyl acid phosphate (0.25 mg/ml) and magnesium sulphate (1.2 mg/ml) in sodium borate buffer (pH 9.7) for 10 min.
- NCM discs were thoroughly washed in 0.15M PBS/T and the NCM discs were fixed in solution containing methanol: acetic acid: water in 5:1:5 proportion.
- A positive reaction was indicated by the development of a purple-to-purple - pink colour in the center of NCM discs.

3.7.3 Detection of antimycobacterial antibody in CSF by Dot-Iba

- 5µl of affinity purified antigen (10µg/ml) was spotted to NCM membrane and incubated overnight at 4°C.
- NCM discs were incubated at 37°C for 2h and were washed repeatedly with PBS-T.
- The unbound sites were blocked with 3% BSA/PBS.
- NCM discs were washed several times with 0.15 M PBS/T and blotted dry.
- 5µl CSF of 'confirmed', 'probable' TBM and control CSF (1:40 - 1:160 dilutions) were added to the NCM discs, incubated at room temperature for 1h and washed with PBS/T
- Subsequently the NCM discs were treated with anti-human IgG-biotin conjugate and Extr-avidin alkaline phosphatase for 1 h respectively.
- The NCM discs were immersed for 10 min in a substrate solution containing o-dianisidine tetrazotised (0.25 mg/ml), β-naphthyl acid phosphate (0.25 mg/ml) and magnesium sulphate (1.2 mg/ml) in 0.06M sodium borate buffer (pH 9.7).
- Reaction was stopped by washing the discs in 0.15M PBS/T and fixed in solution containing methanol: acetic acid: water (5:1:5).
- A positive reaction was indicated by the development of a purple-to-purple - pink colour in the NCM discs.

3.8 DETECTION OF HEAT INACTIVATED MYCOBACTERIAL ANTIGENS IN CSF BY AN IMMUNOBLOT METHOD (156)

2ml of CSF from TBM and control groups were autoclaved at 15 lbs for 30 min. The protein content in the CSF was precipitated by 50% ammonium sulphate and centrifuged at 3500 rpm for 30 min. The precipitate was reconstituted to 200µl in PBS after removing the ammonium sulphate by continuous dialysis for 48h.

- Tuberculin PPD antigen (2 mg/slot) and 200µl heat inactivated CSFs from TBM and control group were subjected to discontinuous SDS-PAGE
- Subsequently transferred onto nitrocellulose membrane.
- After the transfer, the NCM strips were cut at 4 mm width and strips were stored at 4^0C until used for immunostaining
- NCM strips containing Tuberculin PPD antigen, heat inactivated CSF from confirmed, probable TBM and control group were thoroughly washed with 0.15 M PBS
- The strips were then quenched with 2. 5% skimmed milk for 1 h. All the incubations in the assay were carried at 40^0 C.
- The NCM strips were washed with 0.15 M PBS and incubated with (1: 500) polyvalent rabbit IgG to *M tuberculosis* for 2h.
- NCM strips were incubated with anti rabbit IgG – biotin conjugate (1:600) (Sigma chemicals) for 2 h.
- The NCM strips were then washed and incubated with (1: 400) extr avidin HRP (Sigma chemicals) for 2 h.

- The NCM strips were then treated with a substrate containing 3 mg of 4- chloro – 1- napthol in 1 ml of cold methanol and 3μl of 30% hydrogen peroxide in 4 ml of PBS.
- A positive reaction was indicated by development of a clean blue band in the NCM strip

3.9 DEMONSTRATION OF MYCOBACTERIAL ANTIGENS IN THE CSF CYTOSPIN SMEARS USING AN IMMUNOCYTOCHEMICAL METHOD.

Two Cytospin smears were prepared from CSFs of TBM and non -TBM patients using a cytospin (Cytopro WESCOR; USA). Smears were fixed immediately in 90% alcohol. One smear was stained with haematoxylin and eosin stain and examined under the microscope for the presence of lymphocytes and macrophages. Smears that contained adequate number of mononuclear cells and lymphocytes were subjected to immunocytochemical analysis. Smears containing large number of red blood cells were not used. The technical steps involved in immunostaining were

- The alcohol fixed smears were washed in 0.15 M TBS buffer (pH 7.4).
- Smears were treated with 10% normal rabbit serum for 10min at room temperature.
- Smears were then incubated overnight at 4°C with primary antibody, polyvalent rabbit IgG to *M tuberculosis* (20μg/ml)

- Subsequently the smears were incubated with anti rabbit IgG- biotin (Dako LSAB2 system, peroxidase), followed by Streptavidin horse radish peroxidase, at room temperature for 45 min each and were washed thoroughly with TBS-T after each incubation.
- Smears were incubated in the substrate, consisting of Diaminobenzidine tetrachloride (DAB) dissolved in 0.05M Tris buffer (pH 7.4) (Dako) and 3% H_2O_2, at room temperature for 5-10 min.
- Finally the smears were counterstained with hematoxylin, dried, cleared in xylene and mounted.
- Presence of brownish-pink granular material in the cytoplasm of the monocytes cells is indicative of positive reaction.

3.10 MOLECULAR BIOLOGICAL METHODS IN THE DIAGNOSIS OF TUBERCULOUS MENINGITIS - POLYMERASE CHAIN REACTION (PCR)

3.10.1 Standardisation of PCR

Prior to application of PCR for the laboratory diagnosis of TBM, following standardisation experiments were performed.

a. Lowest number of *M tuberculosis bacilli* that can be detected by PCR.

b. Lowest detection limit of mycobacterial DNA.

3.10.1.1 (a) Standardisation of PCR to detect the lowest number of tubercle bacilli.

A loopful of *M tuberculosis* bacilli from L J slope was transferred into 2 ml eppendorf tube containing 1 ml sterile 0.15 M PBS and glass beads (0.5mm diameter) in. The suspension was vortexed for 2 min and allowed to settle for 30min at room temperature. The supernatant was diluted 5 times further in 0.15M PBS and vortexed. The number of bacilli in the supernatant was counted using an erythrocyte counting chamber and reconstituted to 80000 bacilli/µl. This was then serially diluted in the order 8000, 800, 80, 8, 4, 2 and 1. Mycobacterial DNA was isolated from the above samples.

3.10.1.2 (b) Standardisation of PCR using different concentration of *M tuberculosis* DNA

Mycobacterial DNA was isolated from a loopful of *M tuberculosis* bacilli. Accordingly the concentration of *M tuberculosis* DNA obtained was 6,77 µg/ml. A master dilution of 100 ng/ml was reconstituted and was then serially diluted in the order, 10ng, 1ng, 100pg,

10pg, 1pg, 100fg, 80fg, 60fg, 40fg, 20fg, and 10fg/ml respectively. DNA was extracted and PCR was performed as per the protocol described.

3.10.2 PCR protocol (97)

3.10.2.1 Mycobacterial DNA extraction

- 100µl of 5 % lysosyme (50 mg/ ml stock) was added into a 2ml sterile tube containing 500µl of sample. It was then vortexed and incubated at 37°C for 20 min.
- To the above, 100 µl of 2 % Proteinase K was added, vortexed and incubated at 55°C for 1h.
- This was followed by the addition of 50µl of 4% Triton X 100 and 100µl of sodium hydroxide. It was again vortexed and boiled for 5 min.
- The above solution was brought to room temperature and 50µl of 1M Tris HCl was added.
- Equal volumes of phenol was added, mixed thoroughly by vortexing and centrifuged at 12000g for 5 min.
- The upper aqueous layer was transferred to a new sterile tube and equal volumes of chloroform isoamyl alcohol (24:1) were added. It was then vortexed and centrifuged as above.
- Equal volumes of isopropanol were added to the aqueous phase, mixed well and incubated at 4°C. It was followed by centrifugation at 4°C, 15000g for 15 min.

- The supernatant was decanted.
- The pellet was washed repeatedly using 70 % and 100 % ethanol. The supernatant was decanted and the pellet dried in a vacuum dryer.
- The pellet was resuspended in 10 μl of Tris-EDTA buffer (pH 8).
- The DNA was quantitated with spectrophotometer (Biospec 1601, Shimadzu; Japan) at 260 nm.

3.10.2.2 PCR amplification

- The autoclaved PCR microtubes were labelled as positive control, negative control and others according to the number of samples available.
- 10x PCR buffer, dNTPs and primers were thawed on ice.
- Master mix (PCR Cocktail) consisted of

  10x PCR buffer (Tris buffer pH 8.3)

  2.5mM $MgCl_2$

  dNTPs 200μM each

  Primers 50 pmol each.

  T_4 5' CCT GCG GTA GGC GTC GG 3' IS6110 (47)
  T_5 5' CTC GTC CAG GGC CGC TTC GG 3'

  devRf 5' GGT GAG GCG GTT GGG TCGC 3' Dev (151)
  dev Rr 5' CGC GGC TTG CGT CCG ACGTTC 3'

  Taq polymerase 1 Unit

  Sterile ddH_2O

- The master mix was vortexed briefly and spinned down to collect mixture. 45µl was dispensed into each tube and capped tightly to prevent cross contamination.
- 5 µl of DNA sample was added, starting from negative control (reaction mix without DNA), then samples and finally the positive control [*M tuberculosis* DNA from $H_{37}Rv$ strain (1ng/ml)].
- The tube was vortexed briefly and spinned down. One drop of mineral oil was overlayered in each tube and capped tightly. All the tubes were placed on ice at all time to avoid non-specific amplification.
- PCR was performed on a Perkin-Elmer 480 thermocycler. Amplification conditions were

Initial denaturation	94˚C	5 min
Denaturation	94˚C	1min
Annealing	60˚C	1min
Extension	72˚C	1min
Final extension	72˚C	12 min

3.10.2.3 Analysis of PCR products using agarose gel electrophoresis

- 1.5% agarose was prepared in 1x TBE buffer containing ethidium bromide to a final concentration of 0.5 µg/ml.
- 10 µl of each PCR product was loaded into each well and the gel was run at 60V until bands had migrated through half.
- The gel was visualised under UV illumination. (Bio-Rad; USA)

3.10.2.4 Southern blot

- The gel was denatured for 20 min in denaturation buffer with slow agitation. The solution was decanted and rinsed with ddH_2O.
- Neutralization solution was added and the gel was agitated for another 20 min, the solution was decanted and rinsed with ddH_2O.
- The gel was blotted onto a piece of nylon membrane (cut to the same size) by capillary transfer method.
- The membrane was wrapped in a clean filter paper and baked for 2 h at 80˚C to fix the DNA onto the membrane.

3.10.2.5 Oligonucleotide hybridisation

- The probe was endlabelled using ^{32}P (5' CTG CCC AGG TCG ACA CAT 3').
- The membrane was then treated with pre-hybridisation buffer for 1h at 42˚C.
- It was followed by incubation with hybridisation buffer containing radiolabelled oligonucleotide probe, overnight at 42˚C.
- The membrane was subjected to thorough washing in 5x SSC, 1x SSC and 0.5x SSC buffer containing 0.1% and 0.5% SDS, three times for 15 min each, at 28˚C, 37˚C, and 65˚C respectively.
- The membrane was sealed in saran wrap and exposed to X-Ray film for 48h at –70˚C.

3.10.3 Polymerase Chain Reaction of CSF in TBM patients

Molecular diagnostic method for the detection of *M tuberculosis* DNA in CSF specimen was used, which amplified 123 bp sequence in insertion sequence IS6110 as well as 513 bp sequence in the Dev gene in the genome of *M tuberculosis* complex. Different steps involved in PCR are sample preparation, preparation of reaction mix, amplification and southern blot hybridisation. DNA was extracted and PCR was performed as per the protocol described.

3.11 DEMONSTRATION OF MYCOBACTERIAL ANTIGENS IN FORMALIN FIXED PARAFFIN EMBEDDED SECTIONS FROM INTRACRANIAL TUBERCULOMAS USING IMMUNOHISTOCHEMCAL METHOD (131)

- Tissues were directly fixed in 10% buffered formalin for 24 h and processed using standard protocol.
- 5μ thick paraffin sections from tuberculous and non-tuberculous intracranial granulomatous lesions were cut using a rotatory microtome (Leica 800; Germany) and the sections were deparaffinised by treating in xylene, rehydrated in 100%, 70%, 50% ethanol and finally in double distilled water.
- Sections were subjected to antigenic retrieval by boiling in 0.01M Sodium citrate buffer (pH 6) three times with an interval of 10min in a microwave oven.
- Sections were incubated in 3% H_2O_2 for 15-30 min, in order to remove the endogenous peroxidase activity in tissues.

- The sections were treated with 10% normal rabbit serum in 1% BSA / 0.15M TBS for 30min, in order to eliminate the non-specific reaction.
- Sections were then incubated overnight at 4°C with primary antibody, polyvalent rabbit IgG to *M tuberculosis* (20μg/ml)
- Subsequently the sections were incubated with anti rabbit IgG- biotin (Dako LSAB2 system Denmark), followed by Streptavidin horse radish peroxidase, at room temperature for 45 min and were washed thoroughly with 0.15M TBS-T after each incubation.
- Sections were incubated in the substrate, consisting of DAB dissolved in 0.05M Tris buffer (pH 7.4) (Dako) and 3% H_2O_2, for 5-10 min at room temp.
- Finally the sections were counterstained with hematoxylin, dehydrated passing the section through ddH_2O, 50%, 70%, 100% ethanol, and mounted.

In order to evaluate the specificity of the reaction, normal rabbit IgG was substituted instead of rabbit IgG to *M tuberculosis.* This substitution gave consistently negative results in all specimens of intracranial tuberculoma. This would suggest that the localisation of mycobacterial antigen in the granulomatous lesion was specifically due to rabbit IgG to *M tuberculosis.*

3.12 POLYMERASE CHAIN REACTION FOR THE DETECTION OF *M TUBERCULOSIS* DNA IN PARAFFIN EMBEDDED FORMALIN FIXED TISSUES FROM INTRCRANIAL TUBECULOMAS (99)

3.12.1 DNA Extraction from tuberculoma

- 20 µ paraffin sections from each block of tuberculous and fungal granuloma were cut using a rotatory microtome (Leica 800; Germany).
- Carry-over contaminating DNA was prevented by using a fresh disposable blade for each test and control samples and the microtome was cleaned with xylene and 100% ethanol after processing each specimen.
- Sections were cut and collected in 1.5 ml microcentrifuge tubes and were melted at 65°C for 10 min.
- The sections were deparaffinised treating with 1 ml of xylene, vortexed the mixture, incubated at room temperature for 30 min and centrifuged at 12000 x g for 5 min. The supernatant was discarded and the procedure repeated.
- One milliliter of absolute alcohol was added, vortexed and pelleted by centrifugation at 12000 x g for 5 min. Supernatant was removed and the pellet was dried in a vacuum drier.
- The pellet was resuspended in 300 µl of digestion buffer containing proteinase K and SDS. The mixture was incubated under rocking conditions for 24- 48h, until most of the tissue was disintegrated.
- It was followed by inactivation of proteinase K by incubating the samples at 95°C for 10 min.

- Equal volumes of phenol was added mixed thoroughly by vortexing and centrifuged at 12000g for 5 min.
- The upper aqueous was transferred to a new sterile tube and equal volume of phenol: chloroform (1:1) was added, vortexed and centrifuged as above.
- Equal volume of chloroform: isoamyl alcohol (24:1) was added to the aqueous layer, vortexed and centrifuged as above.
- DNA was precipitated by the addition of sodium acetate at a final concentration of 0.2 M and 99% ice- cold ethanol (500 µl). The supernatant was vortexed, - incubated overnight at -20°C and centrifuged at 15000 x g for 15 min at 4°C .
- The pellet was vacuum dried and resuspended in 25 µl of Tris EDTA buffer (pH 8).
- The DNA was quantitated using a spectrophotometer (Biospec 1601, Shimadzu) at 260 nm.

3.12.2 DNA amplification

- The autoclaved PCR microtubes were labelled as positive control, negative control and others according to the number of samples available.
- 10x PCR buffer, dNTPs and primers were thawed on ice.
- Master mix (PCR Cocktail) consisted of

  10x PCR buffer (Tris buffer pH 8.3)

  2.5mM $MgCl_2$

  dNTPs 200µM each

  Primers 50pmol each

T_4 5' CCT GCG GTA GGC GTC GG 3' IS6110

T_5 5' CTC GTC CAG GGC CGC TTC GG 3'

devRf 5' GGT GAG GCG GTT GGG TCGC 3' Dev

dev Rr 5' CGC GGC TTG CGT CCG ACGTTC 3'

Taq polymerase 1 Unit

Sterile ddH_2O

- Master mix was vortexed the briefly and spinned down to collect mixture. 45μl was dispensed into each tube and capped tightly to prevent cross contamination.
- 5 μl of DNA sample was added starting from negative control (reaction mix without DNA), then samples and finally the positive control (reaction mix along with DNA from $H_{37}Ra$ strain).
- The tube was vortexed briefly and spinned down. One drop of mineral oil was overlayered to each tube and capped tightly. All the tubes were placed on ice at all time to avoid non-specific amplification.
- PCR was performed on a Perkin-Elmer 480 thermocycler. Amplification conditions were

Initial denaturation	94°C	5 min
Denaturation	94°C	1min
Annealing	60°C	1min
Extension	72°C	1min
Final extension	72°C	12 min

3.12.3 Analysis of PCR products by agarose gel electrophoresis

- 1.5% agarose gel was prepared in 1x TBE buffer containing ethidium bromide to a final concentration of 0.5 μg/ml.
- 10 μl of each PCR product was loaded into each well and the gel was run at 60V until bands have migrated through half.
- The gel was visualized under UV illumination.

RESULTS AND DISCUSSION

CHAPTER-IV

4.1 CYTOLOGICAL FEATURES OF CSF IN TBM AND NON-TBM GROUPS

In 3 TBM patients, the CSF cytospin smear showed only occasional lymphocytes. In 16/40 patients, the CSF cytospin smear showed predominantly lymphocytosis. In the remaining 21 patients, the CSF cytospin smear showed pleocytosis, composed by admixture of lymphocytes, plasmacytoid lymphocytes and monocytes (Fig: 4.1). These monocytes showed abundant granular cytoplasm and central or eccentrically placed nuclei. Some of the monocytes (40%) showed degenerative changes in their nuclei and cell wall. In occasional microscopic fields, several monocytes were seen in aggregates. These were referred as "wandering tubercle" (Fig: 4.2).

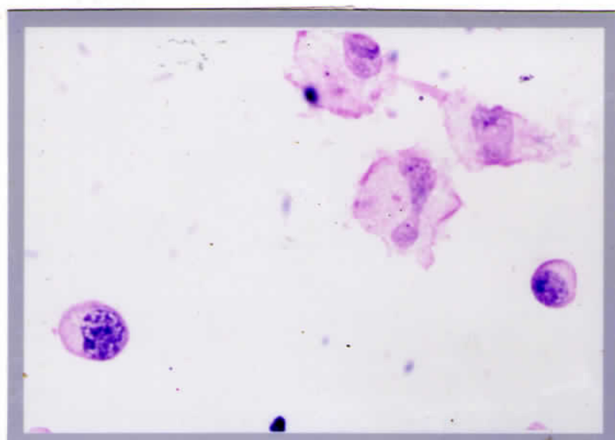


Fig 4. 1: Photomicrograph of CSF-cytospin smear showing admixture of monocytoid cells and lymphocytes in a TBM patient. (Hematoxylin and Eosin x 400)

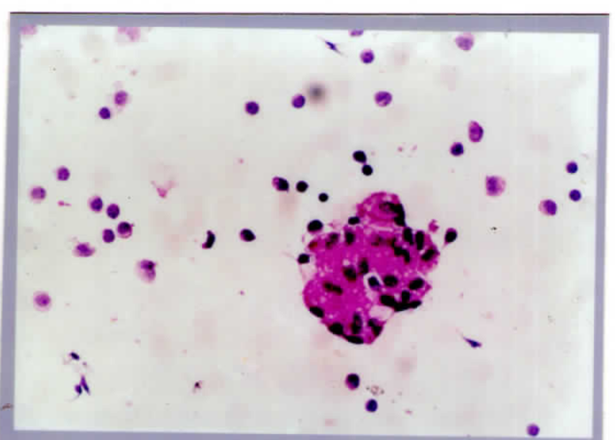


Fig 4.2: Photomicrograph of C SF-cytospin smear in a TBM patient, showing aggregates of monocytoid cells-"wandering tubercle" (Hematoxylin and Eosin x 400)

The CSF cytological features in disease control group (non-TBM) showed pleocytosis in 18/40 patients, lymphocytosis in 18/40 patients. In 4 patients, the

cytological examination revealed admixtures of neutrophils and lymphocytes. Characteristic aggregations of monocytes as seen in TBM group were not observed. However, overall cytological features in CSF specimen did not differentiate TBM from disease control group.

4.2 RESULTS OF BACTERIOLOGICAL METHOD FOR THE LABORATORY DIAGNOSIS OF TBM

CSF smears examined for acid-fast bacilli in Z- N stain	n=40
CSF studied for culture of *M tuberculosis* in L-J medium	n=40
Positive results by Z-N stain	0/40
Positive cultures	5/40
Sensitivity in Z-N smear method (%)	0
Sensitivity in L-J culture method (%)	12.5

4.3 CHARACTERISATION OF CFA, AFFINITY COLUMN PURIFIED MYCOBACTERIAL ANTIGEN AND PPD BY SDS-PAGE

The electrophoretic pattern of CFA, affinity purified mycobacterial antigen and tuberculin PPD are shown in Fig: 4.3. CFA demonstrated 20 bands when stained with Commassie brilliant blue stain. The molecular weight of these bands ranged between 10 - 110 kDa.

The mycobacterial antigen isolated from CFA using an immunoabsorbent affinity chromatography showed a single band and possessed a molecular weight of 14 kDa.

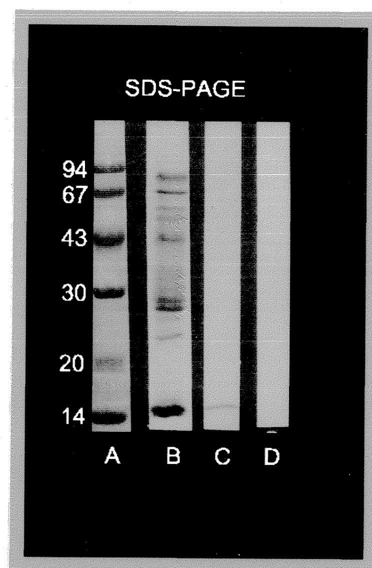


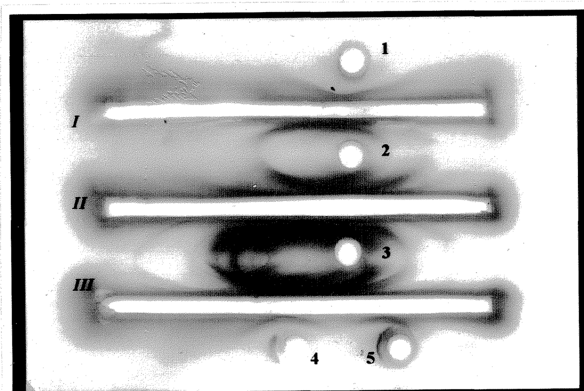
Fig-4.3: SDS PAGE: Lane A-Molecular weight standard; Lane B- CFA; Lane C-- 14kDa antigen; Lane D- Control.

Tuberculin PPD antigen demonstrated four stainable bands and possessed molecular weights of 35, 45, 66 and 82 kDa respectively (Fig: 4.6).

4.4 CHARACTERISATION OF RABBIT ANTIBODY TO *M TUBERCULOSIS* AND AFFINITY COLUMN PURIFIED MYCOBACTERIAL 14 KDA ANTIGEN USING IMMUNOELECTROPHORESIS (IEP) AND ELISA.

4.4.1 Results in IEP:

These are represented in Fig: 4.4



IEP: Well No 1- 14kDa antigen; Well No. 2&3 – CFA; Well No. 4&5 – 14 kDa antigen; Trough I – Rabbit antibody to 14kDa antigen; Troughs 2&3 – Rabbit antibody to CFA.

- Multiple precipitin arcs were demonstrated with CFA and rabbit polyvalent antibody to *M tuberculosis*.
- Single precipitin arc was observed with 14 kDa and rabbit antibody to CFA of *M tuberculosis*.
- Single precipitin arc was observed with CFA and rabbit antibody to 14 kDa mycobacterial antigen.
- Single precipitin arc was seen with 14 kDa antigen and rabbit antibody to 14 kDa mycobacterial antigen.

Interpretation:

Results of IEP indicated that

- 14 kDa is one of the immunoreactive antigens of CFA.
- Rabbit antibodies to CFA and 14 kDa antigen react with each other and gave a single precipitin line in IEP.

4.4.2 Evaluation of rabbit antibodies to mycobacterial antigen by ELISA

Results:	End-point titers
CFA Vs rabbit polyvalent antibody to *M tuberculosis*	1: 390,625
CFA Vs rabbit antibody to 14 kDa mycobacterial antigen	1: 3125
14 kDa Vs rabbit polyvalent antibody to *M tuberculosis*	1: 3125
14 kDa Vs rabbit antibody to 14 kDa	1: 3125

CFA Vs normal rabbit serum	nil
14 kDa Vs normal rabbit serum	nil
CFA Vs anti rabbit IgG- biotin conjugate	nil
14 kDa antigen Vs anti rabbit IgG - biotin conjugate	nil

4.5 RESULTS OF IMMUNOLOGICAL BASED ASSAYS FOR THE DIAGNOSIS OF TBM

4.5.1 Results of detection of antimycobacterial antibody by Dot-Iba in CSF of patients with TBM

Table 4-1 represents the results of Dot-Iba for the detection of antimycobacterial antibody in TBM and control groups. The assay gave positive results at 1: 160 in all the five culture positive CSF specimens. No false negative reaction was recorded in any of the culture proven patients with TBM. Thus the sensitivity of Dot-Iba in the confirmed cases of TBM was 100%. Of the 35 patients with probable TBM, the assay yielded positive results in 28 patients at a titer of 1:160. In the remaining 7 patients, the colour reaction was recorded between 1:40 - 1:80 titer. In the control group, no colour reaction was observed in the CSF at the titer of 1: 160. The cut-off titer was taken at 1:160 as this gave the best discrimination between tuberculous and non-tuberculous subjects. Using this criterion, Dot-Iba had a specificity of 100%. The L-J culture method invariably required 8-10 weeks to grow *M tuberculosis* in CSF of patients with TBM. The sensitivity of culture method was only 12.5%. In Z-N stained smears, acid-fast bacilli could not be

demonstrated even in culture positive CSFs and therefore CSF smear examination cannot be regarded as a useful method for the routine laboratory diagnosis of TBM.

Table 4-1: **Detection of antibody to 14 kDa mycobacterial antigen in CSFs by Dot-Iba**

Patient group		Antibody titer		
		1:40	1:80	1:160
TBM group	Confirmed (n=5)	5	5	5
	Probable (n=35)	0	7	28
Disease control group	Bacterial meningitis (n=15)	8	0	0
	Partially treated pyogenic meningitis (n= 10)	0	7	0
	Others (n= 15)	3	0	0

It is well established that, successful isolation of *M tuberculosis* in the CSF specimen in patients with TBM by the conventional bacteriological method is often infrequent. There are several factors that account for the infrequent isolation of *M tuberculosis* from the CSFs of patients with TBM. The CSFs from patients with TBM are usually obtained by the lumbar route and the lumbar CSF contains lower concentration of *M tuberculosis* than ventricular or cisternal CSF (128). Secondly, tubercle bacilli get embedded in the dense exudates in the basal cisternae or leptomeninges and the exudates form a barrier, preventing tubercle bacilli from circulating into lumbar CSF. Thirdly and perhaps more importantly, most patients with TBM received a course of ATT before being referred to specialized centers for neurological diseases. The CSF in partially treated patients with TBM is seldom found to contain *M tuberculosis* and thus the culture is invariably negative. In the absence of

definitive diagnostic criteria, alternative laboratory methods other than the bacteriological methods are essential for the clinical diagnosis of TBM.

A localised immunological response within the CNS is known to occur with several infective diseases of CNS including TBM. As a consequence of humoral immune responses, a prospective intrathecal synthesis of antibody in a patient with TBM will be higher in CSF than in serum. Elevated antimycobacterial antibody in patients with TBM suggests that the intrathecal synthesis of mycobacterial antibody can occur during the active stages of the disease. Some of these antibodies react specifically with mycobacterial antigens while other antibodies are non-specific and they may yield false positive results in immunoassays. Therefore, it will be essential to use a specific mycobacterial antigen that will react only with a specific antibody present in the CSF specimens. Such a diagnostic approach will eliminate the false-positive results.

In order to meet the above, we isolated a specific mycobacterial antigen from CSF of patients with TBM using immunoabsorbent affinity chromatography method. This unique method of isolation of mycobacterial antigen has not been reported in the earlier published studies for the laboratory diagnosis of TBM. The value of this qualitative test lies in its potential to differentiate patients with TBM from partially treated pyogenic meningitis where the clinical and cytological parameters closely resemble that of patients with TBM. The Dot-Iba as applied in our study has several advantages over conventional ELISA method. NCM used in Dot-Iba possess better binding capacity than polystyrene plates usually used in ELISA. The results of Dot-Iba can be sent within 6 h after the receipt of the specimens in the laboratory. The results can be visually read and there is no

need for equipments like ELISA reader. More importantly, the NCM disc coated with 14kDa antigen can be stored at 4°C for several weeks and many CSF samples can be handled at a time by a single laboratory staff. However, the diagnostic significance of detection of antibodies to *M tuberculosis* has the following limitations a) The assay cannot distinguish active stages of the disease from chronic stages b) passive transfer of antibodies into the CSF from serum can occur even in non-TBM patients due to the impairment of the blood brain barrier.

4.5.2 Results of antigen based assays in CSF of patients with TBM

Three distinct methods were used in this study and they will be discussed under **(I) Dot-Iba, (II) Immunoblot, (III) Immunocytological methods.**

4.5.2.1 Results of detection of mycobacterial antigens in CSF by a Dot-immunobinding assay (Dot-Iba)

Standardisation of the Dot-Iba indicated that the sensitivity of the assay is 50 ng antigen/ml. NCM disc containing the 14 kDa (50 ng/ml) mycobacterial antigen was used as a positive control whenever a batch of CSF specimens was assayed.

Dot-Iba gave positive results in all the five culture positive patients with TBM. Thus the sensitivity of Dot-Iba for patients with confirmed TBM was 100%.

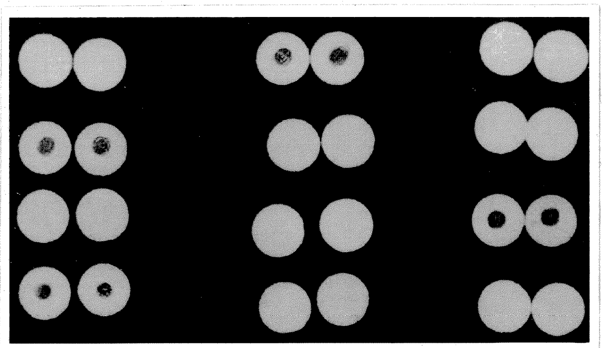


Fig 4.5: Dot-Iba showing positive and negative results in TBM and control groups.

26 out of 35 CSF specimens from patients with probable TBM were positive by Dot-Iba. In 9 out of 35 probable patients with TBM, Dot-Iba gave negative results. In these nine patients with TBM, the microbiological studies for pyogenic bacteria and fungi were also negative. In order to confirm the diagnosis in these nine patients, PCR for tuberculosis was performed and the results were again negative. The repeat CSF studies in these nine patients continued to show elevated protein and leuckocyte cell-count and negative Dot-Iba results. Therefore the precise cause of meningitis in these nine patients could not be established. For the 40 CSF samples from the patients with non-tuberculous neurological diseases, the Dot-Iba gave constantly negative results. Thus the specificity of the assay in the CSF samples from the subgroup studied was 100%. The results for the patients with TBM and non-TBM control groups were reproducible when the assay was performed at different occasions and there was no variation in the results.

Earlier immunoassays described in the literature for the detection of mycobacterial antigens in CSF from patients with TBM include a latex particle agglutination test with anti plasma membrane antibody (82), a sandwich ELISA with anti *M bovis* BCG antibody (138), an inhibition ELISA with polyvalent antibody against *M tuberculosis* (102) and a competitive ELISA with anti *M bovis* BCG antibody (133). Mastroianni et al (101) applied a dot blot method in their study of 38 patients with TBM. They used anti *M bovis* BCG antibody in their study and their assay detected mycobacterial antigen at a concentration of 100 ng/ml. The assay detected the presence of mycobacterial antigen in all the eight culture positive TBM patients. Two out of 25 patients with non-tuberculous meningitis gave false positive results in their study. Neither

the biochemical nor the immunological properties of the antigen detected in the CSF specimens were highlighted. It would be worthwhile to note that in all the studies mentioned above, the antibodies used in the assay to detect mycobacterial antigens in CSF specimens were either commercial products or were induced in other species. However in our study we have used specific human CSF-IgG to *M tuberculosis*, to isolate a mycobacterial antigen from the culture filtrate of *M tuberculosis.* Immunosorbent affinity chromatography was applied to isolate a specific mycobacterial antigen and this mycobacterial antigen showed a single band by SDS-PAGE and had a molecular weight of 14 kDa. Based on the data, we consider that a positive Dot-Iba result has definite diagnostic value in a culture negative patient with TBM and a negative Dot-Iba result would suggest that ATT should not be continued in patients with probable TBM. The Dot-Iba also did not give false positive results for any of 40 patients with non-tuberculous neurological diseases.

Dot-Iba established in our laboratory is rapid and specific for the detection of mycobacterial antigens in CSF. More importantly it can be readily performed in a routine clinical laboratory and does not require any sophisticated equipment and the result can be easily interpreted by visual examination of the NCM discs. The entire procedure requires only 6 h after the receipt of the CSF specimen in the laboratory. The technical aspects of the Dot-Iba in our study can be performed in a simple way and a large number of CSF specimens can be easily handled by the staff of a single laboratory. NCM discs containing 14 kDa antigen can be routinely stored at 4°C for at least six months. The reagent used in the assay has a shelf life of more than 6 months and more importantly the

assay is reproducible, we therefore consider this approach to be most suited to the laboratories in developing countries, where TBM is still prevalent.

Table 4-2: **Comparison of results of Dot-Iba and bacteriological methods in patients with TBM**

		Dot-Iba		AFB-Smear	Culture
		Antigen detection	Antibody detection		
No. of patients		40	40	40	40
Patients with positive results		31	33	0	5
Sensitivity (%)	Confirmed TBM	100	100	0	100
	Probable TBM	74	80	0	0
Overall sensitivity (%)		77.5	82.5	0	12.5
Specificity		100	100	100	100

4.5.2.2 Detection of mycobacterial antigen by Immunoblot method

Fig: 4.6 depicts the results of immunoblot method for the detection of mycobacterial antigen in CSF of patients with TBM.

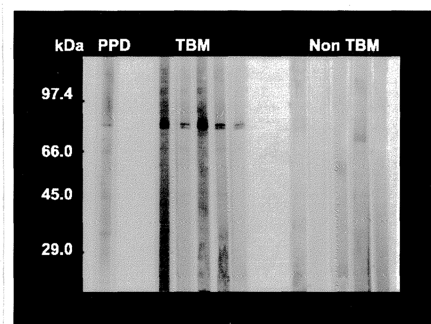


Fig 4.6 Immunoblot of PPD and heat inactivated CSFs of TBM & non- TBM groups. Note the presence of 82-kDa antigen in CSFs of TBM patients.

The heat inactivated mycobacterial antigen standard i.e. PPD, demonstrated four mycobacterial antigens, bearing the molecular weights of 35, 45, 66 and 82 kDa. The CSFs from five 'confirmed' TBM patients contained 45, 66 and 82 kDa antigens, of

which 82 kDa antigen appeared most prominent in NCM. In 16 out of 25 patients with 'probable' TBM, 82 kDa antigen was also demonstrated in the NCM strips. In the remaining 9 patients in the 'probable' TBM, 82 kDa antigen was absent. Some of the CSFs from the control group showed 35 and 66 kDa antigens but 82 kDa was absent in all of them. Based on this observation, the presence of 82 kDa antigen in the heat inactivated CSF in a patient of meningitis would distinguish tuberculous from non-tuberculous aetiology. All TBM patients with 82kDa antigen in their heat-inactivated CSFs were regarded as positive for tuberculous aetiology. Accordingly, five 'confirmed' patients with TBM and 16/25 patients with 'probable TBM were treated with ATT during their hospital stay and they showed optimal neurological recovery at the time of discharge from the hospital. Nine out of the 25 'probable' TBM did not show 82 kDa antigen in their CSFs. Hence they were not regarded as TBM and ATT was withdrawn. These nine patients were extensively investigated to establish the cause of meningitis. Despite of that the aetiology of meningitis remained undetermined in them.

The data of immunoblot studies for the detection of mycobacterial antigen highlighted three relevant observations. Firstly, heat inactivation of CSF specimen prior to the assay reduced the risk of handling the infectious material in the laboratory. Secondly, the assay gave a high specificity, as no false positive results were observed in the disease control group. Thirdly, the assay can easily be performed in any routine clinical laboratory. We consider that this approach is novel and can be applied for the rapid laboratory diagnosis of TBM in developing world.

4.5.2.3 Demonstration of mycobacterial antigens in CSF cytospin smears using an immunocytochemical method

Sixteen out of 22 CSF cytospin smears from TBM patients showed admixtures of lymphocytes and monocytoid cells in the hematoxylin and eosin stained smears. 30-40% of monocytes in the cytospin smear showed positive immunostaining for mycobacterial antigen in the form of brownish-pink granules in the cytoplasm (Fig: 4.7). Some of the macrophages showed intense immunostaining while other macrophages in the smear showed varying degrees of immunostaining. This may be attributed to different stages of intracellular phagocytosis of the tubercle bacilli by the macrophages. Also seen in the CSF cytospin smears are aggregates of extracellular granular immunostained particles (Fig: 4.8) and we regard this as the breakdown products of tubercle bacilli.

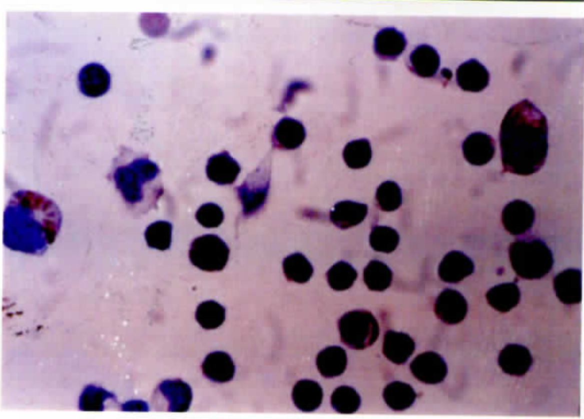


Fig 4.7: Photomicrograph of CSF-Cytospin smear showing positive immunostaining for mycobacterial antigens in the cytoplasm of monocytoid cells in a TBM patient. (ABC method x 400)

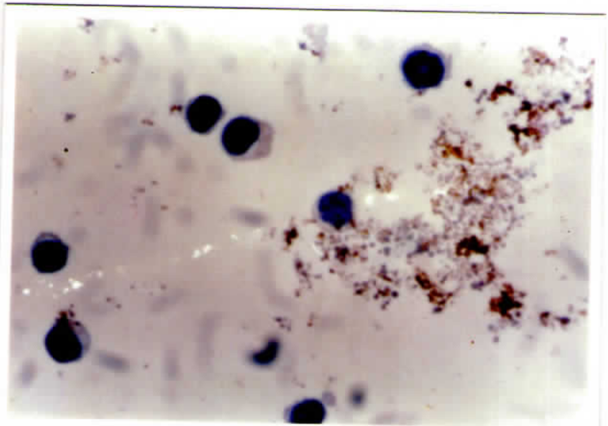


Fig 4.8: Photomicrograph of CSF-Cytospin smear in a TBM patient showing extracellular granular immunostained particles suggestive of break down products of tubercle bacilli. (ABC method x 400)

We also regard that, these circulating mycobacterial antigens can occur both in particulate and soluble forms, which can be detected by the immunoassays. Positive

immunostaining in the monocytoid cells was also seen in those three CSFs of TBM in which *M tuberculosis* were isolated by culture. This assay also gave positive results in 13 culture negative CSF from patient with TBM. In six CSF smears of TBM patients, the immunostaining was negative, because in these cases, the CSF smears only showed few lymphocytes.

The CSF cytospin smears from none of the disease control showed these characteristic immunostaining in the cytoplasm of monocytoid cells (Fig: 4.9).

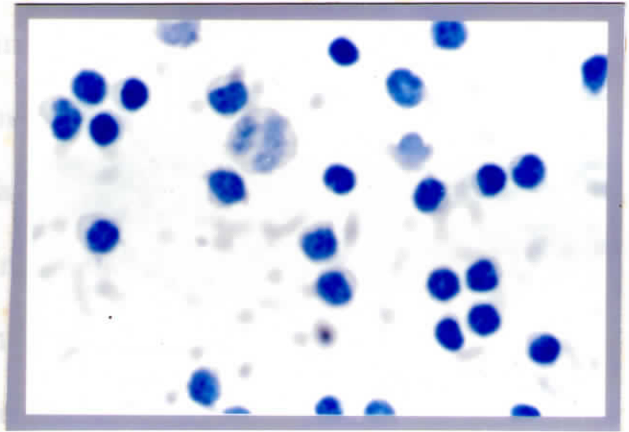


Fig 4.9: Photomicrograph of CSF-Cytospin smear showing negative immunostaining for mycobacterial antigens in the cytoplasm of monocytoid cells in a non-TBM patient.(ABC method x 400).

TBM is a potentially curable infectious disease of CNS. As the bacteriological methods are less sensitive and time consuming, there is a need to design an alternative diagnostic method for the early laboratory diagnosis of TBM, so that an effective therapeutic modality can be instituted in patients with TBM. In order to meet the above objective, we devised a simple immunocytological method in this study. The principle of this assay is that, the CSF in patients with TBM during active stages of the disease contains monocytoid cells (macrophages) and lymphocytes. The function of these monocytes is to phagocytose the tubercle bacilli and process the antigenic component of

the bacilli. Thus the cytoplasm of the monocytes in patients with TBM during the active stages contains mycobacterial antigens. Presence of mycobacterial antigens in the cytoplasm of the monocytes has been demonstrated by an immunocytochemical method in this study. We have used rabbit IgG to *M tuberculosis* as the primary antibody to demonstrate mycobacterial antigens in the CSF smears. 16 out of 22 CSF cytospin smears from patients with TBM gave positive immunostaining while in cytospin smears from six TBM patient gave negative immunostaining because there was paucity of monocytoid cells in the CSF smears. There were no false positive immunostaining in the CSF smears from the disease control group. Similar immunocytochemical studies for the demonstration of mycobacterial antigen in the CSF cytospin smear have not been reported earlier in the literature. This method is unique, simple, rapid and also carry a high degree of specificity. Hence, this method is best suited to laboratories in developing world. The primary antibody i.e. rabbit polyvalent antibody to *M tuberculosis* is relatively easy to prepare and can be easily preserved at 4°C for atleast six months. The results of this test can be visualised by an ordinary microscope. However this technique has the following limitations a) presence of sufficient number of monocytoid cells (macrophages) in the CSF is mandatory. These monocytoid cells are present only in active stages of the disease. b) Secondly, these monocytoid cells tend to disappear during the course of ATT. In tertiary referral center like our Institute, most patients with TBM are referred from other outlying hospitals where these patients have received ATT. c) Thirdly, there are degenerative changes in the monocytoid cells and this can result in

shrinkage of cytoplasm of macrophages. These structural artifacts can affect quality of immunocytochemical staining for mycobacterial antigens in the CSF. d) Presence of large number of red blood cells in the CSF will result in background staining. Despite of these limitations, immunocytological studies in cytospin smear holds a promise in the routine laboratory diagnosis of TBM.

4.6 RESULTS OF PCR IN THE DIAGNOSIS OF TBM

4.6.1 Standardisation of PCR

(a) In this study the lowest detection limit of mycobacterial DNA in our study was 10fg (Fig: 4.10). (b) The lowest number of *M tuberculosis bacilli* that could be detected using PCR was 8 tubercle bacilli (Fig: 4.11).

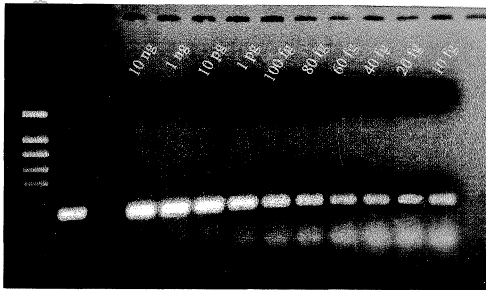


Fig 4.10: Ethidium bromide stained gel showing amplification of *M tuberculosis* $H_{37}Ra$ DNA Lane1: Marker*; Lane 2: Positive control**; Lane 3: Negative control; Lane 4-13: *M tuberculosis* DNA ranging from 10ng-10fg.

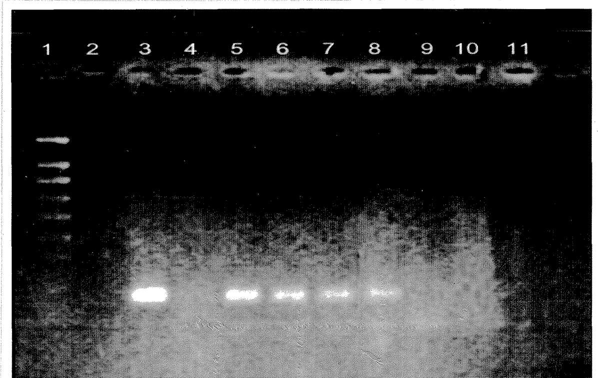


Fig 4.11: Ethidium bromide stained gel showing amplification of *M tuberculosis* $H_{37}Ra$ DNA Lane 1: Marker *; Lane 2 : unloaded ; Lane 3: Positive control ** Lane 4: Negative control; Lane 5-11: *Mt* t*uberculosis* DNA extracted from 8000, 800, 80, 8, 4,2,1 mycobacteria respectively.

*Marker: pUC 18 cleaved by Taq I & Sau IIIA. ** Positive control: *M tuberculosis* DNA from $H_{37}Rv$.

4.6.2 Results of PCR in CSF in TBM and non-TBM patients

PCR gave positive results in 3 out of 5 patients with confirmed TBM and 13 out of 35 patients with probable TBM. Thus the sensitivity of PCR in confirmed and probable TBM group were 60% and 37% respectively. The sensitivity of PCR with IS6110 (123 / 240 bp) and Dev primers (513 bp) did not show any difference. PCR also gave negative results in all the 40 CSFs from the disease control group.

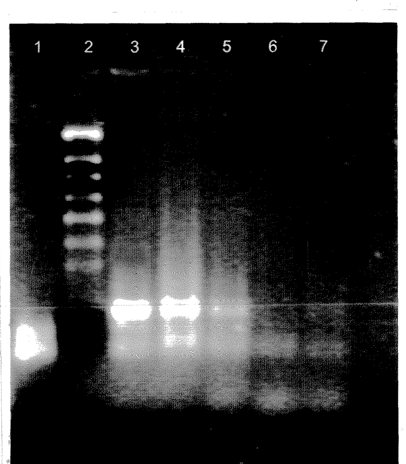


Fig 4.12: Ethidium bromide stained gel showing amplification of *M tuberculosis* DNA (using IS611 0 primers) in CSF samples of TBM and non-TBM patients..Lane1: Negative control Lane 2: Marker ; Lane 3: positive control; Lane 4-7: Amplified products from CSF samples showing positive and negative results.

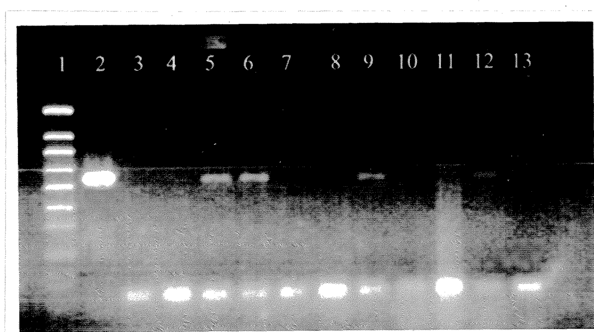


Fig 4.13: Ethidium bromide stained gel showing amplification of *M tuberculosis* DNA using (dev primers) from CSF samples of TBM and non-TBM patients. Lane1: Marker ; Lane 2: Positive control ; Lane 3: Negative control; Lane 4-13 amplified products from CSF samples showing positive and negative results.

*Marker: pUC 18 cleaved by Taq I, Sau IIIA. ** Positive control: *M tuberculosis* DNA from $H_{37}Rv$.

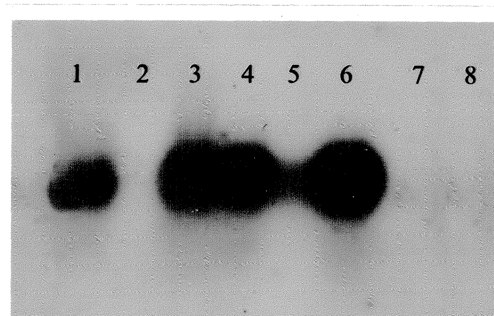


Fig 4.14: Southern hybridisation with IS6110 probe. Lane 1: Positive control ; Lane 2: Negative Control ; Lane 3-7: CSF samples showing positive and negative results.

4.7 COMPARISON BETWEEN BACTERIOLOGICAL METHOD, DOT-IBA AND PCR FOR THE LABORATORY DIAGNOSIS OF TBM

The results obtained in 40 CSF specimens from patients with TBM by Ziehl- Neelsen staining, bacterial culture, Dot-Iba for mycobacterial antigen and PCR for detection of *M tuberculosis* are summarised in Table 4-3.

The Ziehl -Neelsen stained CSF smears did not demonstrate acid - fast bacilli in any of the 40 patients with TBM. Conventional bacterial culture grew *M tuberculosis* in 5 patients (12.5%). The Dot-Iba detected mycobacterial antigen in all the five culture positive patients with TBM. Twenty six out of 35 CSF (74.3%) specimens from patients with probable TBM were positive by Dot-Iba. In 9 patients with probable TBM, the assay did not detect the mycobacterial antigen. The PCR gave positive results in 3 out of 5 patients with confirmed TBM and 13 out of 35 patients with probable TBM. Thus the sensitivities of PCR in confirmed and probable TBM group were 60% and 37% respectively. Nine CSFs, which gave negative results in the Dot-Iba, were also negative by PCR. All the 13 CSFs, which gave positive results in PCR, were also positive by Dot-Iba. However among 13 CSFs from probable TBM, which were negative in PCR, yielded positive results with Dot-Iba. For the 40 CSF specimens from control group, the Dot-Iba gave negative results. Thus, specificity of Dot-Iba with CSF samples from the subgroup studied was 100%. Results in patients with TBM and control groups were reproducible when the assay was performed at two different occasions.

Table 4-3: **Data showing the results of bacteriological methods, PCR and Dot-Iba in CSF of TBM and non-TBM patients.**

Sl No	Patient group	Z-N staining of CSF smear	Culture for *M tuberculosis*	PCR	Dot -Iba
1	TBM(n=40) Definite (n=5)	0	5(100)	3 (60)	5 (100)
	Probable (n=35)	0	0	13(37)	26(74.3)
2	Disease control (n=40)	0	0	0	0
3	Overall Specificity (%)	0	100	100	100

* Figures in parentheses indicate sensitivity of the assay in percentage.

Considerable data are currently available for the detection of *M tuberculosis* by PCR in clinical specimens such as sputum, pleural fluid etc. in patients with PT. However the precise use of PCR for the routine laboratory diagnosis in patients with TBM still remains a subject of conjecture. A review of published studies on PCR for the laboratory diagnosis of TBM indicated that the sensitivity of PCR ranged from 33-90.5%. In this study the overall sensitivity of PCR for the diagnosis of TBM was 37%. The low sensitivity of PCR for TBM could be due to following reasons. (a) Volume of CSF: Larger volume of CSF (> 2 ml) yielded more positive PCR results than smaller volume of CSF (< 1ml). Bonington et al. (12) stated that increasing the volume of CSF enhanced the sensitivity of PCR (b) The lumbar CSF in patients with TBM contains fewer tubercle bacilli than cisternal and ventricular CSF (128) and therefore the yield of mycobacterial DNA in lumbar CSF samples will be sub-optimal. (c) The yield of target DNA is sub-optimal because of the tough cell wall of *M tuberculosis*. (d) The presence of inhibitors in

the CSF during amplification. (e) The effect of ATT prior to PCR because ATT has an effect on the bacterial load and in turn results in the reduced yield of DNA.

Due to the above factors as well as requirement of a clean and specific laboratory area, sophisticated instrumentation, expensive chemicals and storage of radioactive materials, PCR is regarded to be inappropriate for routine laboratory application in developing world. Finally in order to get reliable and accurate results in PCR, technical expertise is essential. Development of simple, reproducible, inexpensive diagnostic tests for tuberculosis is essential in view of its global increase in the incidence and prevalence. Thus, we consider that Dot-Iba developed in our laboratory is not only rapid but also simple and it can be carried out in routine laboratories with limited resources.

Table 4-4: **Comparison between Dot-Iba and PCR for the laboratory diagnosis of TBM**

Sl No:	Criteria		PCR	Dot-Iba
1	Routine application in developing countries		Not feasible	Feasible
2	Duration of assay		48-72h	6-8h
3	Presence of *M.tuberculosis* in CSF		Essential	Not Essential
4	Sophisticated Laboratory Facilities		Required	Not required
5	Quantity of CSF required		>1-2ml	100μl
6	Sensitivity in TBM	Confirmed	60%	100%
		Probable	37%	74.3%
7	Specificity in TBM		100%	100%
8	Cost effectiveness		Unaffordable in developing countries	Affordable

4.8 DEMONSTRATION OF MYCOBACTERIAL ANTIGENS IN FORMALIN FIXED PARAFFIN EMBEDDED SECTIONS OF INTRACRANIAL TUBERCULOMA BY IMMUNOHISTOCHEMICAL METHOD

IHC yielded positive results in 8 out of 10 cases of intracranial tuberculoma. In the paraffin sections, mycobacterial antigens were characteristically located within the cytoplasm of several macrophages and Langhan's giant cells of tuberculous granuloma. They appeared as diffusely stained granular brownish pink material (Fig: 4.16). Immunostaining was not present within the macrophages or giant cells in the granuloma. This may be attributed to different stages of intracellular ingestion and phagocytosis of tubercle bacilli within the granuloma.

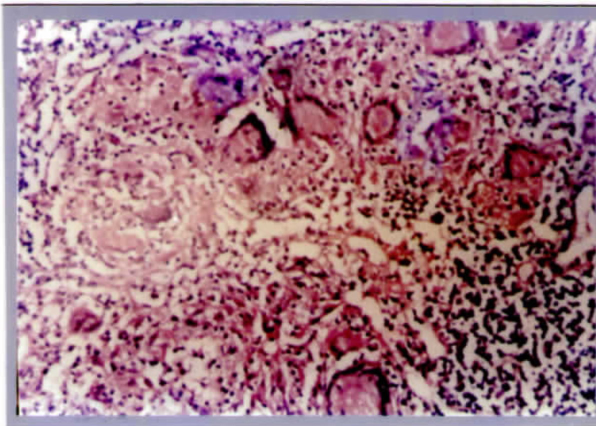


Fig 4. 15: Photomicrograph of intracranial granulomatous lesion composed by Langhan's type of giant cells, epitheliod cells and lymphomononuclear cells. (Hematoxylin & Eosin x 200)

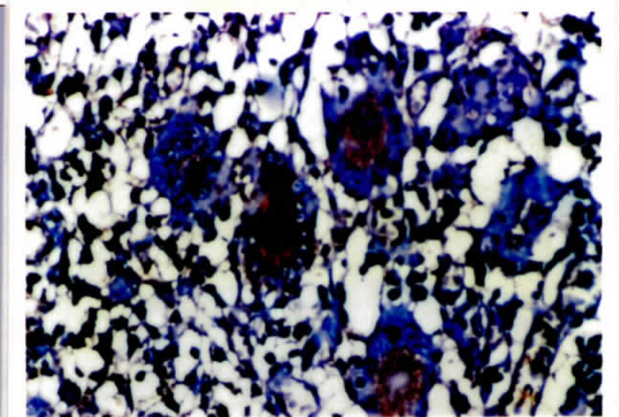


Fig 4. 16: Photomicrograph showing positive immunostaining for mycobacterial antigens in the cytoplasm of Langhan's giant cells in a tuberculous granuloma. (ABC method x 400)

In none of the tuberculoma, characteristic morphological appearance of acid-fast bacilli was identified. This may be as a result of phagocytosis and subsequent disintegration of the tubercle bacilli within the granulomatous lesions.

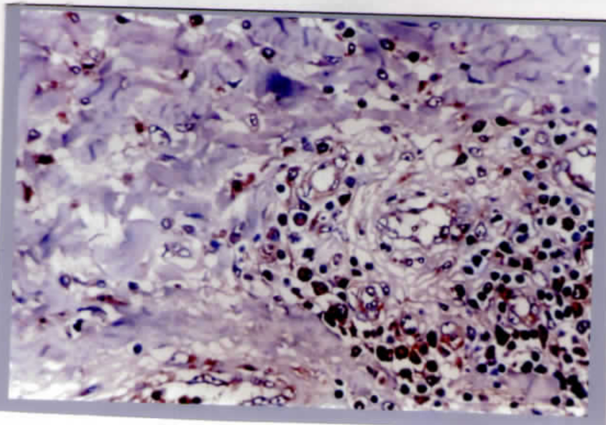


Fig 4. 17: Photomicrograph showing positive immunostaining for mycobacterial antigens in the cytoplasm of macrophages of intracranial granulomatous lesion.
(ABC method x 400)

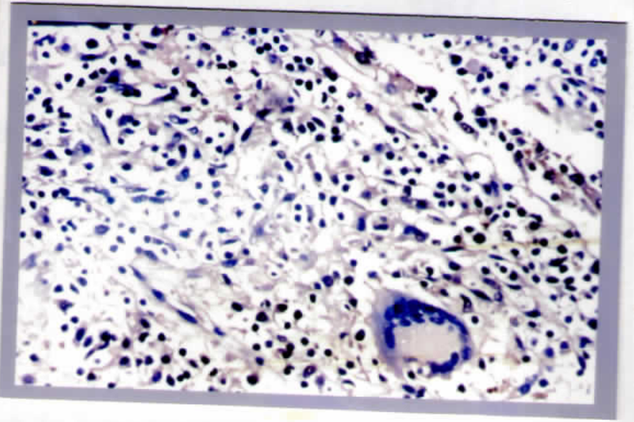
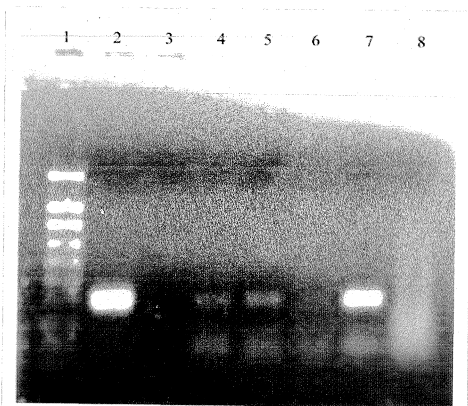


Fig 4. 18: Photomicrograph showing negative immunostaining for mycobacterial antigens in the cytoplasm of macrophages and Langhan's giant cells in a fungal granuloma.
(ABC method x 400)

In two cases of intracranial tuberculoma, mycobacterial antigens were not demonstrated, because there was extensive caseation and paucity of macrophages and giant cells within the lesions. Thus the sensitivity of the IHC depends upon the presence of macrophage and giant cells within the granuloma. In none of the paraffin sections of ten intracranial fungal granuloma, characteristic intra-cytoplasmic localisation of mycobacterial antigens was demonstrated. In other words, the rabbit IgG to *M tuberculosis* did not cross-react with fungal antigen present within the macrophages and giant cells in fungal granuloma. Thus the specificity of IHC method was 100% in this study.

4.9 DEMONSTRATION OF MYCOBACTERIAL DNA BY PCR IN FORMALIN FIXED PARAFFIN EMBEDDED SECTIONS OF INTRACRANIAL TUBERCULOMA

Six out of ten intracranial tuberculous granuloma gave positive results by PCR (Fig: 4.19 & Fig: 4.20). All the positive cases showed a single band in ethidium bromide stained agarose gel electrophoresis, which corresponded to the positive control (mycobacterial DNA). Interestingly enough, PCR showed positive results in two intracranial tuberculoma, which gave negative results in IHC. In these two cases extreme caseation with minimal cellular reaction were present in hematoxylin and eosin stained paraffin sections. These two cases also showed the presence of AFB in the Ziehl-Neelsen smear.



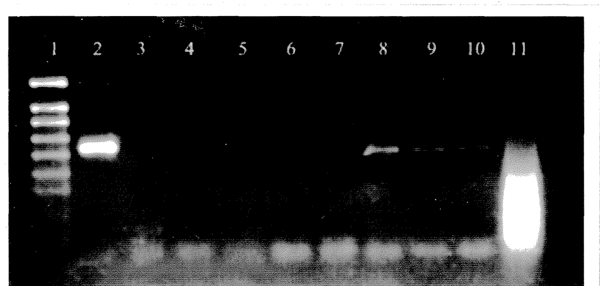


Fig 4.19 Agarose gel electrophoresis of PCR products amplified using IS6110 primers showing Lane 1: Marker* Lane 2: Positive control**; Lane 3: Negative control; Lane 4-7: DNA isolated from Intracranial tuberculoma showing positive and negative results in PCR; Lane 8: Fungal granuloma showing negative result.

Fig 4.20 Agarose gel electrophoresis of PCR products amplified using Dev primers showing Lane1: Marker*; Lane2: :Positive control**; Lane 3: Negative control; Lane 4-10: DNA isolated from Intracranial tuberculoma showing positive and negative results in PCR; Lane 8: Fungal granuloma showing negative Result.

*Marker: pUC 18 cleaved by Taq I, Sau IIIA. ** Positive control: *M tuberculosis* DNA from $H_{37}Rv$.

4.10 COMPARISON BETWEEN BACTERIOLOGICAL METHODS, IHC AND PCR FOR THE DIAGNOSIS OF INTRACRANIAL TUBERCULOMA.

Table 4-5 represents the results of IHC, PCR and bacteriological methods in ten cases of intracranial tuberculoma. The results of bacteriological culture in all the ten cases were available 6-8 weeks after the inoculation of the specimen onto LJ medium and *M tuberculosis* was isolated only in one specimen, while the acid-fast bacilli were demonstrated in two specimens. IHC yielded positive results in 8 out of 10 cases of intracranial tuberculoma. While only 6 out of 10 cases of intracranial tuberculous granuloma were positive by PCR. Comparative analysis between PCR and IHC in patients with intracranial tuberculoma showed that IHC to be having a sensitivity and specificity of 80% and 100% while PCR gave sensitivity of 60% and specificity of 100%. The IHC required 12-16h to perform the test while PCR require 48-72h. IHC can be undertaken in routine laboratory and require only small laboratory space while PCR requires a well-equipped laboratory, require individual station for isolation, preparation of amplicor, also require elaborate instrumentation, technical expertise and skill. PCR can be performed only in a small number of cases in a day, while IHC can be done in large number of cases. Thus, IHC method for the demonstration of mycobacterial antigens in intracranial tuberculous granuloma possesses several operational advantages over PCR.

Absolute criteria to establish the laboratory diagnosis of tuberculous granuloma depends upon the demonstration of *M tuberculosis* either by bacteriological culture or by demonstration of acid-fast bacilli in Z-N stain of paraffin sections. Bacteriological method to isolate *M tuberculosis* from the specimen requires several weeks and more

importantly the culture may be negative in vast majority of instances. There could be two important reasons for the low sensitivity of bacteriological methods for the isolation of *M tuberculosis*. Firstly, majority of patients with intracranial tuberculoma received antituberculosis chemotherapy prior to surgery, as a result of which there is a paucity of viable tubercle bacilli in the lesions. Secondly, the acid-fast bacilli are engulfed and phagocytosed by the macrophages within the granulomatous lesions. As a result of which the morphology of the bacilli gets distorted and only fragments of bacilli are present in the lesions. In Z-N preparation, fragments of tubercle bacilli are not identified. The same phenomenon can also explain the frequent negative culture in the specimens of intracranial tuberculoma. In the absence of a confirmatory bacteriological diagnosis, alternate methods to establish mycobacterial aetiology in an intracranial caseating granulomatous lesion has become essential. This has clinical relevance because caseating granuloma caused by other aetiological agents such as fungi can mimic those of tuberculous granuloma. In an attempt to distinguish tuberculous from fungal aetiology, in an intracranial granuloma, we have used two methods namely IHC and PCR in our study. Both PCR and IHC did not yield false positive results in fungal granuloma and carried high specificity.

Detection of *M tuberculosis* by polymerase chain reaction has been found to be useful for the laboratory diagnosis of extra pulmonary and pulmonary tuberculosis. Clinical specimens from patients with tuberculosis subjected to PCR include sputum, pleural fluid and cerebrospinal fluid. Apart from this, PCR has also been applied for the identification of mycobacterial DNA in the formalin fixed paraffin sections. Most of the

PCR studies on formalin fixed paraffin sections are reported in the lung (99) and lymph node (118). Published reports regarding the utility of PCR in formalin fixed paraffin sections of intracranial tuberculoma are only few and most of them are case reports. In one report (65), PCR was useful in establishing tuberculous aetiology in a patient in whom intracranial tuberculoma resembled that of meningioma. In other two reports, (115,151), PCR was performed in CSF specimens to establish tuberculous aetiology in patients with intracranial tuberculoma. Hitherto, reports on larger series highlighting the utility of PCR in intracranial tuberculoma have not been published. In our data, PCR yielded positive results in 6 out of 10 surgical specimens of intracranial tuberculoma. It is well recognized fact that the effectiveness of PCR for detection of *M tuberculosis* DNA, in formalin fixed paraffin embedded tissue is dependent on a) fixation time of tissue in formalin b) DNA extraction procedure, c) length of PCR target, d) concentration of target DNA amplified, e) PCR protocol itself. Some of the above factors may be responsible for the relative low sensitivity of PCR in the intracranial tuberculoma. On the contrary, IHC method is simple, reproducible, and possesses operational advantage over PCR. The immunological reagents used in IHC method can be safely stored for one year at 4°C and this technique does not require any elaborate instrumentation and technical expertise. Thus we consider IHC is best suited to laboratories in developing countries where there is constraint in laboratory resources and technical expertise.

Table 4-5: **Data showing the results of bacteriological methods, IHC and PCR in intracranial tuberculoma.**

SI No.	CASE (Age/Sex)	BACTERIOLOGICAL		IHC	PCR
		CULTURE	Z-N STAIN		
1.	40/M	-	-	+	+
2.	28/M	+	+	-	+
3.	8M	-	+	-	+
4.	12F	-	-	+	+
5.	18/F	-	-	+	-
6.	60/M	-	-	+	-
7.	5/M	-	-	+	-
8.	11/M	-	-	+	-
9.	40/M	-	-	+	+
10.	36/F	-	-	+	+

4.9 DEMONSTRATION OF MYCOBACTERIAL DNA BY PCR IN FORMALIN FIXED PARAFFIN EMBEDDED SECTIONS OF INTRACRANIAL TUBERCULOMA

Six out of ten intracranial tuberculous granuloma gave positive results by PCR (Fig: 4.19 & Fig: 4.20). All the positive cases showed a single band in ethidium bromide stained agarose gel electrophoresis, which corresponded to the positive control (mycobacterial DNA). Interestingly enough, PCR showed positive results in two intracranial tuberculoma, which gave negative results in IHC. In these two cases extreme caseation with minimal cellular reaction were present in hematoxylin and eosin stained paraffin sections. These two cases also showed the presence of AFB in the Ziehl-Neelsen smear.

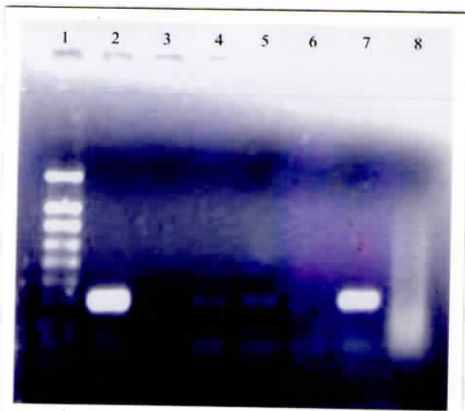


Fig 4.19 Agarose gel electrophoresis of PCR products amplified using IS6110 primers showing Lane 1: Marker* Lane 2: Positive control**; Lane 3: Negative control; Lane 4-7: DNA isolated from Intracranial tuberculoma showing positive and negative results in PCR; Lane 8: Fungal granuloma showing negative result.

Fig 4.20 Agarose gel electrophoresis of PCR products amplified using Dev primers showing Lane1: Marker*; Lane2: :Positive control**; Lane 3: Negative control; Lane 4-10: DNA isolated from Intracranial tuberculoma showing positive and negative results in PCR; Lane 8: Fungal granuloma showing negative Result.

*Marker: pUC 18 cleaved by Taq I, Sau IIIA. ** Positive control: *M tuberculosis* DNA from $H_{37}Rv$.

SUMMARY AND CONCLUSIONS

CHAPTER V

5.1 RESTATEMENT OF THE PROBLEM IN THE LABORATORY DIAGNOSIS OF TBM

TBM is one of the common infectious diseases of the CNS among hospitalised patients for neurological diseases in our country. TBM is a potentially curable infectious disease of CNS. However a complete neurological recovery in a patient with TBM depends upon early laboratory diagnosis as well as prompt institution of appropriate ATT. Any delay in making the diagnosis of TBM and the commencement of ATT will invariably result in neurological complications as well as sequelae of the disease. The "Gold Standard" applied for the laboratory diagnosis of TBM is the demonstration of the causative agent of the disease *M tuberculosis* in the CSF specimens by the bacteriological methods (i.e. Z-N staining method and cultures in L-J medium). Despite of the best possible technical efforts, *M tuberculosis* was isolated only in 12.5% of patients with TBM in this study. In the remaining 87.5% patients with TBM, the CSFs were analysed on more than one occasion and *M tuberculosis* could not be demonstrated by bacteriological methods. Besides the lower sensitivity, the conventional bacteriological methods are cumbersome, time-consuming and require at least 8-10 weeks for culturing *M tuberculosis*. Therefore the bacteriological methods are not useful for establishing the early laboratory diagnosis of TBM. Because of the delay in the laboratory diagnosis as well as the delay in the institution of the appropriate ATT, a significant percentage of patients with TBM often manifest neurological complications and sequelae from this disease. These facts may account for the high mortality and morbidity rates that are

currently prevalent in patients with TBM. Thus alternate methods other than bacteriological methods are essential for the early laboratory diagnosis of TBM so that mortality and morbidity rate in TBM can be minimised.

5.2 DESCRIPTION OF PROCEDURE AND MAJOR FINDINGS

In order to achieve the above objective we developed immunodiagnostic as well as molecular biological methods in our laboratory to detect the mycobacterial antigens, antimycobacterial antibodies to mycobacterial antigen and mycobacterial DNA in CSF of patients with TBM. The sensitivities of both immunodiagnostic and molecular biological techniques were critically evaluated in culture proven patients with TBM and specificity was assessed in the CSF specimens of patients with non-tuberculous neurological diseases.

5.2.1 Direct demonstration of mycobacterial antigens in CSF cytospin smears.

In this unique and novel method, two cytospin smears of CSF in patients with TBM and control groups were prepared. One of the smears was stained with hematoxylin and eosin, and examined under the microscope. Cytological features in CSF smears such as lymphocytes, monocytoid cells (macrophage), eosinophils and neutrophils were recorded. The other smear was utilised for immunocytochemistry. Conventional immunocytochemical method [primary antibody rabbit IgG to *M tuberculosis;* secondary antibody anti rabbit IgG-biotin conjugate, streptavidin peroxidase and substrate

diaminobenzidine tetrachloride (DAB)] was performed to demonstrate mycobacterial antigen in the cytoplasm of the monocytoid cells. Presence of mycobacterial antigen was indicated by the development of brownish-pink granular material in the macrophages. This method demonstrated the presence of mycobacterial antigen in 16 / 22 CSF-cytospin smears of patients with TBM. In all of the culture proven CSF from TBM patients, the immunocytological assay gave positive results. More importantly this method gave negative results in all the CSF smears in the control groups. The technical steps of this assay are simple and required only 6 h to get results after the receipt of the specimens in the laboratory. Elaborate instrumentation and technical expertise are not essential. Reviewing the entire literature of immunodiagnosis of TBM, such a diagnostic approach has not been previously described so far. The only limitation of this test is that the CSF should not be contaminated with RBC and presence of adequate number of monocytes is mandatory in the cytospin smears.

5.2.2 Isolation of specific mycobacterial antigen by an immunoabsorbent affinity chromatography.

We collected 10 ml CSF (cisternal) at autopsy from a culture proven patient of TBM. The CSF-IgG was recovered by passing the CSF through Protein-A-Sepharose column. This CSF-IgG showed an end point antibody titer to CFA 1:64000 in ELISA. The CSF-IgG (3mg/ml) was coupled with Cynogen bromide-Sepharose 4B and thus an immunoabsorbent column was prepared. The CFA was passed through the immunoabsorbent column. The specific mycobacterial antigen in the CFA, bound to

immunoabsorbent column was eluted with 4 M urea. The SDS-PAGE showed that this affinity purified antigen possessed a molecular weight of 14 kDa. Antibody to 14 kDa was raised in rabbits and this was further evaluated using IEP. This approach is again unique and has not been described in the literature.

5.2.3 Dot - immunobinding assay in TBM

Using this 14 kDa antigen, a simple Dot-Iba was standardised in NCM for the detection of mycobacterial antigens and antimycobacterial antibody in CSF of patients with TBM and non-TBM. Specific immunological reaction was demonstrated by incubation with antirabbit IgG-biotin conjugate, extr-avidin alkaline phosphatase and DAB. Development of a purple colour in the center of NCM discs indicates positive reaction. This test can be done in an ordinary clinical laboratory and does not require elaborate instrumentation as required in other immunoassays such as ELISA, western blot assays. A single laboratory staff can examine large number of CSFs in a day.

5.2.4 Detection of mycobacterial antigen by Immunoblot assay

Immunoblot method was performed on heat inactivated CSF specimens from TBM and non-TBM patients, for the detection of mycobacterial antigen. Heat inactivation of CSF, prior to the assay reduced the risk of handling the infectious material in the laboratory. The assay carried a sensitivity of 70% and a specificity of 100%.

5.2.5 Polymerase chain reaction in TBM

The PCR was performed in 40 CSFs from TBM patients, using a standard PCR protocol. IS6110 and Dev primers were used to amplify the mycobacterial DNA present in the CSF samples. The PCR products were analysed by agarose gel electrophoresis and Southern blot hybridisation. The assay showed a sensitivity of 37% in probable TBM patients and 60% in confirmed TBM patients.

5.2.6 IHC and PCR in intracranial tuberculoma

Besides the application of Dot-Iba and PCR in CSFs, an attempt was also made in this study, to demonstrate mycobacterial antigen and mycobacterial DNA in the intracranial tuberculous lesion by IHC and PCR method. This becomes essential because *M tuberculosis* could not be demonstrated in the tuberculous lesion by bacteriological methods. The results of IHC and PCR in the formalin fixed paraffin sections of tuberculoma were 80% and 60% respectively. Both methods were useful to distinguish a tuberculous granuloma from fungal granuloma. The distinction between two diseases is difficult at light microscopic level and secondly, the chemotherapy for both diseases differs from each other.

5.3 CONCLUSIONS

- The bacteriological method in this study showed a sensitivity of 12.5% for the laboratory diagnosis of TBM.
- A unique immunocytochemical method was developed in this laboratory to demonstrate the mycobacterial antigen in the CSF specimen. This method has definite diagnostic significance in the laboratory diagnosis of TBM.
- Using an affinity chromatography, a specific mycobacterial antigen (14 kDa) was isolated from the CFA of *M tuberculosis* using human specific CSF-IgG to *M tuberculosis.*
- A simple Dot-Iba was developed in the laboratory to detect the mycobacterial antigen and antimycobacterial antibody in the CSF of patients with TBM. The assay carries a sensitivity of 76% and a specificity of 100%.
- PCR for *M tuberculosis* in CSF of TBM showed a sensitivity of 37% in TBM.
- Immunohistochemical method was useful to demonstrate a mycobacterial antigen in the formalin fixed paraffin sections of intrathecal tuberculoma and showed a sensitivity of 80%.
- PCR was performed simultaneously in the formalin fixed paraffin sections of intracranial tuberculoma and gave a sensitivity of 60%.
- Both IHC and PCR were helpful in distinguishing tuberculous granuloma from fungal granuloma in those cases, which gave positive results in the assay.

- Because the bacteriological method is less sensitive, development immunodiagnostic methods are essential for the laboratory diagnosis of neurotuberculosis. Immunoassays like direct immunocytochemical and Dot-Iba methods developed in our laboratory will meet the above requirements and are best suited to laboratories in developing world.

5.4 RECOMMENDATION FOR FUTURE INVESTIGATION

- The results of this study indicate that, the direct demonstration of mycobacterial antigen in the CSF cytospin-smears using an immunocytochemical method hold definite promise for the rapid laboratory diagnosis of TBM. It is being suggested to undertake this study in a larger number of CSF cytospin smears of TBM and non-TBM (disease control) groups to assess the sensitivity and specificity.
- Using an affinity chromatography, 14 kDa mycobacterial antigen was isolated from the culture filtrate of *M tuberculosis.* This mycobacterial antigen was used for the laboratory diagnosis of TBM. It will be essential to characterise the antigenic epitopes and immunochemical properties of 14 kDa antigen with reference monoclonal antibodies to *M tuberculosis*.
- In this study, the PCR for TBM showed a low sensitivity. IS6110 negative strains of *M tuberculosis* have been reported in Kerala State (64). In order to enhance the sensitivity of the PCR for the diagnosis of TBM, newer sets of primers should be

used. Attempts should be made to prepare in-house primers, which will amplify the mycobacterial DNA in CSF even at very low concentrations.

- The sensitivity of PCR in formalin fixed tuberculoma was 60%. In order to enhance the sensitivity of PCR in tuberculoma, an attempt should be made to isolate mycobacterial DNA in the unfixed specimens of intracranial tuberculoma. An attempt has to be made to asses the sensitivity of PCR in the fixed and unfixed specimens of intracranial tuberculoma from the same patient.
- Routine application of immunohistochemical method for the laboratory diagnosis of intracranial tuberculous granuloma is advocated particularly in those cases in which AFB and culture did not confirm the diagnosis.

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APPENDICES

APPENDIX - A

PUBLICATIONS FROM THIS THESIS

Published papers

1. **Sumi MG**, Mathai A, Sarada.C and Radhakrishnan VV.
   Rapid diagnosis of tuberculous meningitis using Dot-immunobinding assay to detect mycobacterial antigen in cerebrospinal fluid specimens. ***J Clin Microbiol*** 1999; 37: 3925-3927.

2. **Sumi MG**, Mathai A, Sarada.C and Radhakrishnan VV
   Rapid diagnosis of tuberculous meningitis by a Dot-immunobinding assay. ***Acta Neurol Scand*** 2000; 101:61-64.

3. Mathai A, Radhakrishnan VV, **George SM**, Sarada C.
   A newer approach for the laboratory diagnosis of tuberculous meningitis. ***Diagn Microbiol Infect Dis*** 2001; 39(4): 225-8.

4. **Sumi MG**, Mathai A, Sheela R, Radhakrishnan NS, Radhakrishnan VV, Indhulekshmy R and Sathish Mundayoor.
   Diagnostic utility of Polymerase Chain reaction and immunohistochemical techniques for the laboratory diagnosis of intracranial tuberculoma. *Clin Neuropathol* 2001; 20:176-180.

Accepted for publication

1. **Sumi MG**, Mathai A, Reuben S, Radhakrishnan V.V, Sasikumar S, Jayapal V and Felix J.A Dot-Immunobinding Assay (Dot-Iba) for the rapid diagnosis of Pulmonary Tuberculosis. (Accepted in ***Indian Journal of Experimental Biology***)

Under publication

1. **Sumi MG**, Mathai A, Reuben S, Sarada C, Radhakrishnan VV. An immunocytochemical method for the early laboratory diagnosis of tuberculous meningitis. (***Clinical Diagnostic and laboratory Immunology***)

2. **Sumi MG**, Mathai A, Reuben S, Sarada C, Radhakrishnan VV, Indulakshmi R, Sathish M, Ajaykumar R, Manju Y K. A Comparative Evaluation of Dot Immunobinding Assay (Dot -Iba) and Polymerase Chain Reaction (PCR) for the Laboratory Diagnosis of Tuberculous Meningitis. (***Diagnostic Microbiology and Infectious Diseases***)

3. Mathai A, **Sumi MG**, Sarada C, Radhakrishnan VV. Detection of heat inactivated mycobacterial antigens in CSF of patients with TBM. (***Neurology India***)

APPENDIX - B

PROTOCOL FOR THE PREPARATION OF REAGENTS

B.1 Acrylamide solution

Acrylamide - 25.2 g

Bisacrylamide - 0.8 g

Dissolved in 70 ml of deionised water. When the acrylamide was completely dissolved, water was added to a final volume of 100 ml. The solution was filtered under vacuum through a 0.45 membrane. Stock acrylamide was stored at 4° C in a dark bottle.

B.2 Carbonate bicarbonate buffer

Sodium carbonate - 1.59 g

Sodium bicarbonate - 2.93 g

Sodium azide - 0.2 g

Total volume was made upto 1 liter by dissolving in distilled water. (pH 9.6)

B.3 Chloroform : Isoamyl alcohol (24:1)

Chloroform and Isoamyl alcohol was mixed in a proportion of 24:1. The above solution was stored under 100mM Tris-Cl (pH-0.8) at 4 °C in dark glass bottle.

B.4 Denaturation buffer

5N NaCl

1N NaOH

Distilled water

B.5 Denhardt's reagent (50X)

Ficoll	-5 g
Polyvinylpyrrolidone	-5 g
Bovine serum albumin	-5g

The above reagents were dissolved is 500ml of distilled water. It was filtered and stored at -20°C.

B.6 Diethanolamine buffer

Diethanolamine	- 97 ml
Sodium azide	- 0.2 g
Magnesium chloride $6H_2O$	- 0.1 g

Dissolved in 800 ml distilled water. The pH was adjusted to 9.8 by dilute HCl and made upto 1 litre.

B.7 Digestion buffer

50 mM Tris Cl (pH 7.5)

10 mM EDTA

500 mM NaCl

100ml of the buffer was prepared using the above solutions, sterilized by autoclaving and stored at 4° C. 1% SDS as well as Proteinase K (300µg/ml) was added at the time of use.

B.8 Ethidium Bromide:

10mg of ethidium bromide was added to 1ml of distilled water and stirred on a magnetic stirrer for several hours to ensure that the dye has dissolved properly. It was stored in a dark bottle at room temperature

B.9 Hybridisation buffer

5XSSC

0.5% SDS

100 µg/ml denatured fragmented Salmon sperm DNA

50% formamide

Distilled water

B.10 Lysozyme:

A stock solution of lysozyme was prepared at a concentration of 50mg/ml in distilled water. It was dispensed into aliquots and stored at -20°C. The aliquots were discarded after each use.

B.12 Neutralisation buffer

5N NaCl

1M Tris Cl (pH 7.5)

Distilled water

B.13 Phenol

Phenol was distilled at 160°C to remove oxidation products. Hydroxyquinone was added to a final concentration of 0.1%. It was then equilibrated with equal volumes of buffer (0.5 M Tris-Cl pH=8.0). The mixture was stirred on a magnetic stirrer for 15 minutes. When the layers separated, the upper aqueous layer was aspirated. This procedure was repeated until the pH of phenol was >7.8. After equilibration, the phenol was stored under a layer of 0.1M Tris-Cl (pH = 0.8) containing 0.2% β mercaptoethanol.

B.14 Phenol: Chloroform (1:1)

Equal volumes of phenol and chloroform was mixed and equilibrated by extracting several times with 0.1 M Tris-HCl. The equilibrated mixture was stored under an equal volume of 0.01M Tris-HCl (pH7.6) at 4 °C in dark glass bottle.

B.15 Phosphate buffered saline:

Disodium hydrogen phosphate.$2H_2O$	- 1.44 g
Potassium chloride	- 0.2 g
Sodium chloride	- 8.0 g

Total volume made upto 1 litre by dissolving in distilled water. (pH 7.4)

B.16 Phosphate buffered saline -Tween 20

Phosphate buffered saline	- 1000 ml
Tween –20	- 0.5 ml

B.17 Prehybridisation buffer

5X SSC

0.5% SDS

100 μg/ml denatured fragmented Salmon sperm DNA

5X Denhardt's solution

B.18 Proteinase K:

Stock solution was made by adding 20 mg of proteinase K to 1ml of distilled water; aliquoted and stored at -20°C. Concentration of proteinase K for each reaction was 50μg/ml.

B.19 Sample loading buffer:

Bromophenol Blue	- 25 mg
Xylene cyanol	- 25 mg
Glycerol	- 4 ml

4ml of glycerol was added to 6 ml of autoclave water and the solution was mixed thoroughly. Bromophenol blue and xylene cyanol was dissolved in the above solution and stored at 4 °C.

B.20 Sodium acetate (0.1M)

8.2 g of sodium acetate was dissolved in 800 ml distilled water. The pH was adjusted to 5.0 by the addition of glacial acetic acid. The final volume was made upto 1000 ml and stored at 4 °C.

B.21 Sodium acetate [(3M) pH 5.2 and pH 7]

408.1 g of sodium acetate .3 H_2O was dissolved in 800 ml of distilled water. The pH was adjusted to 5.2 with glacial acetic acid or the pH was adjusted to 7.0 with

dilute acetic acid. The volume was adjusted to 1000 ml with distilled water. It was dispensed into aliquots and sterilized by autoclaving.

B.22 Sodium bicarbonate buffer (0.1 M)

8.4 g of sodium bicarbonate was dissolved in 800 ml of distilled water. The pH was adjusted to 9.0 by 0.1 M sodium carbonate. The final volume was made up to 100 ml and stored at 4 °C.

B.23 Sodium borate buffer (0.1M)

Disodium tetraborate 38.14 g was dissolved in 800 ml of distilled water. The pH was adjusted to 9.0 by the addition of dilute HCl. The volume was made upto 100 ml and stored at 4°C.

B.24 Sodium dodecyl sulfate (10% SDS)

100 g of electrophoresis grade SDS was dissolved in 900 ml of water. It was heated at 68°C to assist dissolving SDS in water. The pH was adjusted to 7.2 by adding a few drops of concentrated HCl. The volume was adjusted to 1000 ml with water and dispensed into aliquots.

B.25 SSC (20 X)

Trisodium citrate - 88.2 g

NaCl - 175.3 g

Distilled water - 1000ml

175.3 g of NaCl and 88.2 g of Trisodium citrate was dissolved in 800 ml of distilled water. The pH was adjusted to 7.0 with 10 N NaOH solution. The volume was adjusted to 1000ml with distilled water. Sterilize by autoclaving.

B.26 Tris borate- EDTA Buffer (10 X TBE):

Tris base -108 g

Boric acid -58 g

0.5 M EDTA (pH=8.0) -40 ml

Tris base and boric acid was dissolved in 800ml of distilled water and 40ml of 0.5M EDTA was added to it. The volume was adjusted to 1000ml with distilled water.

B.27 Tris buffered saline

Tris base -6g

NaCl -17.4g

Tween 20 -0.1%

6g of Tris base was dissolved in 800 ml of distilled water. The pH was adjusted to 7.6 using concentrated HCl. 17.4g of NaCl was added to the above solution. 0.1 %

of Tween 20 was added to the solution and made upto 1000 ml. It was stored at 4°C.

B.28 Tris EDTA buffer

10 mM Tris Cl (pH 8)

1mM EDTA (pH 8)

B.29 Tris-Cl Buffer:

12.114g of Tris base was dissolved in 800ml of distilled water. The pH was adjusted to 8.0 using concentrated HCl. The volume was made upto 1000ml with distilled water.

APPENDIX- C

ABBREVIATIONS

ABC	Avidin-Biotin complex
Ab	Antibody
ADA	Adenosine deaminase
AFB	Acid fast bacilli
Ag	Antigen
AIDS	Acquired immune deficiency syndrome
AMTD	Amplicor *Mycobacterium tuberculosis* direct test
ATT	Antituberculosis therapy
bp	base pair/s
BCG	Bacille Calmette-Guerin
BSA	Bovine serum albumin
cm	centimeter
°C	Degree centigrade
CFA	Culture filtrate antigen
CIC	Circulating immune complex
CMI	Cell mediated immunity
CNS	Central nervous system
CSF	Cerebrospinal fluid
CT	Computer tomography

ddH_2O	double distilled water
dNTP	deoxyribonucleotide triphosphate
DAB	Diamino benzidine tetrachloride
DNA	Deoxyribonucleicacid
Dot-Iba	Dot immunobinding assay
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme-linked immunosorbent assay
fg	Fentogram
g	gram/s
h	Hour/s
HCl	Hydrochloric acid
H_2O	Water
H_2O_2	Hydrogen peroxide
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
IEP	Immunoelectrophoresis
IFA	Incomplete Fruend's adjuvant
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IHA	Indirect haemagglutination assay
IHC	Immunohistochemistry

kDa	Kilodalton
lbs	pounds
LAM	Lipoarabinomannan
LJ	Lowenstein-Jensen
μg	microgram
μl	microliter
μM	micromoles
mg	milligram
min	minute/s
ml	milliliter
mM	millimole
M	Molar
M	*Mycobacterium*
MAb	Monoclonal antibody
MAF	Macrophage activating factor
$MgCl_2$	Magnesium chloride
MIF	Macrophage inhibitory factor
MRI	Magnetic resonance imaging
MTD	*Mycobacterium tuberculosis* direct test
MTSE	*Mycobacterium tuberculosis* saline extract
ng	nanogram

nm	nanometer
N	Normal
NAP	p-nitro α-acetylamino-3-hydroxy propiophenone
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NCM	Nitrocellulose membrane
NCP	Nitrocellulose paper
OT	Old tuberculin
pg	picogram
pmol	picomole
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline-Tween.
PEG	Polyethylene glycol
PCR	Polymerase chain reaction
PT	Pulmonary tuberculosis
PPD	Purified protein derivative
rpm	rotations per minute
RaHG	Rabbit antihuman gammaglobulin
RBC	Red blood cells
RIA	Radioimmunoassay

SDS	Sodium dodecyl sulphate
SRBC	Sheep red blood cells
SSC	Sodium citrate - Sodium chloride
TBE	Tris-Borate- Ethylene diamine tetra acetic acid
TBM	Tuberculous meningitis
TBS	Tris buffered saline
TBS-T	Tris buffered saline-Tween
TE	Tris- Ethylene diamine tetra acetic acid
TRC	Tuberculosis Research Center
TSA	Tuberculostearic acid
USA	United States of America
UV	Ultra violet
V	Volt
Vs	Versus
Z-N	Ziehl-Neelsen

APPENDIX - D

PROFORMA FOR RECORDING THE CLINICAL DATA AND INVESTIGATIONS IN PATIENTS WITH TUBERCULOUS MENINGITIS, NON-TUBERCULOUS MENINGITIS AND NON-MENINGITIC NEUROLOGICAL DISEASES

Name of the patient H. No. Age Sex

Date of Admission Date of Discharge/Death

1. Symptoms

   Fever

   Headache

   Vomiting

   Convulsions

   Altered sensorium

   Focal Neurological Deficits

   History of Tuberculosis

2. General examinations

   Temperature / Pulse / BP

   Lymphadenopathy

3. CNS Examination

   Consciousness

   Cranial nerve palsies

Fundus

Other neurological deficits

Signs of meningeal irritation

4. <u>CVS / GIT / RESP System</u>

5. <u>Investigations</u>
    1. TLC
    2. Hb
    3. ESR
    4. Blood sugar
    5. Serum albumin
    6. Serum IgG
    7. Bacteriology of any site of infection
    8. Chest X-Ray
    9. Mantoux test
    10. Biopsy if any
    11. CT scan head
    12. MRI scan

6. <u>CSF Chart</u>
    1. Pressure
    2. Total count
    3. Lymphocytes
    4. Protein
    5. Sugar
    6. Albumin
    7. Immunoglobulin-G

8. CSF-IgG Index
9. Mycobacterial Antibody Index
10. Gram stain /AFB stain / India ink
11. Culture for pyogenic /AFB / Fungus
12. ELISA for *M tuberculosis*
13. Blot
14. PCR

7. <u>Treatment and duration</u>

Anti-tubercular drugs

Anti pyogenic drugs

8. <u>Hospital course</u>

Improved / Deteriorated / Expired

9. <u>Autopsy findings</u>

10. <u>Diagnosis of TBM</u>

1. Confirmed by AFB isolation of CSF
2. Presumed by TB focus in the body
3. Presumed by therapeutic response
4. Confirmed at autopsy
5. By ELISA test
6. By Blot method
7. By PCR technique

8. CSF-IgG Index
9. Mycobacterial Antibody Index
10. Gram stain /AFB stain / India ink
11. Culture for pyogenic /AFB / Fungus
12. ELISA for *M tuberculosis*
13. Blot
14. PCR

7. <u>Treatment and duration</u>

Anti-tubercular drugs

Anti pyogenic drugs

8. <u>Hospital course</u>

Improved / Deteriorated / Expired

9. <u>Autopsy findings</u>

10. <u>Diagnosis of TBM</u>

1. Confirmed by AFB isolation of CSF
2. Presumed by TB focus in the body
3. Presumed by therapeutic response
4. Confirmed at autopsy
5. By ELISA test
6. By Blot method
7. By PCR technique