A T cell based assay using specific *Mycobacterium tuberculosis* antigens, ESAT-6 and CFP 10, for the diagnosis of Tuberculous Meningitis

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CERTIFICATE

This is to certify that the study **"A T cell based assay using specific *Mycobacterium tuberculosis* antigens, ESAT-6 and CFP 10, for the diagnosis of Tuberculous Meningitis"** is a bona fide work of Dr. Maya Mary Mathew submitted in fulfillment of the DM Branch 1 - Neurology examination conducted by the Dr M.G.R Medical University, Chennai, Tamilnadu in February 2007. Her work was carried out under the guidance of **Dr. Chandran Gnanamuthu**, Professor in Neurology.

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INTRODUCTION

Tuberculous meningitis is the most dangerous form of infection with *Mycobacterium tuberculosis.* Despite chemotherapy, it is fatal in 25% of adults and causes neurological sequelae in 25% of survivors [1]. Delay in diagnosis and treatment are regarded as the major contributing factors in the high mortality. The diagnosis and treatment of tuberculous meningitis continues to challenge physicians throughout the world.

Commencing treatment for tuberculous meningitis prior to the onset of alteration of sensorium is the only way to ensure improved outcome. Unfortunately, three factors make this difficult. First, the presenting clinical features of the disease are non-specific. Second, small numbers of bacilli in the cerebrospinal fluid (CSF) reduce the sensitivity of conventional bacteriology. Third, alternative diagnostic methods are incompletely assessed [2].

The current practice for diagnosis of tuberculous meningitis is based on clinical features (including CSF analysis), radiology, bacteriology and molecular methods. Several diagnostic criteria are in use for initiating antituberculous therapy. There are problems with these criteria. First, the criteria vary according to the prevalence of tuberculosis. Second, if antituberculous chemotherapy is started on the basis of one diagnostic variable, more than half will be wrongly treated unnecessarily exposing them to the risks of drug toxicity. If treatment is only begun in the presence of more than one variable, nearly half will not receive appropriate treatment and these patients will eventually die or be left with severe neurological sequelae.

The definitive diagnosis of tuberculous meningitis depends upon the detection of the tubercle bacilli in the CSF, either by smear examination or by bacterial culture. Kennedy and Fallon [3] reported tubercle bacilli in 87% of clinically suspected cases. This requires large volumes of CSF to be carefully examined. However, reports from developing countries could achieve bacteriological confirmation in as few as 10% of the cases. Culture of the CSF for tubercle bacilli is not invariably positive. It requires several weeks before culture is positive for mycobacterium bacilli.

Because of frequent difficulty in detecting tubercle bacilli in smears or cultures of CSF, a number of tests have been developed to establish early and definitive diagnosis.

Radioactive bromide partition test and identification of components of the mycobacterial cell wall (e.g., tuberculostearic acid) have been reported to have sensitivity and specificity of over 90%. However, the clinical utility of these has not been found to be satisfactory [4].

Antibodies against tubercle bacilli can be detected with enzyme-linked immunosorbent assay (ELISA) with variable success.

The latex particle agglutination test, which allows rapid detection of tubercle bacillus antigen in CSF, has been reported to be a simple and specific test.

The intradermal tuberculin skin test (TST) is helpful when positive. This has been reported to be negative initially in 50-70% of cases and often becomes positive during therapy.

Mycobacterial RNA or DNA sequences in CSF are identified using cDNA probes by the polymerase chain reactions. These are highly specific and sensitive in the diagnosis of tuberculous meningitis.

Recent advances in *M. tuberculosis* genomics, using subtractive hybridization and DNA microarrays, have identified a genomic segment, RD1, that is present in *M. tuberculosis* complex but absent from all strains of *M. bovis* BCG and almost all environmental mycobacteria [5]. RD1 gene products offer the potential for the development of new diagnostic tests that might differentiate *M.tuberculosis* (MTB) infection from BCG vaccination and exposure to environmental mycobacteria. Early secretory antigen target – 6 (ESAT-6) and culture filtrate protein 10 (CFP 10) are two such gene products [6].

The enzyme-linked immunospot assay (ELISPOT) detects interferon-γ (IFN-γ) secreting T cells specific for these two antigens [7]. Applied to blood samples this assay has high sensitivity and specificity for the diagnosis of tuberculous infection, but does not distinguish between latent tuberculous infection and active tuberculosis[8]. Recently it has been shown that ESAT-6-specific IFN-γ-secreting $CD4^+$ T cells are concentrated at sites of active tuberculosis [9]. Using ELISPOT these cells can be detected in pleural fluid, suggesting that this assay may be a rapid, sensitive and specific marker

for active tuberculosis at specific anatomical sites. However, detection of functional MTB-antigen-specific T cells in CSF is scientifically challenging given the rapid activation-induced death of CSF T cells in tuberculous meningitis.

Given the possibility of using this T cell assay for diagnosis of active tuberculous infection, we aimed to determine whether IFN-γ-secreting MTB-antigen-specific T cells are present in the CSF of patients with tuberculous meningitis, whether the use of ELISPOT on CSF samples is feasible, and whether ELISPOT has a clinically useful diagnostic sensitivity for active tuberculous meningitis.

AIM

To determine the presence of interferon γ-secreting *Mycobacterium tuberculous* antigen-specific T cells in the cerebrospinal fluid of patients with tuberculous meningitis using the enzyme linked immunospot assay (ELISPOT).

OBJECTIVES

To evaluate usefulness of the enzyme linked immunospot assay (ELISPOT), a T cell based assay using specific *Mycobacterium tuberculosis* antigens, ESAT-6 and CFP 10, for the diagnosis of tuberculous meningitis.

LITERATURE REVIEW

Historical background

Tuberculosis has stalked humankind for centuries. The characteristic lesions caused by the rod-like

tubercle bacillus *Mycobacterium tuberculosis* have been discovered in Neolithic skeletons in Europe, and in Egypt in mummies dating back to 3700 BC. Pulmonary tuberculosis, or 'leprosy of the lung', was described and known as phthisis by Hippocrates [10]. At the height of the tubercular epidemic of 1882, the self trained Prussian bacteriologist, Robert Koch isolated and identified the tubercle bacillus, thereby proving the contagious nature of the disease [11]. In 1933, Rich and McCordock, after a series of post-mortem experiments in rabbits and children, found that tubercular meningitis developed after the release of bacilli from old focal lesions in communication with the meninges [12].

The only treatment for tuberculosis then was a long stay in a sanatorium with complete rest, nutritious diet and fresh air. Despite the discovery of the BCG (Bacilli-Calmette-Guerin) in 1906, there was no cure for tuberculosis until the introduction of streptomycin in the late 1940s. Despite advances in recognition of the disease, improved living conditions and effective chemotherapeutic drugs, tuberculosis continues to manifest in humans in different forms.

Epidemiology

Tuberculosis is the world's second commonest cause of death from infectious disease, after HIV/AIDS. There were an estimated 8 – 9 million new cases of tuberculosis in 2000, fewer than half of which were reported; 3 – 4 million cases were sputum – smear positive, the most infectious form of the disease [13]. Sub-Saharan Africa has the highest incidence rate (290 per 100 000 population), but the most populous countries of Asia have the largest number of cases: India, China, Indonesia, Bangladesh and Pakistan together account for more than half the global burden. The average prevalence of all forms of tuberculosis in India is estimated to be 5.05 per thousand, prevalence of smear-positive cases being

2.27 per thousand and average annual incidence of smear –positive cases 84 per 1,00,000 annually [14]. It has been estimated that approximately 10% of all patients with tuberculosis have CNS involvement. Tuberculous meningitis accounts for 70 to 80% of cases of neurological tuberculosis [15].

Pathology and Pathogenesis of tuberculous meningitis

The macroscopic consequences of infection have been researched post mortem and, more recently, through CT and MRI of the brain [16]. The early meningeal exudate setting off the whole process of basal tuberculous meningitis is believed to arise from outward extension of a small focus in the cortical surface, often referred to as the 'Rich focus'. Alternatively, there may be miliary TB of the brain, and one of the miliary tubercles may rupture outwards to produce similar extensive meningitis.

In summary, three processes cause most of the common neurological deficits: the adhesive exudates can obstruct CSF causing hydrocephalus and compromise cranial nerves; granulomas can coalesce to form tuberculomas (or an abscess) which, depending on their location, cause diverse clinical consequences; and an obliterative vasculitis can cause infarction and stroke syndromes [2].

Molecular and Cellular pathogenesis

The main route of entry of the causative agent is the respiratory route and hence alveolar macrophages are the important cell types, which combat the pathogen. *M tuberculosis* replicates slowly but

continuously and spreads via the lymphatic system to the hilar lymph nodes. In most infected individuals, cell-mediated immunity develops 2 to 8 weeks after infection. Activated T lymphocytes and macrophages form granulomas that limit further replication and spread of the organism. *M tuberculosis* is in the centre of these caseating granulomas, but is usually not viable. Unless there is subsequent defect in cell-mediated immunity, the infection generally remains contained and active disease may never occur [17].

The development of cell-mediated immunity against *M tuberculosis* is associated with the development of a positive result in the tuberculin skin test. At the cellular level, an effective host immune response occurs as follows [18]. Alveolar macrophages infected with *M tuberculosis* interact with T lymphocytes via several important cytokines. The infected macrophage releases interleukins 12 and 18, which stimulate T lymphocytes (predominantly CD4 positive T lymphocytes) to release interferon γ. This cytokine, in turn, stimulates the phagocytosis of *M tuberculosis* in the macrophage. Interferon γ does not directly stimulate the killing of *M tuberculosis* by the macrophage, at least partly because the organism inhibits the cytokine's transcriptional responses. Interferon γ is, however, crucial for the control of *M tuberculosis* infection, and it also stimulates the macrophage to release tumour necrosis factor α, which is important in granuloma formation and control of the extent of infection. The T-lymphocyte response is antigen specific and is influenced by the major histocompatibility complex.

Kinetics of immune response is used to understand the pathophysiology and prognosis of patients with tuberculous meningitis [56]. CSF concentrations of lactate, interleukin-8, and interferon γ were high before treatment and then decreased rapidly with antituberculosis chemotherapy. However, significant immune activation and blood-brain barrier dysfunction were still apparent after 60 days of treatment. Death was associated with high initial CSF concentrations of lactate, low numbers of white blood cells, in particular neutrophils, and low CSF glucose levels.

Several factors trigger subsequent development of active disease. HIV is the greatest single risk factor for progression to active disease in adults. Other medical conditions that can compromise the immune system and predispose to development of active disease include poorly controlled diabetes mellitus, renal failure, underlying malignant disease, chemotherapy, extensive corticosteroid therapy, malnutrition, and deficiency of vitamin D or A. These conditions cause defects in the production of tumour necrosis factor α, interferon γ, and interferon γ receptor and interleukin-12 receptor β1.

Clinical diagnosis

Udani et al, in their report, have described the various neurological syndromes caused by tuberculosis [19]. A high index of clinical suspicion is needed to diagnose tuberculous meningitis, as the initial symptoms may be non-specific. Non-specific symptoms include malaise, anorexia, fatigue, fever, and headache. These may last 2-8 weeks prior to the development of meningeal irritation. This is then followed by appearance of focal neurological deficits. As the disease progresses, increasing evidence of cerebral dysfunction sets in with apathy and irritability progressing to increasing lethargy, confusion, stupor and coma. The terminal illness is characterized by deep coma with decerebrate or decorticate rigidity. The Medical Research Council clinical staging for tuberculous meningitis is as follows [20]:

Stage I: prodromal phase with no definite neurological symptoms

Stage II: signs of meningeal irritation with slight or no clouding of sensorium and minor (cranial nerve palsies), or no neurological deficit

Stage III: severe clouding of sensorium, convulsions, focal neurological deficit and involuntary movements

The single most important determinant of outcome, for both survival and sequelae, is the stage of tuberculous meningitis at which treatment has been started. If treatment is started in stage I, mortality and morbidity is very low, while in stage III almost 50% of patients die, and those who recover may have some form of neurological sequelae.

CSF analysis is an important aspect in the diagnosis of tuberculous meningitis. Usually, there is a predominant lymphocytic reaction (60 –400 white cell per ml) with raised protein levels (0.8 – 4 g/l). In the early stages of infection, a significant number of polymorphonuclear cells may be observed, but over the course of several days to weeks they are typically replaced by lymphocytes. There is a gradual decrease in the sugar concentration of the CSF, which is usually less than 50% of serum glucose concentration, the value ranging from 18 – 45 mg/dl [21].

Chest radiography finds active or previous tuberculosis infection in about 50% of those with tuberculous meningitis, but these findings lack specificity in settings with a high prevalence of pulmonary tuberculosis.

Two studies have tried to identify the clinical and CSF findings predictive of tuberculous meningitis. The first by Kumar et al compared clinical findings at presentation of 110 children with tuberculous meningitis with 94 children who had meningitis which was pyogenic or who recovered with no antituberculous treatment. Five clinical variables were predictive of tuberculous meningitis: report of symptoms for longer than 6 days, optic atrophy, focal neurological deficit, abnormal movements, and neutrophils forming less than half the total CSF leucocytes [22].

The second study by Thwaites et al compared the clinical outcomes of 143 Vietnamese adults with tuberculous meningitis (TM) with 108 who had either a pathogenic bacteria isolated from the CSF or a

CSF glucose to blood glucose ratio less than 0.5 and recovered without antituberculous treatment[23]. A diagnostic rule was developed based on five variables that had a sensitivity of 86% and specificity of 79% when it was tested on a further 75 adults.

Variable	Score
Age (years)	
>36	2
<36	0
Blood WCC (per ml)	
>15000	4
<15000	0
History of illness (days)	
≥6	-5
<6	0
CSF total WCC (per ml)	
≥750	3
<750	0
CSF % neutrophils	

≥90	4
<90	0

WCC = white cell count. Suggested rule for diagnosis: total score ≤ 4 = TM; total score >4 = non –TM (TM – tuberculous meningitis)

The results of these two diagnostic rules are affected by tuberculosis and HIV infection prevalence. Given the fatal consequences of delayed treatment, clinicians should be encouraged to initiate "empirical therapy" in the setting of compatible clinical, epidemiological and laboratory findings.

Radiological diagnosis

Computed tomography (CT) or magnetic resonance imaging (MRI) of the brain may reveal thickening and intense enhancement of meninges, especially in basilar regions [24]. Ventricular enlargement is present in majority of patients. The reported frequency of infarcts demonstrated by CT varies from 20.5% to 38%. Davis et al found tuberculomas in 16% of patients with culture positive or presumptive tuberculous meningitis [25]. It is difficult to differentiate forms of neurotuberculosis from other cerebral disorders radiologically. Kumar et al compared the CT scans of 94 children with tuberculous meningitis with those of 52 children with pyogenic meningitis and found basal enhancement, hydrocephalus, tuberculoma and infarction more common in those with tuberculous meningitis, whereas subdural collections were more common in those with pyogenic meningitis [26]. They suggested basal meningeal enhancement, tuberculoma, or both, were 89% sensitive and 100% specific

for the diagnosis of tuberculous meningitis. Cranial MRI is better than CT for showing brain stem and cerebellar pathology, tuberculomas, infarcts, and the extent of inflammatory exudates.

Tests for detection and identification of mycobacteria

They are broadly divided into direct and indirect tests. The direct approach is concerned with the detection of the mycobacteria by microscopy or culture, detection of tuberculostearic acid (TSBA), biochemical assays, mycobacterial antigen assays and identification of mycobacteria by nucleic acid amplification techniques. The indirect approach includes measurements of host immunity against the bacteria: humoral immunity via the detection of antibodies against the bacteria and cellular immunity via skin tests and T cell based assays.

DIRECT TESTS

Microscopy

Microscopy using Zeihl-Neelsen method or flurochrome procedures with stains such as auramine / rhodamine is rapid, cheap and easy. Sensitivity depends on the source of the sample. The demonstration of AFB in CSF in patients with tuberculous meningitis is less than 20%. A recent study reported a bacteriological diagnosis of tuberculous meningitis in 107 (81%) of 132 adults with the disease; acid-fast bacilli were seen in 77 (58%) patients, and cultured from 94 (71%) patients [27]. They recommend that at least 6 ml of CSF should be collected and examined for 30 minutes to improve the yield.

Detection of TSBA

Tuberculostearic acid (TSBA) is a cell wall fatty acid of mycobacteria and can be detected with gas chromatography. This was reported as a rapid diagnostic test for tuberculous meningitis. It is however, impractical for average routine laboratory because of the equipment and skill needed for the test [28].

Antigen detection

Mycobacterial antigens have been detected by enzyme linked immunosorbent assay (ELISA) in sputum and CSF [29] and by latex agglutination assay in CSF [30].

Culture

Culture for *Mycobacterium tuberculosis* is considered the gold standard in the diagnosis of tuberculosis [31]. Decontamination procedures needed for pulmonary, urinary and gastric juice sample is unnecessary for CSF and other materials collected aseptically, which can be inoculated directly on the media. The recommended practice is to culture on both liquid and solid media. Solid media include egg based media such as Lowenstein-Jensen, Coletsos or agar based media such as Middlebrook 7H10. Liquid media include Kirchner or Middlebrook 7H9 broth. A mean incubation period of 4 weeks is needed before growth can be detected. There are three rapid culture methods, where growth can be picked up in 2 to 3 weeks, these include the radiometric BACTEC method, Septi Chek AFB and microcolony detection on solid media.

Nucleic acid amplification

Nucleic acid (DNA or RNA) amplification techniques can detect and identify mycobacteria directly in clinical samples. The most widely studied is the polymerase chain reaction (PCR). These PCR assays may target either DNA or rRNA and these could be based on conventional DNA based PCR, nested PCR and RT-PCR. Targets include insertion and repetitive elements, various protein encoding genes and ribosomal RNA [32]. A recent systematic review and meta-analysis calculated that the sensitivity and specificity of commercial nucleic-acid-amplification assays for the diagnosis of tuberculous meningitis was 56% (95% CI 46-66) and 98% (97-99) respectively [33]. According to this data, the sensitivity of these assays is too low and may not be better than bacteriology.

INDIRECT TESTS

Antibody detection

These tests use various modifications of the ELISA or immunochromatographic methods to detect different antibody classes. The commonly evaluated tests are based on the detection of IgG or IgA against 38 k Da protein either in the pure or recombinant form. An ELISA test using mycobacterial antigens was devised in this institution. The assay detected mycobacterial antibodies in the CSF of patients with tuberculous meningitis. Three antigens namely PPD, BCG, and *M tuberculosis* were used. With the *M.tuberculosis* antigen, the sensitivity of the assay was 72% and specificity 92% [34].

Tests based on cellular immunity

1.Tuberculin skin test

Since *M. tuberculosis* is an intracellular pathogen, assessment of whether a patient's T cells have been exposed to, and sensitized by, antigens specific to *M tuberculosis* provides an alternative approach to diagnosis and is the principle upon which the classic tuberculin skin test (TST) is based. TST involves intradermal inoculation of purified protein derivative (PPD), a crude precipitate of *M. tuberculosis* culture supernatant that contains more than 200 antigens widely shared among mycobacteria other than *M.tuberculosis*, including M.bovis bacille Calmette-Guerin (BCG) and many environmental mycobacteria [35]. It elicits a delayed-type hypersensitivity response in sensitized individuals. The broad antigenic cross-reactivity of PPD is responsible for the poor specificity of TST. A positive reaction is consistent with BCG vaccination, exposure to environmental mycobacteria, or *M. tuberculosis* infection. Repeated inoculation of tuberculin can itself boost a delayed-type hypersensitivity response to PPD; this booster effect is another cause of false-positive TST reactions. The intrinsic sensitivity of the TST for detecting latent *M.tuberculosis* infection is not known, because there is no definitive test against which to compare it; however, for patients with active tuberculosis, it is 75% -90% sensitive, and its sensitivity falls to <50% in patients with disseminated disease [36].

The tuberculin gamma interferon assay, QuantiFERON-TB (QIFN), is an in vitro test of cell-mediated immunity [37]. Sensitivity was not significantly different for smear-negative or smear-positive cases (80% versus 71%). The QIFN assay is a potential replacement for the Mantoux test.

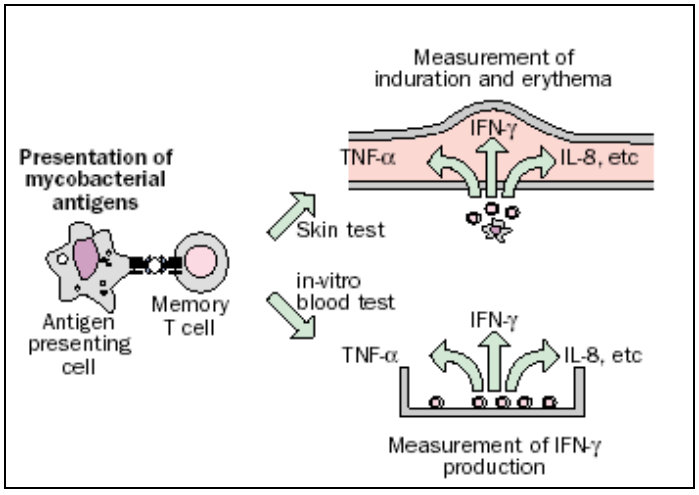
2. T cell based assay

ELISPOT IN THE DIAGNOSIS OF TUBERCULOUS MENINGITIS

Many attempts have been made to identify and isolate specific antigens from

M tuberculosis for use as diagnostic reagents. The identification of regions in the *M tuberculosis* genomes that are absent in BCG and most non-tuberculous mycobacteria provide an opportunity to develop new specific diagnostic tools by rational design. The test used here is an ex-vivo enzyme linked immunospot (ELISPOT) assay for IFN-γ. The basic principle of the test is the same as for the tuberculin skin test. In the in-vitro diagnostic test, mononuclear cells from the site of infection are stimulated in vitro and production of IFN-γ from sensitized T cells is measured by ELISA technique. The only difference being that while in the TST, sensitization to several common mycobacterial antigens are tested; in the immunospot assay, sensitization to one or more antigens specific to only *M.tuberculosis* is tested. A diagrammatic explanation of the in vivo TST and in vitro inmmunospot assay is given below (fig 1).

Fig 1: Diagrammatic representation of in vivo and in vitro T cell based diagnostic tests



Specific antigens of tuberculosis

During the past decade, a major scientific advance has been the identification of antigens that are expressed by *M.tuberculosis,* but not by BCG or by most environmental bacteria. Harboe and colleagues showed the first evidence of tuberculosis complex-specific antigen [38]. They detected the 24-kDa antigen MPB64 (also referred to as MPT64) in *M bovis* and *M tuberculosis* culture filtrates, but

not in those from BCG. This observation was later extended by PCR hybridisation studies, which showed the gene encoding MPT64 to be absent only in some BCG substrains (table below) [39]. More recently, the early-secreted antigenic target 6-kD protein (ESAT-6) was identified from culture filtrate because of its strong recognition in animals infected with *M tuberculosis* [40]. An interspecies assessment showed the gene to be largely restricted to the tuberculosis complex, being absent in all strains of BCG and in environmental isolates with the exception of *M kansasii, M marinum*, and *M szulgai* [41,42,43]. In continuation of this work, the promoter region of the *M tuberculosis ESAT-6* gene was cloned and another antigen designated culture filtrate protein 10 (CFP10), with the same species distribution as *ESAT-6* was identified (next table) [44].

Strain tested	Antigens		
	ESAT-6	CFP 10	MPT 64
Tuberculosis complex			
M tuberculosis	+	+	+
M africanum	+	+	+
M bovis	+	+	+
BCG substrain			
gothenburg	−	−	+
moreau	−	−	+
tice	−	−	+
tokyo	−	−	+
danish	−	−	−
glaxo	−	−	−
montreal	−	−	−
pasteur	−	−	−
Environmental strains			
M abcessus	−	−	−
M avium	−	−	−
M branderi	−	−	−
M celatum	−	−	−
M chelonae	−	−	−
M fortuitum	−	−	−
M gordonii	−	−	−
M intracellulare	−	−	−
M kansasii	+	+	−
M malmoense	−	−	−
M marinum	+	+	−
M oenavense	−	−	−
M scrofulaceum	−	−	−
M smegmatis	−	−	−
M szulgai	+	+	−
M terrae	−	−	−
M vaccae	−	−	−
M xenopi	−	−	−

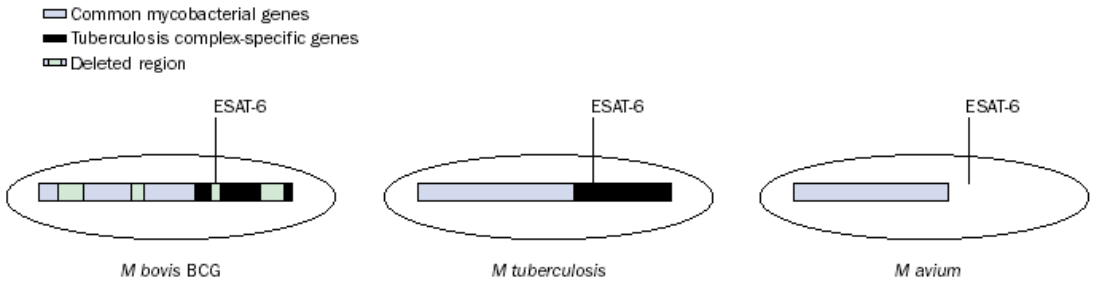
−=not present in species/strain, +=present in species/strain.

Distribution of diagnostic antigens in mycobacterial species

In 1996, Stover and colleagues [45] used subtractive hybridisation to define regions of differences (RD)—stretches of the genome of *M bovis*, which were deleted in BCG during in-vitro passage. These

regions were called RD-1, RD-2, and RD-3, and the genes for ESAT-6, CFP10, and MPT64 (in addition to a number of new potentially interesting antigens) reside in these deleted regions (figure 2). New studies using bacterial artificial chromosome libraries and microarray technology have identified at least eight more regions that have been deleted in BCG. Both ESAT-6 and CFP10 are genomic products of the RD1 gene.

Fig 2: Genes present in mycobacterial strains



Studies on use of ESAT-6 and CFP 10 antigens

The ESAT-6 antigen has so far consistently been reported to be strongly recognised by IFN-γ-secreting lymphocytes from patients with tuberculosis. ESAT-6 evokes murine T-cell responses early during *M tuberculosis* infection and induces stronger responses in patients with tuberculosis than other mycobacterial proteins tested so far [46,47]. The percentage of ESAT-6-responsive patients ranges in studies done in low endemic countries (Denmark, USA, Germany, and Kuwait) from 60% to 80%, whereas reactivity in healthy individuals is virtually absent [46,47, 48, 49]. ESAT-6 contains multiple broadly recognised T-cell epitopes, so that a test based on ESAT-6 should have the potential to induce responses in genetically diverse populations. In this regard, Lalvani and colleagues [50] enumerated ESAT-6-specific T cells by an IFN- specific ELISPOT assay and found a diagnostic sensitivity of more than 90%; however, no response was seen in unexposed healthy donors [50]. CFP 10 is recognised in almost the same high frequency in patients with tuberculosis [51].

Enzyme-linked immunospot assay (ELISPOT)

Lalvani and colleagues first devised this test in patients with past exposure to influenza virus. A sensitive enzyme-linked immunospot (ELISPOT) assay for single cell IFN-γ secretion to detect low frequencies of uncultured influenza peptide-specific CD8 T lymphocytes isolated from peripheral blood was applied. The ELISPOT assay detects secreted cytokine molecules in the immediate vicinity of the cell from which they are derived, while still at a relatively high concentration; each spot in the read-out representing a 'footprint' of the original cytokine-producing cell. Quantitation of these IFN-γ spot-forming cells (SFCs) by this technique is highly sensitive. [52].

ELISPOT assay for detection of latent *M tuberculosis* infection

Lalvani et al used the enzyme linked immunospot assay for rapid detection of *Mycobacterium tuberculosis* infection by enumeration of antigen-specific T cells [53]. In vitro stimulation of human peripheral blood mononuclear cells (PBMCs) by the specific ESAT-6 antigen induces IFN-γ detectable by enzyme-linked immunosorbent assay (ELISA). Four groups were studied. Forty-five of 47 patients with bacteriologically confirmed tuberculosis had ESAT-6 specific IFN-γ secreting T cells, compared with 4 of 47 patients with nontuberculous illnesss, indicating that these T cells are an accurate marker of *M.tuberculosis* infection. This assay thus had a sensitivity of 96% (95% confidence interval 92-100) for detecting *M.tuberculosis* infection in this patient population. By comparison, of the 26 patients with tuberculosis who had a diagnostic TST, only 18 (69%) were positive (p=0.003). In addition, 22 of 26 (85%) TST-positive exposed household contacts had ESAT-6 specific T cells, whereas 0 of 26 unexposed BCG-vaccinated subjects responded. Thus it distinguishes between *M.tuberculosis* infection and BCG vaccination. This has been further confirmed by similar studies by Lalvani et al. [54.55]. ELISPOT test can be used to determine prevalence of latent *Mycobacterium tuberculosis* infection. It

was performed in 100 prospectively recruited healthy adults in Mumbai [60]. Eighty percent responded to more than or equal to one antigen. In contrast, of 40 mostly BCG-vaccinated, United Kingdom-resident healthy adults, none responded to either antigen. This study suggests an 80% prevalence of latent *M.tuberculosis* infection in urban India.

ELISPOT assay for detection of active *M tuberculosis* infection

Recently it has been shown that ESAT-6-specific IFN-γ-secreting $CD4^+$ T cells are concentrated at sites of active tuberculosis [56]. Ten patients with tuberculous pleural effusion had a 15-fold concentration of the T cells relative to their level in peripheral blood. Such cells were absent in 8 control patients with nontuberculous pleural disease [56]. The recruitment of ESAT-6-specific T cells to inflamed tuberculous tissue demonstrates their function in vivo and suggests a novel way to diagnose tuberculous pleuritis. It has also been used to detect latent and active tuberculosis infection in HIV-positive individuals, sensitivity of the test being 92% [57].

ELISPOT assay for diagnosis of tuberculous meningitis

Detection of functional MTB-antigen-specific T cells in CSF is scientifically challenging given the rapid activation-induced death of CSF T cells in tuberculous meningitis.[58]. There is no published data on using this test for diagnosis of tuberculous meningitis. This study was undertaken as a pilot study to determine whether IFN-γ-secreting MTB-antigen-specific T cells are present in the CSF of patients with tuberculous meningitis, whether the use of ELISPOT on CSF samples is feasible, and whether ELISPOT has a clinically useful diagnostic sensitivity for active tuberculous meningitis.

MATERIALS AND METHODS

This was a prospective study carried out in the department of Neurology, Christian Medical College, Vellore, India, from March 2004 to May 2006. Subjects were also recruited from the departments of Medicine. Subjects were divided as cases and controls. Cases were patients with clinical features highly suggestive of tuberculous meningitis presenting consecutively to the study physician. Controls were patients with meningitis, but not of tuberculous etiology. Following were the criteria used for recruitment of patients.

Inclusion criteria

Age 16 years and more

Fever, headache or vomiting for more than 2 weeks in cases

CSF picture suggestive of meningitis

Exclusion criteria

Radiological evidence of significant brain edema and impending herniation (cannot get much CSF)

Antituberculous medication use for more than a week

Traumatic CSF (RBC count >1000/cmm)

The diagnosis of tuberculous meningitis was made based on defined criteria [59]. Specific clinical criteria included fever, headache and neck stiffness for more than 2 weeks. Supporting criteria consisted of 1) CSF findings of lymphocytic pleocytosis, raised protein levels and sterile cultures 2) CT/MRI findings of hydrocephalus, granulomas or basal exudates 3) evidence of extra CNS tuberculosis 4) appropriate response to anti-tuberculosis chemotherapy. Patients with specific clinical criteria were classified as "highly probable tuberculosis" if 3 supporting criteria are present or "probable tuberculosis" if only 2 were present. Only "probable" and "highly probable" cases were included in the study.

CSF (5ml) by lumbar puncture and heparinised peripheral venous blood (10ml) were collected from each of these patients at the time of the initial testing, at admission. Samples for ELISPOT assay were processed within 6 hours of collection. If collected overnight, they were kept in an air-conditioned room and sent to the lab for processing in the morning. All CSF samples were also subjected to estimation of sugar, protein content and to microscopy of centrifuged CSF with Gram, Ziehl-Neelsen and India ink stains, with PCR for mycobacterium tuberculosis (using IS6110 primers), and bacterial, mycobacterial and fungal culture.

The *ex-vivo* IFN-γ ELISPOT assay incorporating overlapping peptides spanning ESAT-6 and CFP10 was performed using $2.5X10^5$ peripheral blood mononuclear cells (PBMC) or CSF cells incubated overnight in complete medium in single wells of pre-coated interferon-γ ELISPOT plates, as described below. The reagents of the ELISPOT assay were obtained from the Tuberculosis Immunology Group, Nuffield Department of Medicine, University of Oxford.

Methodology of performing the MTB ELISPOT assay [60]

Add 100µl per well of R10 medium is added into the ELISPOT wells and placed in the incubator at 37^0C for 1 hour.

Collect 10ml of whole blood in a 50ml centrifuge tube containing 25µl of heparin (Heparin at a concentration of 5000U/ml).

Dilute the entire 10 ml of whole blood with 8 ml of RPMI1640 medium.

Gently layer 9ml of whole blood onto 5ml of Ficoll in a 15 ml Falcon tube.

Turn brake off and spin blood at 2000 rpm for 22minutes.

Transfer 2ml of the separated plasma and transfer into a clean 15ml Falcon tube and then add PBMC layer.

Bring up to 12ml with R10 medium.

Spin cells at 1700rpm for 7 minutes.

Remove supernatant from cells.

Gently resuspend pellet in R10.

Make up to 10ml with R10.

Mix and remove 10µl for counting chamber.

Spin at 1350 rpm for 7 minutes.

Resuspend pellets such that there are 2.5x 10^6 cells per ml.

Add 50µl "positive control" (PHA – phytoheamagglutinin), "panel A" (ESAT-6 pool) and "panel B" (CFP-10 pool) to appropriate wells. Add nothing to negative control.

Incubate the wells overnight at 37^0C in a 5%CO_2 incubator.

Wash 6 times with 150µl per well of PBS-Tween20 buffer. Tap on paper towels to remove remaining liquid.

Add secondary antibody at 1µgm per well. Dilute it in PBS. Add 50ul per well. (5µl per ml of PBS).

Incubate at room temperature for 90 minutes.

Wash 6 times with 150µl per well of PBS-Tween 20 buffer and tap plate on paper towels to remove remaining liquid.

Add 50µl per well of BCIP chromogen and allow spots to develop at room temperature for 20 minutes.

Once the spots are clearly seen in the wells containing PHA stop the reaction by washing the wells in tap water 3 times.

Procedure for processing CSF

Spin CSF in a centrifuge tube at 1500rpm for 10 minutes in a refrigerated centrifuge.

Remove supernatant using a pipette tip (Do not decant by tipping the tube, use only 200µl pipette). The cell pellet is usually not seen.

Resuspend cell pellet (invisible) in 5ml of RPMI1640.

Take out 10µl for counting.

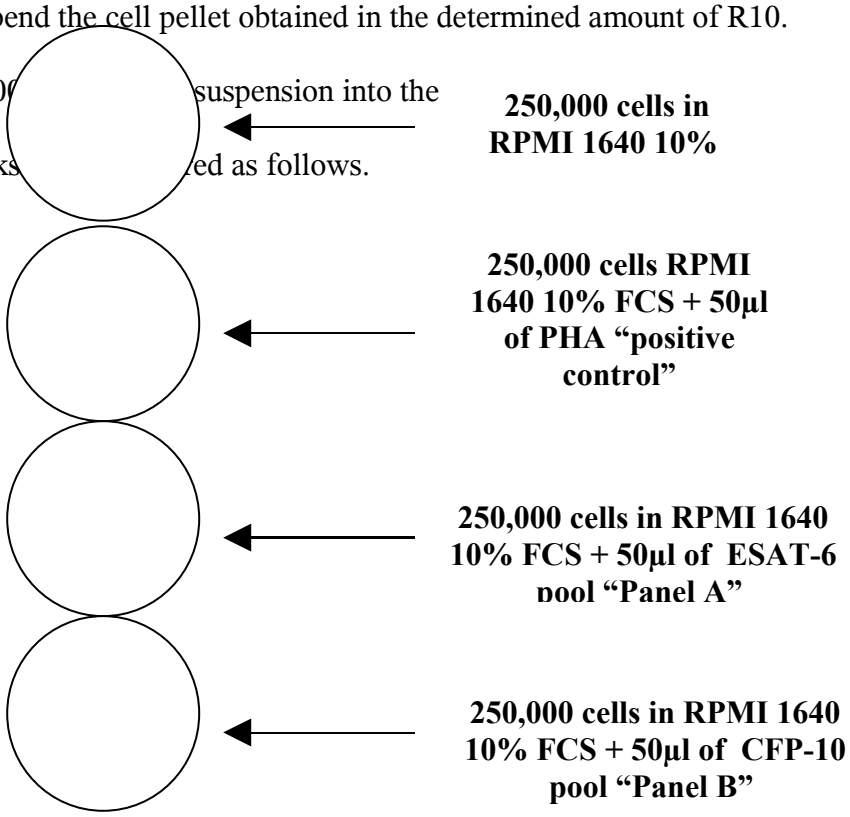
Spin the suspended cells at 1200rpm for 5minutes.

Determine the amount of R10 to be used for final suspension of the cells by counting, while the spinning is going on.

Resuspend the cell pellet obtained in the determined amount of R10.

Add 10(suspension into the

A works ed as follows.



RPMI – Roswell Park Memorial Institute (Nutritive liquid medium),

FCS – Fetal calf serum (Nutritive liquid medium)

PHA – Phytohemagglutinin,

ESAT-6 – Early secretory antigen 6,

CFP 10 – Culture filtrate protein 10

Well 1 – negative control

Well 2 – positive control

Well 3 – contains ESAT-6 antigen

Well 4 – contains CFP 10 antigen

Procedure for counting cells

Dilute 10µl of the cell suspension in 90µl of 1XPBS. After mixing thoroughly, take 10µl of the cell suspension and place it on the counting grid of the hemocytometer.

Count the total number of cells in the four outer large squares.

Average number of cells = Total number of cells counted in the 4 squares

Total cell count = Average number of cells $x10^4x10$ (dilution factor) x volume from which the initial 10µl for cell counting is drawn (This may be 5ml in the case of CSF or 10 ml in the case of whole blood).

Adjust the cell concentration such that there are 250,000 cells in every 100µl.

Spot counting

ELISPOT plates were scored by eye, using a hand lens. Thresholds for a positive response were more than 5 (for peptides) or 10 (for phytohemagglutinin (PHA): positive control) spot forming cells (SFCs) and at least twice the frequency of, the negative control wells, as previously described [6, 9].

Background numbers of SFC in negative control wells were always ≤5/well. The person performing and reading the assays was blind to personal identifiers and clinical and microbiological data. The test was carried out in the Nephrology diagnostics lab of Christian Medical Hospital by the technologist posted there.

The clinician (myself) blind to the results of ELISPOT assays prospectively assessed case records of each patient on a proforma.

Sensitivity and confidence intervals were calculated using a statistical package SPSS.

RESULTS

During the study period, a total of 24 patients were recruited, the number of the cases being 14 and 10 controls. The age and sex distribution of the subjects is as given in table 1 and 2 respectively.

Table 1. Age distribution of cases and controls

Age (years)	CASES (n=14)	CONTROLS (n=10)
Mean	39.6 yrs	42.8 yrs
Median	37 yrs	42 yrs
Range	16 – 69 yrs	15 – 73 yrs
SD	39.6 ± 15.4	42.8 ± 20.1

Table 2. Sex distribution of cases and controls

Sex	CASES	CONTROLS
Male	8 (57.1%)	9 (90%)
Female	6 (42.8%)	1 (10%)
Total	14	10

Table 3. Clinical Diagnosis in cases and controls

Diagnosis	Number	Percentage
CASES (n=14)		
Highly probable TBM	8	57.1%
Probable TBM	6	42.8%
CONTROL (n=10)		
Partially treated pyogenic meningitis	5	50%
Aseptic meningitis	3	30%
Carcinomatous meningitis	2	20%

Out of the 14 cases of tuberculous meningitis, 8 were diagnosed as 'highly probable' and 6 as 'probable'. None of them were confirmed cases as none had bacteriologically proven tuberculous meningitis.

The duration of clinical symptoms in all the cases was more than 2 weeks. In the controls, the duration ranged from 2 days to 4 months, the longest duration being for the patient with carcinomatous meningitis.

Table 4. Clinical symptoms in cases and controls

Symptoms	CASES (n=14)	CONTROLS (n=10)
Fever	8 (57.1%)	7 (70%)
Headache	11 (78.5%)	4 (40%)
Vomiting	6 (42.8%)	3 (30%)
Seizures	3 (21.4%)	1 (10%)
Altered sensorium	3 (21.4%)	6 (60%)
Visual disturbance	6 (42.8%)	0

Table 5. Clinical signs in cases and controls

Signs	CASES (n=14)	CONTROLS (n=10)
Neck stiffness	3 (21.4%)	6 (60%)
Papilledema	4 (28.5%)	1 (10%)
Cranial nerve 3,4, 6 palsy	6 (42.8%)	1 (10%)
Other cranial N palsy	2 (14.2%)	0
Hemi/Quadriparesis	0	2 (20%)
Paraparesis	2 (14.2%)	0
Cerebellar signs	1 (7.1%)	0
Extra CNS TB	3 (21.4%)	0

The commonest presenting symptoms in both cases and controls were fever and headache. The controls were more sick at admission, 6 (60%) of them being in altered sensorium as compared to the cases where only 3 (21.4%) of them were in coma. Only 3 of the cases had close contact with tuberculosis either within the household or within the work place.

Table 6. Peripheral blood total white cell count in cases and controls.

Total White cell count per cu mm	CASES (n=14)	CONTROLS (n=10)
>15,000	0	3 (30%)
<15,000	14 (100%)	7 (70%)

Total white cell count is one of the variables used in the diagnostic score for clinical diagnosis of tuberculous meningitis. All cases had total white cell count less than 15 000 as one expected. The 3 controls that had high white cell count were those with pyogenic meningitis.

Table 7. Cerebrospinal fluid variables in cases and controls

CSF variable	CASES (n=14) Median (90% range)	CONTROL (n=10) Median (90% range)
Total WCC (per ml)	115 (2 – 750)	200 (30 – 6400)
% Lymphocytes	97 (65 – 100)	95 (40 – 99)

CSF/ blood glucose ratio	0.4 (0.2 – 0.57)	0.49 (0.1 – 0.89)
CSF protein (mg/ml)	159 (21- 900)	104 (49 – 450)

WCC – white cell count

All cases except one had CSF pleocytosis more than 750 per cu mm and all had lymphocytic predominance. Even among the controls, only 3 (30%) had very high CSF count, as most of the controls were partially treated pyogenic meningitis. CSF glucose to blood glucose was low in most of the cases (78%). CSF protein was high in all cases.

Culture and PCR results

Routine cultures for all cases and controls showed no growth. CSF for AFB smear and culture were negative in all cases.

TB PCR on CSF was positive only in one patient; however, the ELISPOT test in the same patient was negative due to low CSF cell count. In the rest of the cases, TB PCR was negative.

Table 8. Chest X ray in cases and controls

Chest X ray	CASES (n=14)	CONTROLS (n=10)
Normal	10 (71.3%)	8 (80%)
Abnormal	4 (28.5%)	2 (20%)

Only 4 cases (28.5%) had chest X ray evidence of tuberculosis in the form of pleural effusion, perihilar lymphadenopathy and apical opacities. On the other hand, 20% of the controls also had abnormal chest X ray that was suggestive of aspiration pneumonia.

Table 9. Neuroradiology in cases and controls

CT/MRI Brain	CASES (n=14)	CONTROLS (n=10)
Normal	6 (42.8%)	8 (80%)
Abnormal	8 (57.2%)	2 (20%)

Table 10. Abnormal Neuroradiology findings in cases

Abnormality	Number (total =14)	Percentage
Tuberculoma	1	7.1%
Spinal arachnoiditis	1	7.1%
Vertebral disc disease	1	7.1%
Cord myelitis	1	7.1%
Meningeal enhancement	3	21.4%
Cortical venous sinus thrombosis	1	7.1%
Infarcts	1	7.1%
Hydrocephalus	1	7.1%

All patients in the study had either CT or MRI of the brain prior to performing the CSF analysis. The various MRI abnormalities present were meningeal enhancement on post contrast scan (21.4%); tuberculomas (7.1%) and one patient had multiple infarcts (7.1%). Some of the abnormal MRI findings are shown.

Image 1. T1 W post contrast axial MRI brain images showing multiple confluent ring enhancing lesions in trigonal region of left ventricle with minimal subependymal and parenchymal extension with extensive edema.

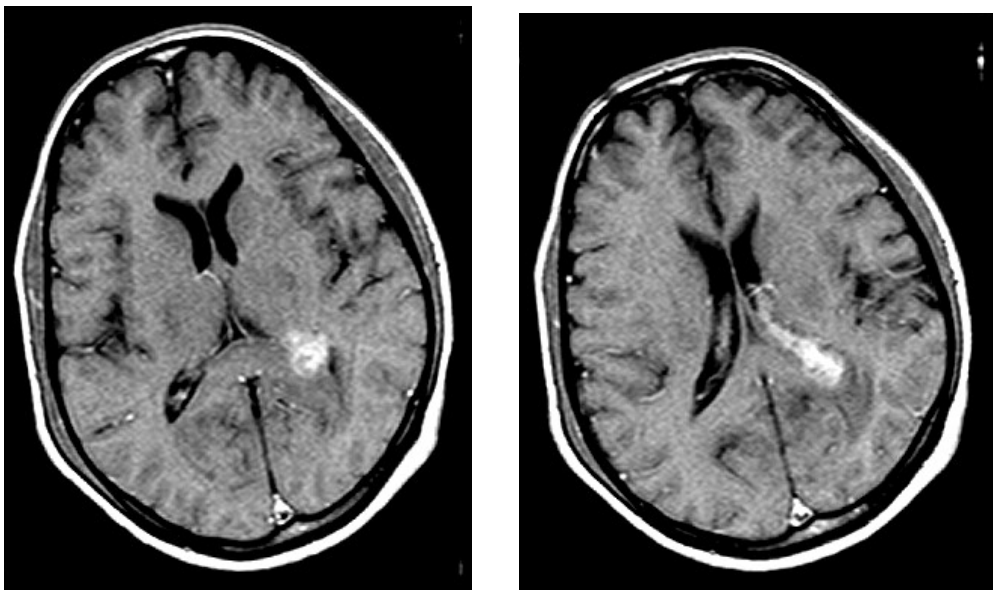


Image 2. T1 W post contrast axial MRI brain images of the above patient on treatment with ATT showing confluent thick walled, ring-enhancing lesions predominantly centered in the right thalamus. Enhancement of the ependyma and the choroid plexus of the left lateral ventricle compared to the last MRI has totally resolved. Appearance of new tuberculomas while on treatment with ATT is well known.

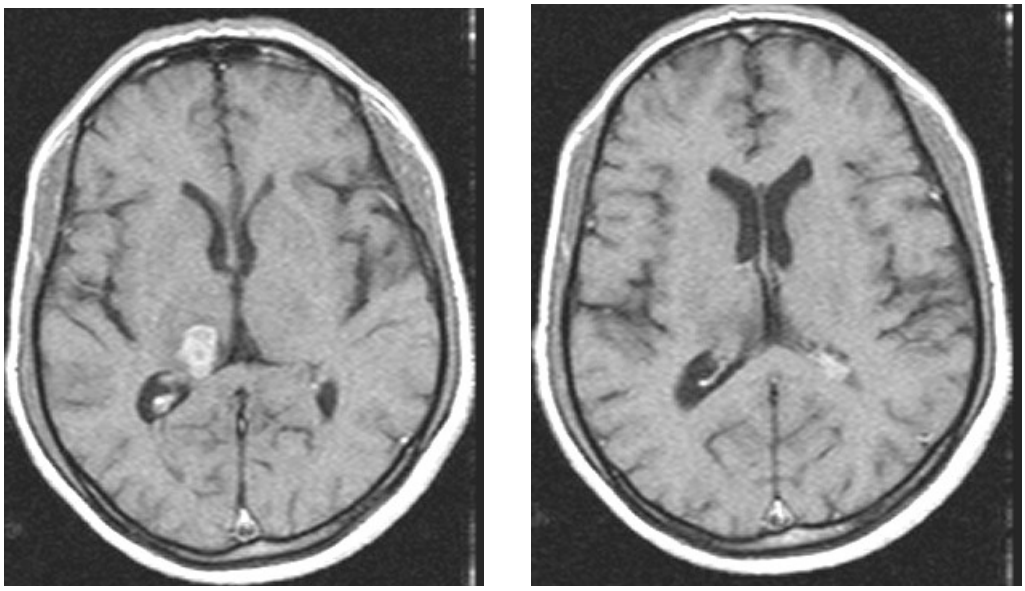


Image 3. T1 W post contrast sagittal images of MRI brain showing diffuse thickening and enhancement of the tentorium, falx cerebelli, dura along the floor of posterior cranial fossa, petrous apex and parasellar regions.

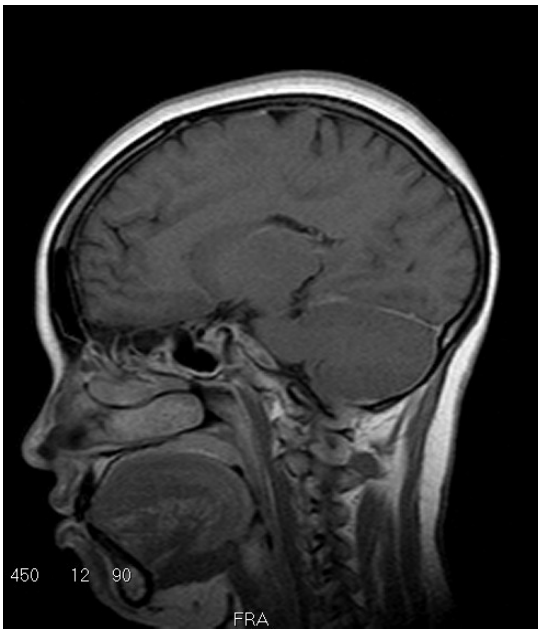


Image 4. T1 W post contrast sagittal images of MRI brain of the above patient post treatment with antituberculous chemotherapy showing complete resolution of the meningeal enhancement.

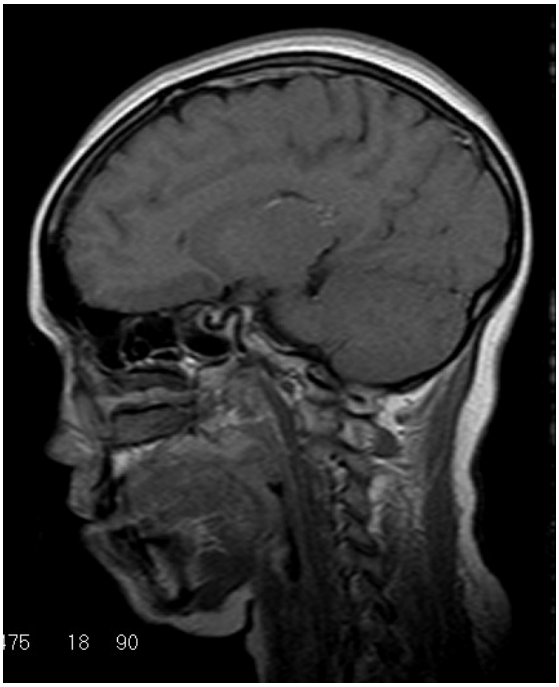
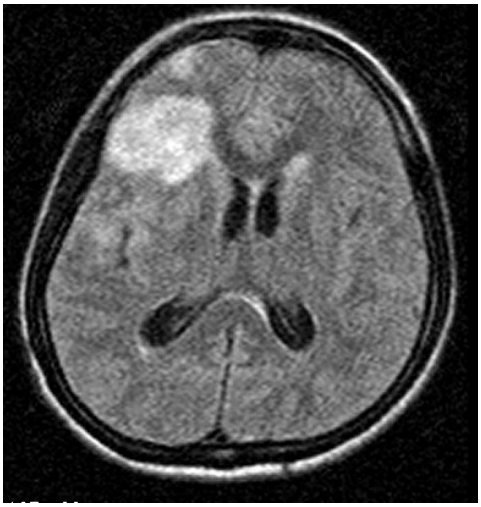


Image 5. T2 W and T1 W post contrast MRI brain axial images showing wedge shaped area of altered signal intensity (hypo in T1 and hyper in T2) in the right frontal lobe involving the inferior frontal gyrus and adjacent white matter with no post contrast enhancement, mild adjacent leptomeningeal enhancement and mild mass effect suggestive of subacute infarct due to tuberculous arteritis.



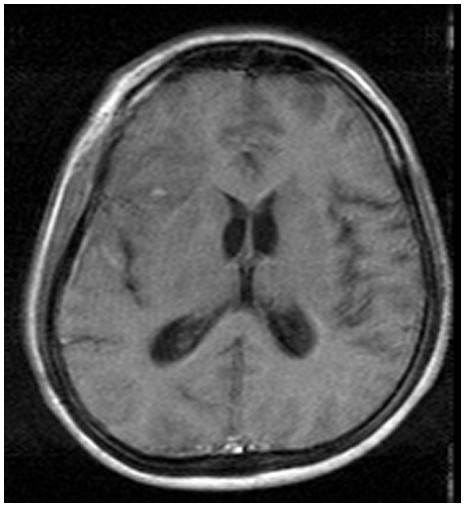


Table 11. ELISPOT ASSAY in cases and controls

Test	CASES (n=14)	CONTROLS (n=10)
Positive in blood only	3	2
Positive in CSF only	1	0
Positive in CSF & Blood	6	0
Negative in CSF & Blood	2	7
Failed assay	2	1

Low CSF counts in 4 cases.

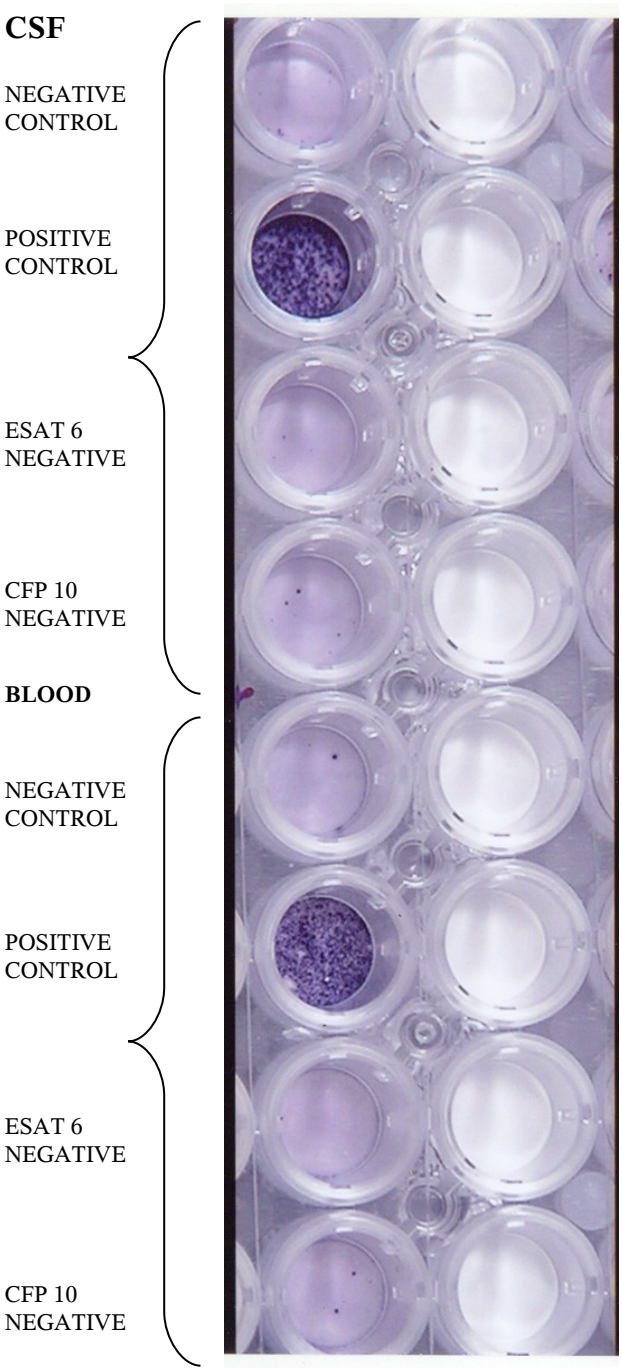
Only 8 cases were ideal for both CSF and blood analysis for the ELISPOT assay. Out of these 8 cases, 6 were positive in both blood and CSF and one only in CSF.

In two of the 14 cases, the assay failed, i.e., the positive control also showed no response, indicating that the lymphocytes isolated were not viable and did not react with phytohemagglutinin. One of them had received steroids prior to the assay and this could probably have caused lymphocytes to degenerate. There were no identifiable reasons for the other case having failed. Three of the 14 cases that were positive in blood but negative in CSF had low CSF cell count (less than 25 cells per cu mm) and hence enough lymphocytes could not be isolated. One of the case that was negative in CSF and blood also had low CSF cell count. Thus 4 cases had negative CSF due to low CSF white cell count (white cell count below 25).

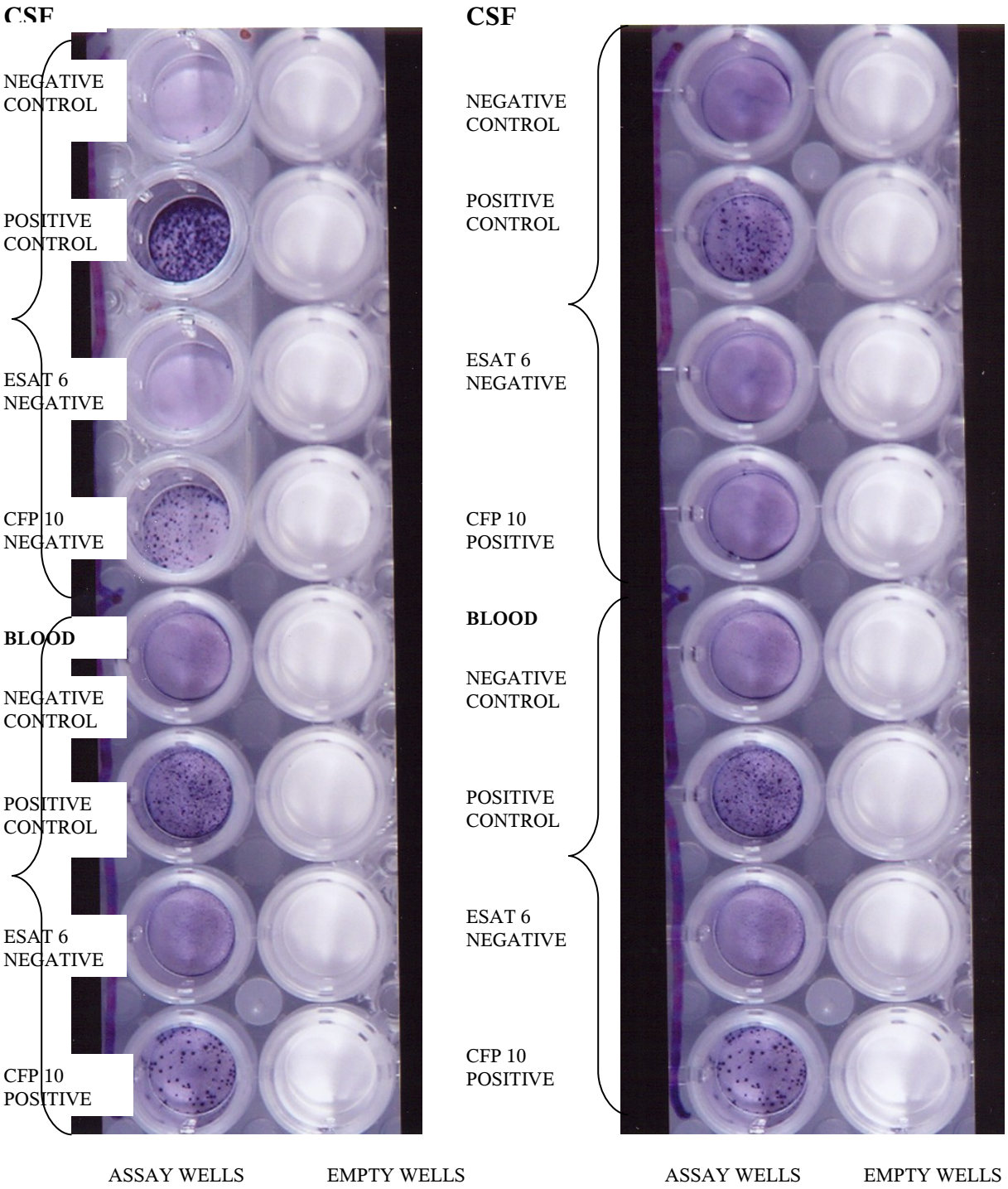
Among the controls, one of the assays was invalid both for blood and CSF. The CSF assay probably failed as there was contamination of the CSF with blood and blood is known to interfere with lymphocyte viability. All controls had enough CSF cell count for adequate lymphocyte separation. CSF was negative in the 9 controls. The test was positive in blood only in 2 controls, which could mean that the person had been exposed to the tuberculous antigen and need not necessarily mean infection. Both these controls were not exposed to BCG vaccine.

The sensitivity of the test calculated was 87.5% and specificity 100% with positive predictive value of 100% and negative predictive value 90%. The significance calculated by the Fischer exact test was 0.00041, this being highly significant.

Image 6. ELISPOT wells showing negative results for CSF and blood
The top 4 wells are for CSF and the lower 4 wells for blood. The 1st well has the negative control, the 2nd positive control and the 3rd and 4th wells has the specific peptides (ESAT-6 and CFP10 respectively). Here the positive control is showing spots and not the other wells, indicating that T cells are viable but not reactive for the TB peptides.



Images 7 & 8. Image ASSAY WELLS T EMPTY WELLS tive for blood and negative for CSF. Image 8 shows the assay being positive for blood and CSF.


CSF
NEGATIVE CONTROL
POSITIVE CONTROL
ESAT 6 NEGATIVE
CFP 10 NEGATIVE
BLOOD
NEGATIVE CONTROL
POSITIVE CONTROL
ESAT 6 NEGATIVE
CFP 10 POSITIVE
ASSAY WELLS
EMPTY WELLS
CSF
NEGATIVE CONTROL
POSITIVE CONTROL
ESAT 6 NEGATIVE
CFP 10 POSITIVE
BLOOD
NEGATIVE CONTROL
POSITIVE CONTROL
ESAT 6 NEGATIVE
CFP 10 POSITIVE
ASSAY WELLS
EMPTY WELLS

DISCUSSION

Tuberculosis is a disease of increasing significance. Tuberculous meningitis is the most serious form of tuberculosis and needs to be attended to as a medical emergency. Delay in diagnosis and treatment are regarded as the major contributing factors in the high mortality. Despite chemotherapy, it is fatal in 25% of adults and causes neurological sequelae in 25% of survivors [1]. Delay in diagnosis and treatment are regarded as the major contributing factors in the high mortality.

The initial diagnosis of tuberculous meningitis is based on the neurological symptoms, signs and CSF analysis. Various diagnostic criteria have evolved [22, 23] because of the lack of a sensitive test for confirmation of tuberculous meningitis. Definitive diagnosis is based on isolation of mycobacterium from the CSF. However this being a paucibacillary form of tuberculosis, demonstration of AFB in CSF in patients with tuberculous meningitis is less than 20%. A recent study reported a bacteriological diagnosis of tuberculous meningitis in 107 (81%) of 132 adults with the disease; acid-fast bacilli were seen in 77 (58%) patients, and cultured from 94 (71%) patients [27]. They recommend that at least 6 ml of CSF should be collected and examined for 30 minutes to improve the yield. A microbiological confirmation could not be obtained in any of our patients.

This pilot study was undertaken to determine the feasibility of a T cell based assay for the rapid diagnosis of tuberculous meningitis.

The test was performed in 14 cases with tuberculous meningitis - diagnosis based on standard diagnostic criteria [60] and 10 patients with non-tuberculous meningitis that served as the control samples. All cases were classified as having either 'highly probable' or 'probable' tuberculous meningitis. All of them had responded to anti tuberculous chemotherapy at follow up except for one patient who had shown response in the initial month of chemotherapy and then was lost to follow up. The controls included patients with partially treated pyogenic meningitis (50%), aseptic meningitis (30%) and carcinomatous meningitis (20%). Three of them died, 2 with the carcinomatous meningitis and one with pyogenic meningitis who was elderly (73yrs) and also had multiorgan failure. The rest of the control subjects improved during the hospital stay itself with standard treatment based on the diagnosis.

The median age of the patients with tuberculous meningitis was 37 years and the mean was 39.6 years. Tuberculous meningitis is classically described as a disease of the young adults, which is in keeping with this study. In the diagnostic criteria for diagnosis of tuberculous meningitis devised by Thwaites et al [23], the cut off age for likelihood of tuberculous meningitis is below 36 years. Majority of our patients were males.

Only 'highly probable' (n = 8; 57.1%) and 'probable cases' (n = 6; 42.8%) of tuberculous meningitis were included in the study. The control subjects clearly had no clinical evidence for tuberculous meningitis.

The commonest clinical symptom for patients with tuberculous meningitis was headache (78.5%). The other symptoms were fever (57.1%), visual disturbances (42.8%) in the form of blurring of vision, diplopia, photophobia and vomiting (42.8%). This is in keeping with studies from other centers [15,16,19] where headache and fever were the most common presenting symptoms (50 to 80%).

The commonest clinical sign was focal neurological deficits (78.3%) in the form of cranial nerve palsies, paraparesis (due to tuberculous myelitis) and cerebellar signs. Only 21.4% of cases had neck stiffness. Other studies show that the presence of neck stiffness ranged from 40 to 80% [15].

Only 3 cases (21.4%) had evidence of extra CNS tuberculosis - pulmonary tuberculosis in 2 and Pott's spine in the other. Extra CNS tuberculosis has been found in about 10 - 22% of non HIV- infected patients with tuberculous meningitis [22] but on the other hand, HIV- infected patients presenting with tuberculous meningitis have 41 – 68 % incidence of extra CNS tuberculosis [61].

The CSF abnormality in patients with tuberculous meningitis showed pleocytosis (median being 115 cells per ml) with lymphocyte predominance (median 97%), low CSF glucose to blood glucose ratio (median 0.4) and high protein (median 159 mg per dl). One patient had normal CSF despite the MRI showing pachymeningitis. CSF pleocytosis with lymphocyte predominance and low CSF glucose to blood glucose ratio are two important variables in the clinical diagnostic criteria for tuberculous meningitis [22,23]. CSF analysis thus forms an important aspect in the diagnosis of tuberculous meningitis. However, in certain forms of CNS tuberculosis as in tuberculomas, CSF may be normal. It is in these situations that diagnosis becomes difficult unless one opts for tissue diagnosis, which is very invasive.

We were unable to obtain microbiological confirmation of TB in any of our patients. This was not surprising, as this is a paucibacillary form of TB in which microbiological confirmation is frequently not obtained, especially in the setting of a routine service laboratory. A bacteriological gold standard is too insensitive to be used alone for evaluation of new diagnostic tests, which may be more sensitive than culture and Ziehl-Neelsen [23]. Therefore, like other investigators[22,23,59] we used composite

reference standards for diagnosis that use culture, Ziehl-Neelsen, clinical, radiological and therapeutic outcome criteria.

All cases had either CT or MRI of the brain with contrast. It was abnormal in 57.2% (n=6) of the cases while in the controls it was abnormal in 20% (n=2). Other studies report abnormality on neuroimaging in 68 – 80 %. Since the findings are non-specific, radiology alone cannot be relied on for the diagnosis of tuberculous meningitis. However it forms a reliable supporting evidence for diagnosing the disease. The abnormal imagings in the controls were for the patient with post trauma meningitis who had fracture of the left maxillary sinus and frontal bone. The other abnormal imaging was for the patient with carcinomatous meningitis with multiple cranial nerve palsies.

T cell based assays have been used for diagnosis of latent tuberculous infection [54,55,60] and also in active tuberculous infection [56]. Recently it has been shown that ESAT-6-specific IFN-γ-secreting $CD4^+$ T cells are concentrated at sites of active tuberculosis [56]. Using ELISPOT these cells can be detected in pleural fluid [56], suggesting that this assay may be a rapid, sensitive and specific marker for active TB at specific anatomical sites.

This pilot study done showed the presence of interferon γ-secreting *Mycobacterium tuberculous* antigen-specific T cells in the cerebrospinal fluid of patients with tuberculous meningitis using the enzyme linked ELISPOT assay. Further, these cells were absent in the CSF of patients with non-tuberculous meningitis. The ELISPOT assay for diagnosis of tuberculous meningitis, as per this study, showed a high diagnostic sensitivity of 87.5%, specificity of 100% with positive predictive value of 100% and negative predictive value 90%. The high sensitivity and speed of CSF ELISPOT suggest it could be a useful tool for diagnosis of patients with suspected tuberculous meningitis. However in cases with suboptimal cell numbers (<250,000/well), assay sensitivity is adversely affected. Notably, cell numbers were suboptimal in 4 of the 5 negative CSF assays, suggesting low cell numbers are

associated with failure of the assay. None of the control samples were positive in CSF though 2 of them were positive in blood.

There was general concordance of assay results between peripheral blood mononuclear cells (PBMC) and CSF, with the exception of one case in which CSF alone was positive. Frequencies of responding cells were equivalent in PBMC and CSF ($p=0.865$ Wilcoxon signed rank for difference), in contrast to the increased concentration of MTB-antigen-specific T cells at the site of disease in pleural TB[9].

The difficulties encountered with the assay were:

1) Necessity for large volume of CSF (at least 5 ml required), which may be difficult to obtain in the setting of raised intracranial tension when sample is also required for other CSF tests.
2) Since this is a test on live cells, the sample has to be preserved carefully and processed as fast as possible within 6 hours of the CSF collection. This may be difficult when CSF collection is carried out in the late hours of the day and night when the labs may not be accessible.
3) Isolation of the lymphocytes requires expertise and many labs may not have this facility. It requires a minimum period of 6 hours of the technologist to carry out one assay.
4) Low CSF cell count as in the case of tuberculomas and contamination of CSF with blood as in the case of a traumatic tap renders the test insensitive.

This study has demonstrated that ELISPOT is a potential new diagnostic test for tuberculous meningitis, which is rapid, practicable in a resource-poor setting, and appears to have high sensitivity. Large prospective studies are now warranted to validate the clinical utility of CSF ELISPOT in routine practice. Such studies should have better microbiological confirmation, should include children and more controls to obtain robust estimates of the diagnostic specificity as well as sensitivity.

CONCLUSIONS

- This study used a T cell based assay (Enzyme linked immunospot assay – ELISPOT) for diagnosis of tuberculous meningitis. The assay has demonstrated presence of ESAT-6 and CFP 10 specific IFN-γ-secreting $CD4^+$ T cells in the cerebrospinal fluid of patients with tuberculous meningitis.

- The assay was used for diagnosis of tuberculous meningitis. It is a rapid test with the result being available in 24hours. The test has a high diagnostic sensitivity of 87.5% and specificity of 100% with positive predictive value of 100% and negative predictive value 90%.

- The limitations of the assay include necessity for large volume of CSF for the test, careful and quick processing of the CSF sample, lymphocyte isolation from blood and CSF requires expertise, and presence of blood in CSF and low CSF cell count renders the test insensitive.

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ELISPOT FOR DIAGNOSIS OF TUBERCULOUS MENINGITIS

STUDY NO.: HOSPITAL NUMBER:

CASE /CONTROL:

NAME:

AGE: SEX:

ADDRESS:

OCCUPATION:

CLINICAL FEATURES

	Yes/No	**Duration**
Fever		
Headache		
Vomiting		
Seizures		
Altered sensorium		
Meningeal signs		
Focal deficits		
Other complications		
Extra CNS Tuberculosis		
Any other illness		
HIV		
Hypertension		
Diabetes		
Contact with tuberculosis		
BCG Vaccine		

LAB INVESTIGATIONS

WBC count
TC
DC

ESR

CSF TC
- DC
- SUGAR
- PROTEIN

CSF ROUTINE SMEAR & CULTURE
- TB SMEAR & CULTURE
- TB PCR
- FUNGAL CULTURE

MANTOUX

CHEST XRAY

NEUROIMAGING

ELISPOT
- BLOOD
- CSF

FOLLOW UP

ABSTRACT

Title of the abstract: A T cell based assay using specific *Mycobacterium tuberculosis* antigens, ESAT-6 and CFP 10, for the diagnosis of tuberculous meningitis.

Department: Neurology

Institution: Christian Medical College, Vellore, Tamilnadu

Name of Candidate: Maya Mary Mathew

Degree and subject: DM Neurology

Name of the guide: Dr. Chandran Gnanamuthu

Objectives: To evaluate usefulness of the enzyme linked immunospot assay (ELISPOT), a T cell based assay using specific *Mycobacterium tuberculosis* antigens, ESAT-6 and CFP 10, for the diagnosis of tuberculous meningitis.

Methods: This was a prospective study carried out in the department of Neurology, Christian Medical College & Hospital, Vellore, India, from March 2004 to May 2006. Cases were adult patients with clinical features highly suggestive of tuberculous meningitis; controls were age-matched patients with meningitis of non-tuberculous etiology. ELISPOT assay was performed on CSF and heparinised peripheral venous blood. The results were analysed for specificity and sensitivity of the test.

Results: A total of 14 cases and 10 controls were recruited. Out of the 14 cases of tuberculous meningitis, 8 were diagnosed as 'highly probable' and 6 as 'probable'. CSF for AFB smear and culture were negative in all cases. TB PCR on CSF was positive only in one patient. ELISPOT assay in 2 of the cases and one control failed. In the rest 12 cases, it was positive in CSF and blood in 6 and only in CSF in 1. Three cases that were positive in blood and negative in CSF had low cell count. Only 8 cases were ideal for both CSF and blood analysis for the ELISPOT assay and of this 7 were positive. CSF was negative in the 9 controls. The sensitivity of the test was 87.5% and specificity 100% with positive predictive value of 100% and negative predictive value 90%.

Conclusions: The enzyme-linked immunospot assay for detection of *Mycobacterium tuberculosis*-antigen-specific interferon-γ-secreting T cells in cerebrospinal fluid from patients with tuberculous meningitis had a diagnostic sensitivity of 87.5% and specificity 100%. This pilot study demonstrates that this new T cell-based assay is a promising diagnostic test for tuberculous meningitis that is rapid and sensitive.