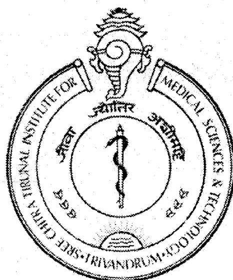
ISOLATION AND CHARACTERIZATION OF LIPID ANTIGENS OF *MYCOBACTERIUM TUBERCULOSIS* AND THEIR APPLICATIONS FOR THE IMMUNODIAGNOSIS OF HUMAN TUBERCULOSIS

ANIE Y

Ph.D THESIS –2008



SREE CHITRA TIRUNAL INSTITUTE FOR
MEDICAL SCIENCES & TECHNOLOGY
THIRUVANANTHAPURAM – 695 012

ISOLATION AND CHARACTERIZATION OF LIPID ANTIGENS OF *MYCOBACTERIUM TUBERCULOSIS* AND THEIR APPLICATIONS FOR THE IMMUNODIAGNOSIS OF HUMAN TUBERCULOSIS

A thesis presented
by

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in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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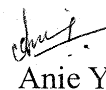
December 2008

DECLARATION

I, **Anie Y**, hereby declare that I had personally carried out the work depicted in the thesis entitled **"Isolation and characterization of lipid antigens of mycobacterium tuberculosis and their applications for the immunodiagnosis of human tuberculosis"** under the direct supervision of Dr. V. V. Radhakrishnan, Professor and Head, Department of Pathology, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala, India, except where external help sought and acknowledged.



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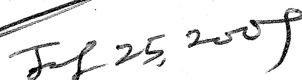
CERTIFICATE

This is to certify that Mrs. Anie Y, in the Department of Pathology of this institute, has fulfilled the requirements of the regulations relating to the nature and prescribed period of research for the Ph.D degree of the Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram. The work relating to her thesis entitled **"Isolation and characterization of lipid antigens of mycobacterium tuberculosis and their applications for the immunodiagnosis of human tuberculosis"** was carried out under my direct supervision.

Dr. V. V. Radhakrishnan



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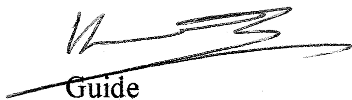

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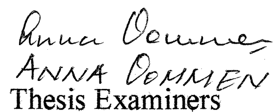

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To God Almighty

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ABBREVIATIONS

ACD	Acid citrate dextrose
ADA	Adenosine deaminase
AFB	Acid fast bacillus
AIDS	Aquired Immunodefeciency Syndrome
ATT	Anti-tuberculosis Chemotherapy
BCG	Bacille-Calmette Guerin
BSA	Bovine Serum Albumin
CF	Cord Factor
CSF	Cerebrospinal fluid
DEA buffer	Diethanolamine buffer
DPX mountant	Dystrene Phthalate in Xylene mountant
ELISA	Enzyme Linked Immunosorbent Assay
HBSS	Hank's balanced salt solution
IFN γ	Interferon gamma
IgG	Immunoglobulin G
LAM	Lipoarabinomannan
MDRTB	Multidrug resistant tuberculosis
OD	Optical density
PBS	Phosphate buffered saline
PMN	Polymorpho-nucleocytes
RBC	Red blood cells
SL-1	Sulfolipid I
TB	Tuberculosis
TBGL	Tuberculosis associated glycolipid
TBGL	Tuberculosis associated glycolipid
ZN staining	Ziehl-Neelsen staining

SYNOPSIS

Introduction:

The global incidence of human tuberculosis in the world has shown increase (1 % a year) despite the control measures viz., effective anti-tuberculosis chemotherapy (ATT) and BCG (Bacille-Calmette Guerin) vaccination. Currently, 1/3rd of the world population is exposed to *Mycobacterium tuberculosis* bacterium, the organism responsible for the disease. India accounts for 1/5th of the global tuberculosis incident cases. According to the latest report of WHO, each year, over 1.9 million people in India develop tuberculosis of which around 0.87 million are infectious cases (WHO, 2007). The reason for our inability to control tuberculosis lies mainly in the emergence of multi-drug resistance tuberculosis, HIV infection and delay in diagnosis. The lack of simple, reliable and cost-effective diagnostic modalities is the main reason for the delay in diagnosis in tuberculosis. Demonstration of *M. tuberculosis* by bacteriological methods is regarded as "gold-standard" in confirming the disease. However, owing to the paucibacillary status of the clinical samples, most often the bacteriological methods fail to establish tuberculous aetiology in these clinical samples. Hence, alternate diagnosis modalities suited for the laboratories of developing countries is quintessential.

Several new techniques have been developed for the diagnosis of tuberculosis and among them; immunodiagnosis generated great interest as it is a rapid, reproducible, user-friendly and non-invasive method. To determine the tuberculosis aetiology in clinical specimens, several mycobacterial antigens have been used but they showed great variability in specificity as well as sensitivity and the search for an antigen with more than 80 % sensitivity and 95 % specificity as recommended by the WHO has not been attained. As the *M. tuberculosis* is known for its very high content of lipids, which is a

unique character among all microorganisms, now, lot of interest have generated to obtain specific mycobacterial lipid antigens. Though extensive immunochemical investigations on the cell wall of *M. tuberculosis* provided a clear picture of the myriad of antigens present on it, the role of these antigens in providing protective immunity or in disease progression is also inconclusive. Their utility in the diagnosis of human tuberculosis is also not thoroughly understood particularly in our population.

In this study, attempts have been made to isolate four major mycobacterial lipid antigens and to study the role of these antigens in the diagnosis of tuberculosis. Efforts were also undertaken to explore the ability of two of these lipid antigens (Cord factor and sulfolipid-I) to attract polymorphonuclear cells, the first type of defence cells recruited towards the infection area during mycobacterial infection.

Objectives of the study:

1. To isolate and characterize four major lipid antigens of *M tuberculosis* viz; (a) Mycolic acid containing glycolipids (tuberculosis associated glycolipid, TBGL), (b) cord factor (trehalose 6, 6' dimycolate), (c) sulfolipid-I (2,3,6,6' tetraacyl α, ά D trehalose 2' sulfate) and (d) lipoarabinomannan (LAM) from *M tuberculosis* bacillus.

2. To evaluate the specific role of these mycobacterial antigens in the immunodiagnosis of tuberculosis by Enzyme-linked immunosorbent assay (ELISA) in patients with pulmonary and pleural tuberculosis.

3. To assess the role of cord factor and sulfolipid-I in the chemotaxis of polymorphonuclear leukocytes (PMNs) during tuberculosis infection.

Methodology:

1. Isolation of antigens:

All four antigens were extracted and isolated from either virulent $H_{37}Rv$ strain or avirulent $H_{37}Ra$ strain of *M. tuberculosis*. These are described in brief.

TBGL: Total mycolic acid containing glycolipid (TBGL) antigen was isolated by extraction with chloroform: methanol (2:1) followed by the treatment with cold acetone.

Cord factor: The cord factor antigen was extracted from *M. tuberculosis* cells at 55°C with chloroform and methanol (2:1). The antigen was then purified using Silica gel flash column chromatography or silica gel thin layer chromatography.

Sulpholipid I: Initially, the envelope lipids were extracted with 0.1 % decylamine in hexane followed by washing with 2N citric acid. From this, the sulfolipid fraction soluble in hot as well as cold ethanol was separated and finally, SL-I was purified by thin layer chromatography and re-chromatographed to assess the purity.

Lipoarabinomannan: LAM antigen was isolated from $H_{37}Rv$ strain of *M. tuberculosis* by phenol: water biphasic partition method after primary extraction with chloroform: methanol: water (10:10:3). The aqueous layer was collected and the phenol was removed by dialysis against distilled water using a membrane with 2500 molecular cut-off. The LAM obtained was analyzed by SDS-PAGE.

2. Diagnostic methods

ELISA for the detection of antibodies in the serum samples of pulmonary tuberculosis patients: Antibody titer against all the isolated antigens was analyzed in the sera samples of pulmonary tuberculosis by a standard indirect ELISA. Among the 102 patients with pulmonary tuberculosis included in the study, 40 patients showed the presence of acid-fast bacillus (AFB) in the sputum samples and hence they were

considered 'confirmed' cases of pulmonary tuberculosis. In the remaining 62 patients, AFB was not detectable by bacteriological methods in the sputum samples, but based on the clinical diagnosis and their positive response to ATT given, they were categorized into 'probable' cases of tuberculosis. 41 patients with respiratory ailments other than pulmonary tuberculosis like lung carcinoma and non-tuberculosis pneumonia were taken as 'disease' control in the study.

ELISA for the detection of antibodies in the pleural fluid of pleural tuberculosis patients: Antibody titer was also analyzed in the pleural fluids of the pleural tuberculosis patients. In this study, 140 patients with clinical and radiological evidences of pleural effusion were selected over a period of two years. Relevant clinical and radiological features were recorded and based on this and their response to ATT; they were categorized into two main groups. Inflammatory cells were observed in 69 patients and among this, 11 patients showed the presence of AFB in the pleural fluids. So they were included in the "confirmed tuberculosis" group. As bacteriological methods did not show the presence of AFB in the pleural fluids in rest of the 58 patients, based on clinical data and response to ATT, they were categorized into "probable tuberculosis" group. 71 patients showed the presence of malignant cells in the pleural effusion and hence they were categorized as patients with malignant pleural effusion.

Comparison of antibody titer in the serum samples and pleural fluid samples: For studying the significance of analyzing the results of antibody titer in pleural effusion for the diagnosis of pleural tuberculosis, pleural fluids and serum samples were collected from 14 patients with tuberculous pleural effusion and 7 patients with malignant pleural effusion and the antibody titer in both pleural fluid and sera samples to anti-TBGL antigen was analyzed by the standardized indirect ELISA.

ELISA for the detection of TBGL antigen in the pleural fluid samples: TBGL antigen was estimated by a modified indirect ELISA. First, antisera to TBGL antigen were raised in two adult female rabbits. The blood was collected from the rabbits and the end-point titer was assessed in the serum. Then, ELISA was standardized with decreasing concentrations of TBGL antigen and rabbit anti-TBGL antibody. A standard graph was prepared with antigen concentration at the 'x' axis and absorbance at the 'y' axis. Pleural fluids were also similarly assayed and concentration of antigen was directly measured from the standard graph.

3. *In vitro* Chemotaxis studies:

Chemotactic property of cord factor and Sulfolipid - I: The ability of the two lipid antigens – cord factor and Sulfolipid – I - to attract polymorphonuclear leukocytes to the infection site was studied. Chemotaxis assay was performed with varying concentrations of cord factor and sulfolipid -I from 5 µg/ml to 50 µg/ml. Well-known chemoattractant casein was taken as a positive control and for obtaining a negative control, chemotaxis was performed in the absence of any chemoattractants.

Inhibition of chemotaxis in the presence of body fluids from tuberculosis patients: In order to find out whether there is any inhibiting factor for chemotaxis of PMNs in the body fluids of tuberculosis patients, chemotaxis assay towards cordfactor/sulfolipid – I/Casein was performed in the presence of sera from tuberculosis patients or diseased controls and pleural fluids from pleural tuberculosis patients and malignant pleural effusion patients.

Results:

1. ELISA for the detection of antibodies in the serum samples of pulmonary tuberculosis patients: The antibody response to different lipid antigens substances in the cell wall varied considerably among tuberculosis patients. The results of the antibody response against the four antigens are shown in the table: 1. From the data, it is concluded that the most suitable antigen in indirect ELISA was TBGL as it is highly sensitive and is sufficiently good in terms of specificity as well. So this test can be used for screening patients for tuberculosis in a large population. The indirect ELISA for the detection of anti-cord factor antibody was 100 % specific and hence may be used for obtaining a confirmed diagnosis. When the results of both these test were combined, the sensitivity was increased to 86.27%.

Table: 1 Result of the indirect ELISAs for the detection of IgG antibody titer to mycobacterial lipid antigens for the diagnosis of pulmonary tuberculosis

Test used	**Efficiency of the test**			
	Sensitivity (%)	**Specificity (%)**	**PPV (%)**	**NPV (%)**
TBGL/IgG	83.33	93.44	95.55	77
CF/IgG	76.47	100	100	71.76
SL-I/IgG	74.51	96.72	93	68.3
LAM/IgG	81.37	88.52	92.2	73.97

2. ELISA for the detection of antibodies in the pleural fluid of pleural tuberculosis patients and antigen detection assay for the diagnosis of pleural tuberculosis: The results of the analysis of antibody titer to these antigens in pleural tuberculosis are given

in table: 2. The result of the antigen detection assay is also included. The results suggest that the best test which can be used routinely is TBGL antigen detection assay. It is 100 % specific and has a considerable sensitivity than the other assays. The cord factor antigen detection assay was also tried but positive rate of the test was very less in clinical samples and hence other antigen detection assays were not included in the study.

Table: 2 Result of the indirect ELISAs for the detection of IgG antibody titer to mycobacterial lipid antigens and the TBGL antigen detection assay for the diagnosis of pleural tuberculosis

Test used	**Efficiency of the test**			
	Sensitivity (%)	**Specificity (%)**	**PPV (%)**	**NPV (%)**
TBGL/IgG	65.21	87.3	83.33	72.1
CF/IgG	59.42	88.73	83.67	69.23
SL-I/IgG	52.17	92.96	88.09	67.34
LAM/IgG	66.67	81.69	77.97	71.6
TBGL antigen detection assay	85.5	100	100	87.65

Comparison of antibody titer in the serum samples and pleural fluid samples: Of the 14 patients with pleural tuberculosis, 6 showed higher antibody titer in pleural fluids than in sera. In 4 patients, the antibody titer was more or less similar in both pleural fluids and sera. In the rest 4 patients, the IgG antibody against TBGL antigen was higher in sera than pleural fluids. In all the 7 patients with malignant pleural effusion, the IgG antibody against TBGL antigen was less in both pleural fluids and sera samples but comparatively, lower titers were seen in pleural fluids. Analyzing these results, it is concluded that

antibody titer analysis in pleural fluids may give additional information in some cases in terms of diagnosis.

3. In vitro Chemotaxis assay:

Chemotactic property of cord factor and Sulfolipid - I: Both cord factor and sulfolipid-I were found to attract PMNs towards them. The chemotaxis of PMNs was found to be greatly influenced by the concentration of cord factor, while the chemotaxis of PMNs was found to be unaffected by the concentration of SL-I in the medium. Migration of cells towards cord factor could be seen even at a very low concentration of 5 µg cord factor/ ml, but the maximum migration was observed at 20µg cord factor /ml concentration. In the case of sulfolipid-I, migrating PMNs was observed at all concentration even after 50µg/ml. But a small decrease in the number of migrating PMNs at 20 µg/ml and a small increase in the number of migrating PMNs at 30 µg/ml were observed.

Inhibition of chemotaxis in tuberculosis patients: The number of migrating cells in the presence of sera from tuberculosis patients was significantly less than the number of migrating PMNs in the presence of sera from non-tuberculosis patients and healthy donors. Just like the inhibition of chemotaxis observed towards the two lipid antigens-cord factor and SL-I; the chemotaxis of healthy PMNs was also inhibited towards the well-known chemo-attractant casein in the presence of tuberculous sera samples. Therefore, the inhibition of migration of PMNs is concluded not solely due to the antibodies raised against these lipid antigens in the patients. And as similar but more intense inhibition was observed in the presence of pleural fluids from tuberculosis patients, certain soluble factor(s); which is excreted either by the host or by the pathogen may be responsible for the inhibition.

Major findings:

1. The lipid antigens were found to be useful in the diagnosis of tuberculosis and it is suited for developing countries as it is

   i. showing sufficient sensitivity and specificity
   ii. reproducible
   iii. cost effective
   iv. needs les expertisation.

2. In pulmonary tuberculosis, TBGL/IgG ELISA can be used for the screening of tuberculosis patients from a large population but a definite diagnosis may be provided by TDM/IgG ELISA

3. In the case of pleural tuberculosis patients, the best test for a definite diagnosis is TBGL antigen detection assay.

4. Detecting antibodies in pleural fluid patients is definitely beneficial in those patients in whom the results of antibody detection in serum is not conclusive.

5. Cord factor and sulfatide exhibit the property of attracting polymorphonuclear leukocytes to the infection site during tuberculosis infection. The response of PMNs towards cord factor was dose-dependent. In tuberculosis patients, the chemotaxis of PMNs is inhibited and this could be due to the secretion of certain soluble factors either by the host or by the pathogen.

CHAPTER.1

INTRODUCTION

1.1 TUBERCULOSIS – THE GLOBAL EMERGENCY

Tuberculosis, the most pervasive, morbid and lethal infectious disease, is responsible for the death of approximately 5000 people per day. According to the latest report of World Health Organization (WHO), currently one-third (2 billion) of the world population is infected with *Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB). The highest rate of incidence per capita is found in Africa, but half of all new cases are in six Asian countries including India, Pakistan, China, Indonesia, Bangladesh and Philippines. In India, 325000 people die due to tuberculosis annually. It is also reported that, each year, over 1.9 million people in India develop tuberculosis of which around 0.87 million are infectious cases (WHO, 2007). Though control measures have been taken through Directly Observed Treatment, Short-course (DOTS) and vaccination, the incidence is still growing 1% a year. The re-emergence of tuberculosis can be attributed to increased incidence of Acquired Immuno Deficiency Syndrome (AIDS) and multi-drug resistance (MDR) tuberculosis. WHO estimated that 0.5 million people are infected with drug resistant strains in 2006 (WHO, 2008). Hence, the disease has been declared a global emergency by WHO in 1993, the only disease ever so designated.

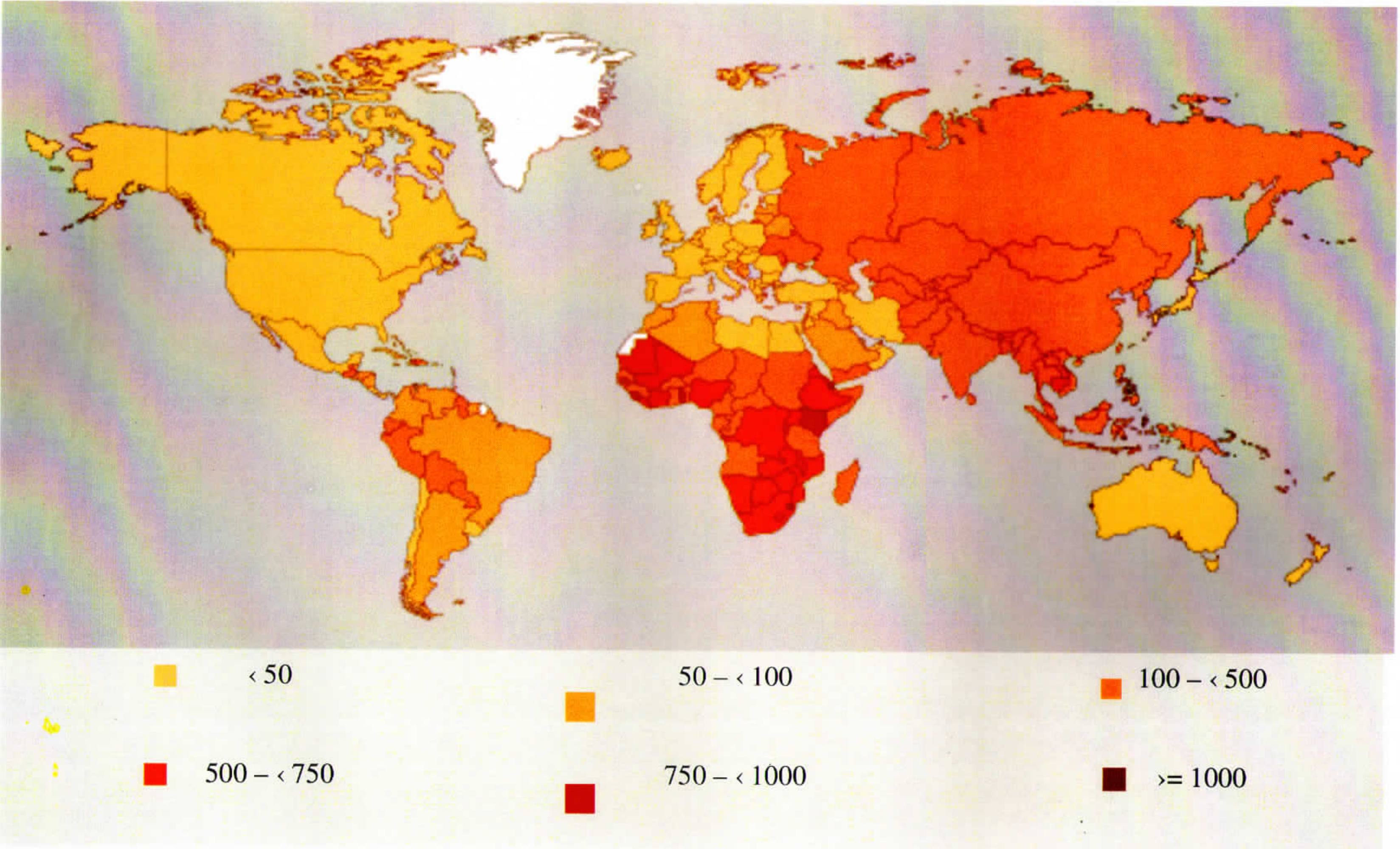


Fig 1.1 Map showing the prevalence of all forms of tuberculosis per 100,000 inhabitants (2005)

http://www.fondation-merieux.org/-Tuberculosis-Map

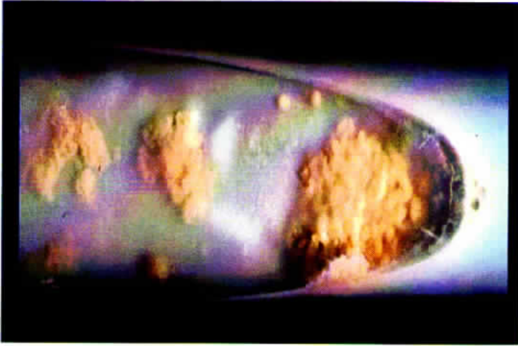
1.1.1 Mycobacterium tuberculosis:

M. tuberculosis is a slow-growing (doubling time is 12-24 hours), non-motile, aerobic bacillus (2-4μm in length), transmitted by the respiratory route. Etymologically, 'Mycobacterium' is derived from the Greek word for fungus (mycus) and small rod (bacteria). The fungus component of the name is derived from the tendency of these micro-organisms to spread diffusely over the surface of the liquid medium in a mould like growth pattern.

Taxonomy of *Mycobacterium tuberculosis (TIGR)*:

Kingdom:	Bacteria
Intermediate Rank 1:	*Actinobacteria*
Intermediate Rank 2:	*Actinobacteridae*
Intermediate Rank 3:	*Actinomycetales*
Intermediate Rank 4:	*Corynebacterineae*
Intermediate Rank 5:	*Mycobacteriaceae*
Genus:	*Mycobacterium*
Species:	*Tuberculosis*

M. tuberculosis is typically a curved or straight rod shaped microbe. Its typical size *in vitro* is 1 to 4 μm in length and 0.3 to 0.6 μm in diameter (Fig: 1.3 a & b). However, *M. tuberculosis* bacilli in the tissue of the host may assume differential morphological characteristics. The bacterium is classified as 'acid and alcohol fast' due to their impermeability to certain dyes and stains. Once stained, acid-fast bacilli will retain dyes when heated and treated with acidified organic compounds (Fig: 1.2 b). *M. tuberculosis* is resistant to drying and chemical disinfectants but sensitive to heat and UV radiation.



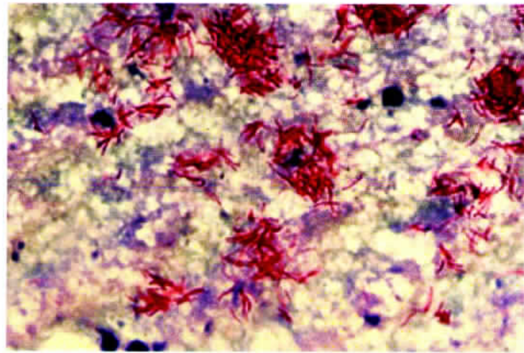
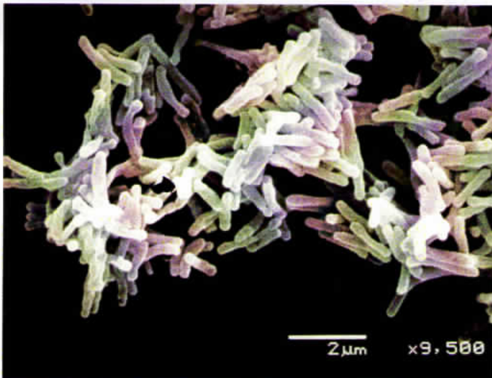


Fig 1.2 a. *Mycobacterium tuberculosis* cultured in Lowenstein- Jensen medium.

Fig 1.2 b Photomicrograph showing acid-fast stained sections with pink-coloured and rod-shaped *M. tuberculosis*



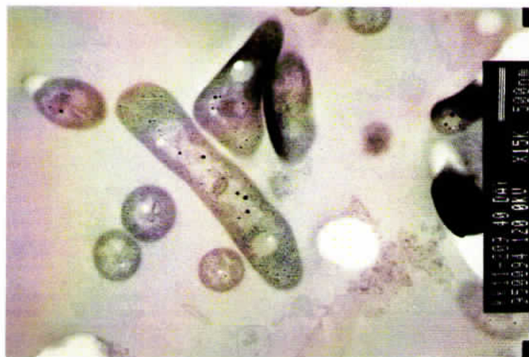


Fig 1.3 a. Scanning electron micrograph showing numerous rod-shaped *M. tuberculosis*

Fig 1.3 b. Transmission electron micrograph of *M tuberculosis*

1.1.2 History of tuberculosis:

It is believed that *M. tuberculosis* originated in soil like other actinomycetes. They evolved to live in mammals and infected human beings through domestication of infected cattle between 10,000 and 25,000 years ago (Anand, 2005). Tubercle bacillus can be traced back as far as 2400 BC, as archeologists found evidence in human bones of Egyptian mummies which showed pathological changes due to tuberculous decay (Zink *et al*, 2001; Zink et al, 2003). Evidence of TB appears in Biblical scripture, in Chinese literature dating back to around 4000 BC and in religious writings in India around 2000 BC (Iseman, 2000).

During 460 BC, Hippocrates identified tuberculosis {known as 'Pthisis' (gk: consumption)} and advised doctors not to attend those patients because all were succumbing to the disease. By the year of 1679, Sylvius described 'tubercles' as characteristic lung changes in consumptive patients. But it was Benjamin Marten, an English physician who first predicted that TB is caused by "wonderfully minute living creatures'. He reported this information in his publication, *A New Theory of Consumption,* in 1720. It was in 1854 that Herman Brehmer proved TB is a curable disease and that considerable healthy climate can cure the patients. He built the first TB sanatorium in the same year.

A major breakthrough in the tuberculosis history was marked by Robert Koch in 1882 when he identified tubercle bacillus with the help of a special staining method developed in his laboratory. He isolated and injected these microorganisms and he could develop tuberculosis in animal models and showed

that it is transmittable. A further significant advance came in 1895 when Wilhelm Konrad von Rontgen discovered the radiation that now bears his name. Thus progress and severity of the disease was able to be accurately reviewed.

The battle against tuberculosis showed significant development during 1900's. In 1921, BCG vaccine was first used in human. The basic work behind the devolopment of vaccine was done by the French bacteriologist Calmette and Guerin. In 1944, Dr. Schatz, Dr. Bugie and Dr. Waksman announced the discovery of an antibiotic called 'Streptomycin' for treating tuberculosis. On November 20, 1944, this antibiotic was administered for the first time to a critically ill tuberculosis patient. After the invention of the streptomycin and other antibiotics that can be used in combination treatment as well as BCG vaccination, though infection rate showed a downward trend; due to the emergence of drug resistant tuberculosis and HIV infection, tuberculosis again became an uncontrollable menace during mid-1980s. Hence, in 1993, WHO declared tuberculosis as a 'global emergency' estimating that one third of the world's population is latently infected with tuberculosis and 7-8 million cases of active tuberculosis occur each year.

As increasing incidence were reported from Africa, in 2004, Nelson Mandela called out for quick diagnosis and treatment for tuberculosis, in order to combat dual infection of AIDS and tuberculosis. The struggle against tuberculosis is still going on as increased number of MDR-TB and AIDS-TB dual infection are being reporting every year.

1.1.3 Pathogenesis and Pathology:

Usually, TB infection is initiated when a person happens to inhale the aerosol droplet containing 1-3 viable bacilli coughed out by an individual suffering from open cases of tuberculous lung disease. On entering the alveolus, the bacilli are phagocytosed by resident alveolar macrophages but the elimination of the tubercle bacillus depends upon the balance between host defense mechanism and the virulence of the pathogen. In most cases, bacillus multiplies within the macrophages and will be carried to the hilar and tracheobronchial lymph nodes where they will establish secondary tubercles. In immunocompetent adults (90%), the disease gets confined in the draining lymph node resulting in latent infection. In them, the antigen presenting cells present mycobacterial antigens to the T-cells which produce pro-inflammatory cytokines that activates macrophages which will be recruited to the sites of infection. The bacillary load is taken up by the activated macrophages which will lead to the formation of granuloma which is a compact organized collection of macrophages, cells like epithelioid cells (transformed macrophages), Langhan's type giant cells (large cells formed by the fusion of epitheloid cells and characterized by numerous nuclei arranged around the cell periphery), lymphocytes, plasma cells and fibroblasts. Granulomas prevent spread of infection by confining bacteria within this compact collection of several types of immune cells. The latent infection may provide the host with an increased resistance to second attacks of this disease but at the same time, it can also constitute an important source of re-activational disease later in life or following an HIV infection.

In approximately 10% of individuals the infection spreads via the blood stream to the spleen, the kidneys, the bone marrow and central nervous system (frequently seen in infants and children whose cellular defenses have not yet become fully developed). The developing infection may continue to disseminate despite the activation of a strong cell-mediated immune response, which nevertheless, seems incapable of limiting the developing infection within the lung. This may lead to extra-pulmonary tuberculosis or severe tissue destruction in the lung leading to 'open' cases of tuberculosis. In the absence of adequate anti-tuberculosis chemotherapy, these people may succumb to death.

1.2 DIAGNOSIS OF TUBERCULOSIS – AN OVERVIEW

Conventional diagnosis of tuberculosis rely on the identification of the microorganism in secretions or tissues from the patient by acid-fast (mainly Ziehl-Neelsen) staining and culture techniques. But the sensitivity of sputum acid-fast stain is low, especially in non-cavitary tuberculosis. The sensitivity of sputum acid-fast staining ranges from 40-60 % (Van Deun A and Portaels F, 1998) in different studies and requires at least 5 x 10^3 bacilli/ml of sputum to give a positive result (Samanich *et al*, 2000). The culture method is more sensitive, but the time needed for results is no shorter than 6 to 8 weeks. Clinical features in tuberculosis are non-specific and chest roentgenographic findings are atypical in more than 30% of patients. Therefore, a rapid diagnostic tool with both high sensitivity and specificity is needed to improve the conventional diagnostic methods.

Several new techniques have been developed to improve the diagnosis of tuberculosis, including newer culture methods, molecular methods (like Polymerase Chain Reaction, nucleic acid hybridization and *in situ* hybridization), chromatography of mycolic acid and serologic tests. Improved culture techniques like Septicheck AFB method, Radiometric Bactec 460 TB method, MB/BACT system and ESP culture II systems gained interest primarily because of their rapidity and accuracy. But most of these bacteriological methods failed to produce adequate sensitivity and requires extensive automation, thus making it more expensive and less reachable to the under-privileged people who are more prone to get affected by tuberculosis.

Polymerase Chain Reaction (PCR) amplifies a specific gene target of *Mycobacterium sps*., and the amplified product is visualized using agarose gel electrophoresis. Though it can detect as less as 1-10 organisms, this method is limited by its inability to distinguish between dead and viable bacilli, and also by the need of expensive instrumentation. In methods like nucleic acid hybridization, nucleic acid probes that can pair to ssDNA or RNA are used to form duplex and used to detect *M. tuberculosis* from conventional solid or broth cultures. *In situ* hybridization methods are also used to detect and locate specific mycobacterial DNA or RNA segment in tissues by using radioactive/fluorescent probes. HPLC is another method used to detect species specific mycolic acids produced by *M. tuberculosis*. All these techniques are rapid but require extensive laboratory facilities as well as technical expertise. Detections of biological compounds like Adenosine Deaminase (ADA), Tuberculostearic acid, IFN-γ etc are some other

diagnostic strategies. But they are limited either by their sensitivity or specificity. Detection of mycobacterial antigens or antibodies directed towards these antigens in the body fluids is another important area that gained attention.

1.2.1 Immunodiagnostic approaches in tuberculosis

Several investigators have indicated that the demonstration of mycobacterial antigens or corresponding antibodies in body fluids may be a clinically useful approach. As mycobacteria are rich in antigens that stimulate antibody production, several assays have been used to detect specific antibody responses in patients with mycobacterial diseases. Using similar techniques, investigators reported ELISA for detecting tuberculous antigens in CSF and pleural and ascitic fluids. Circulating immune complexes have also been described in patients with tuberculosis. ELISA measuring the antibody response to semi-purified mycobacterial antigens has been most commonly used. Recently, crude antigens have been superseded by more purified antigens that are specific to certain mycobacterial species. Many such antigenic materials have been subsequently employed in an attempt to improve both the sensitivity and specificity. The specificity of serological assays can be improved by measuring antibodies directed towards individual antigenic epitopes by competitive inhibition of the binding of monoclonal antibodies.

Immunoreactive properties have been demonstrated in lipid, polysaccharides and protein components of tubercle bacilli. The main protein antigens used in the diagnosis so far are sonicate antigen from BCG and *M.*

tuberculosis, PPD (purified protein derivative) antigen, Antigen 60, Antigen 5 and Antigen 6. They provided sensitivities ranging from 45 – 90 % with widely varying specificity. An important point to be noted is that many bacterial proteins are highly conserved not only within the genus Mycobacterium but also in a broad ranges of other bacterial species and thereby contribute to the antigenic cross reactivity. Many researchers have attempted to isolate the species-specific antigens for use in diagnostic tests, but this task has proved to be very difficult for the following two reasons. First, specific antigenic determinants often occur on the same protein molecule as the shared antigen, therefore making it impossible for purification even by affinity chromatography based on binding with a specific antibody. Second, given determinants may be present on a range of molecules with different physiochemical characters. Thus, preparative techniques based on such differences (gel filtration and ion-exchange chromatography) have not proved very useful. Furthermore, due to antigenic diversity in patients with mycobacterial disease, especially in those with tuberculosis, no single antigen can detect all positive cases. Therefore, search for newer antigenic candidates for serological tests is still going on and now by recombinant DNA technology new proteins are being purified and tested for their utility in the diagnosis of tuberculosis.

1.3 ANTIGENS OF *M. TUBERCULOSIS*:

Research on mycobacterial antigens is mainly aimed at understanding the physiology of the bacteria, their metabolic pathways, enzyme systems and in the study of interactions between individual mycobacterial components and the immune systems of human beings. The goal of the latter effort is to isolate these antigens in their pure form and to identify those that are important in conferring protective immunity against tuberculosis and those that are useful as candidates in the search of improved diagnostic tool.

The antigenic components of *M. tuberculosis* are very complex and they derive either from the cell wall or from the cytoplasm of the bacillus. The cell wall of mycobacteria is unique in the presence of their extraordinary high lipid content constituting up to 60% of the cell wall (Kolattukudy *et al.* 1997). This lipid rich cell wall confers an extremely low permeability barrier on *M. tuberculosis* contributing to its resistance to most common therapeutic agents as well as to its ability to survive within the hostile environment of the macrophages. It also provides 'alcohol-acid' fast nature to the mycobacterium. Cell wall components are also important in disease progression.

1.3.1 The cell envelope:

The mycobacterial cell wall contains components like peptidoglycan

(contains L-alanyl-D-isoglutaminyl-*meso*-diaminopimelyl-D-alanine), arabino-galactan (AG), mycolic acids, lipoarabinomannan (LAM), extractable lipids (including: extractable glycolipids lipooligosaccharides, phenolic glycolipids, glycopeptidolipids, waxes, Glycerophospholipids, acylated trehaloses and sulfolipids) and proteins (Fig: 1,4).

The plasma membrane structure is the first layer of the cell envelope from the inner side of the cell evenly as in other bacteria. A layer of peptidoglycan with a chemically bound polysaccharide arabinogalactan (AG) is found above the plasma membrane. Outer terminuses of AG are esterified by taxonomically specific mycolic acids. The other above mentioned components are linked with these constituents; many of them act as antigens or are toxic (e.g. cord factor). The cells are again surrounded by capsules, most likely of lipid origin, which obviously protect them from lysosomal activity, hydrolytic enzymes and toxic radicals produced by the macrophages. A source of protein antigens is primarily the peptidoglycan layer. Channel forming proteins functionally similar to the well known porins of Gram-negative bacteria have been demonstrated in Mycobacterium.

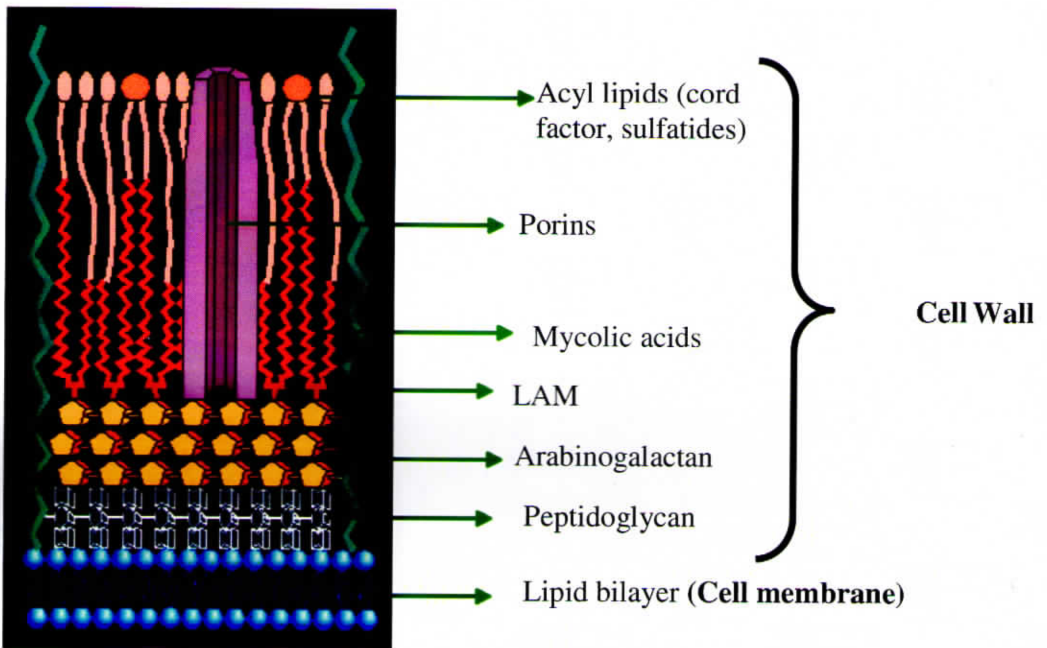


Fig 1.4 Diagrammatic representation of mycobacterial cell wall

1.3.2 Protein and polysaccharide antigens:

Robert Koch in 1890 first reported an antigenic preparation of tubercle bacilli, which he named as old tuberculin or "OT". It consisted of a concentrated sterile filtrate of autoclaved; heat inactivated liquid cultures of *M. tuberculosis*. Seibert *et al* in 1932 purified tuberculous proteins from OT and named it as PPD (purified protein derivative) and subsequently characterized four proteins (designated as A, B, C, and D) and two polysaccharides (designated as polysaccharide I and II) from it. Later, several purified antigens have been prepared from the same antigenic preparation. Daniel and Affronti demonstrated antigen 1, 2, 5 and 6 by immuno-electrophoretic studies from protein A. Protein B contained antigen 1, 2, 5, 6 and 7. Polysaccharide fractions were also further characterized by Birbaun and Affronti. They found that polysaccharide I contained arabinose, galactose and mannose. Polysaccharide II was found to be a glucan and showed antigenicity due to contaminated proteins like antigen 2 (Mathai A, 1993).

The predominant source of polysaccharides is the cell wall of *M. tuberculosis*. Arabinogalactans, arabinomannans, mannans and glucans have been isolated from the cell wall of mycobacterium. These polysaccharides do not elicit the delayed type skin reaction (Mathai A, 1993).

1.3.3 Lipid antigens:

The genus mycobacterium is known for its high content of lipids constituting 40% of its dry weight (Anderson, 1940). These are of two types-

covalently linked compounds and loosely associated molecules that are readily extractable with organic solvents. The former group is mainly composed of mycolic acids and latter group is composed of a mixture of ubiquitous type-specific and species-specific lipids.

As mentioned earlier, the mycobacterial cell wall contains a number of antigens (glycolipids and proteins), that can be used in preliminary serologic diagnosis. Some mycobacterial lipids are shed and might therefore be readily available for loading onto CD1 molecules, whereas others are covalently attached to the mycobacterial cell wall and can thus become available for immune recognition only after the partial degradation of mycobacteria by the host. Mycolic acid was the first lipid antigen that was shown to stimulate specific T cells (Spargo *et al*, 1991). LAM is a dominant liposaccharidic antigen of *M. tuberculosis*. Phenolic glycolipids (PGL) in *M. leprae* and *M. bovis*, glycopeptidolipids in *M. avium* complex or glycolipids (GPL) that contain acylated trehaloses in *M. kansasii*, *M. szulgai*, and *M. malmoense* species are also specific antigens.

1.3.3.1 Mycolic acids:

They are characteristic high molecular weight, alpha branched beta hydroxy fatty acids of the genus mycobaterium (eumycolic acids) and related taxa such as corynebacterium (Corynomycolic acids) and nocardia (nocardo-mycolic acids). Anderson in 1939 isolated this lipid by prolonged saponification of waxes of *M. tuberculosis*. They may contain diverse functional groups such as methoxy,

keto, epoxy, ester group and cyclopropane ring. Based on that, they are classified as alpha mycolic acids, keto mycolic acids, methoxy mycolic acid, carboxy mycolic acids etc. In the bacterial cell, they are present either in lipids, extractable by organic solvents mainly in the form of trehalose 6, 6' dimycolate or as bound esters of arabinogalactan, a peptidoflycan linked polysacharide (Karakousis et al, 2004).

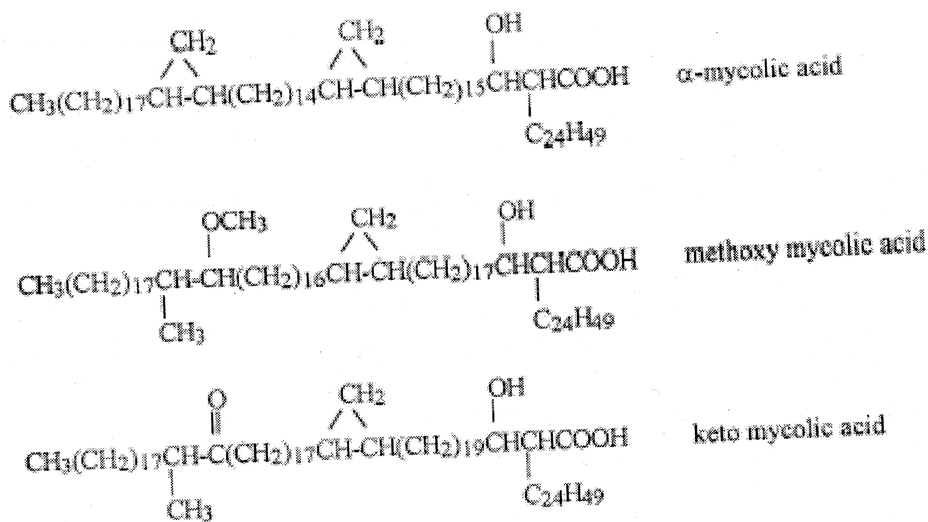


Fig 1.5 Chemical structure of mycolic acids

1.3.3.2 Cord factor:

One characteristic property of *M. tuberculosis* is its tendency to form 'cords' or dense clusters of bacilli in parallel alignment. This quality was noted by Koch in his initial report on the etiological aspect of tuberculosis. The term cord factor was coined by Noll *et al* to describe the glycolipid obtained after a gentle

treatment with petroleum ether extract of *M. tuberculosis* that prevent formation of bacterial cords (Noll *et al*, 1956). It is found in all mycobacterial species except *M leprae*. Bloch isolated cord factor in 1950, and its structure was fully elucidated by Noll *et al* in 1956. It is a glycolipid consisting of two mycolic acid molecules linked with the trehalose by the hydroxyl group of the carbon's 6 and 6' i.e, chemically trehalose 6, 6' dimycolate. They occur as a mixture, differing one from the other by the chemical groups present in the mycolic acid substituents.

Three decades of research on cord factor have uncovered a myriad of biological activities in the pathogenesis of mycobacterial diseases including high toxicity in mice (Kato M, 1973b), granulomagenic activity in mice lung after intravenous administration (Baba *et al*, 1997), adjuvant immunostimulant property (Saito *et al*, 1976) and anti-tumoural activity (Orbach-Arbouys *et al*, 1983). Thus it participates in the host parasite relationship at different levels.

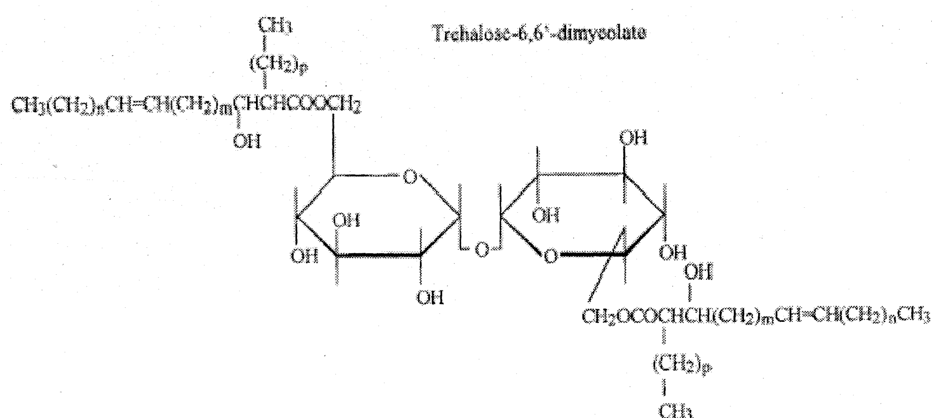


Fig 1.6 Chemical structure of cord factor (trehalose 6, 6' dimycolate)

1.3.3.3 Sulfatides:

In a search for the component of virulent mycobacteria that induced uptake of the dye, Middlebrook and coworkers isolated sulfatides. This family is composed of 5 related molecules SL-I, SL-II, SL-III, SL-IV and SL-V which contained sulfate substituent on position 2'.They differ one from the other by the number and type of acyl substituents and by their positions on trehalose. They are found only in virulent strains of the human pathogen *M. tuberculosis*. It has been correlated with the pathogenicity or virulence of mycobacteria. It is shown to inhibit phagolysosome fusion (Goren et al, 1976), alters phagocyte function (Zhang et al, 1991; Zhang et al 1988) and increases the toxicity of cord factor (Goren et al, 1974).

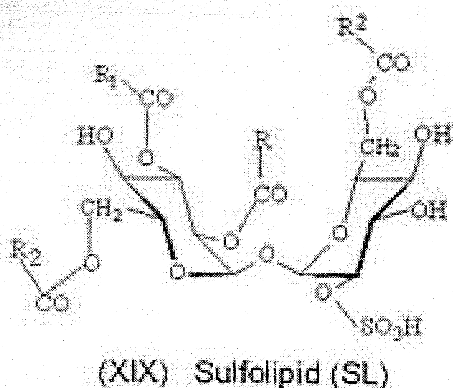


Fig 1.7 Chemical structure of Sulfolipid I

1.3.3.4 Lipoarabinomannan (LAM):

A serologically active polysaccharide isolated from the cell envelope is LAM. It has been used for the diagnosis of different types of tuberculosis. It is a phosphatidyl inositol anchored lipoglycan composed of mannan core with

oligoarabinosyl containing side chains with diverse biological activities. There are generally three classes of LAM – (a) Man LAM which is characterized by extensive mannose capping of the arabinan termini (b) phospho myo inositol capped LAM (PILAM) c) and Ara Lam which lacks mannosylation in its arabinan termini. LAM has been shown to possess a variety of biological activities including gamma-interferon-mediated activation of macrophages, suppression of T cell proliferation, enhancement of TNF-α production by mononuclear cells etc.

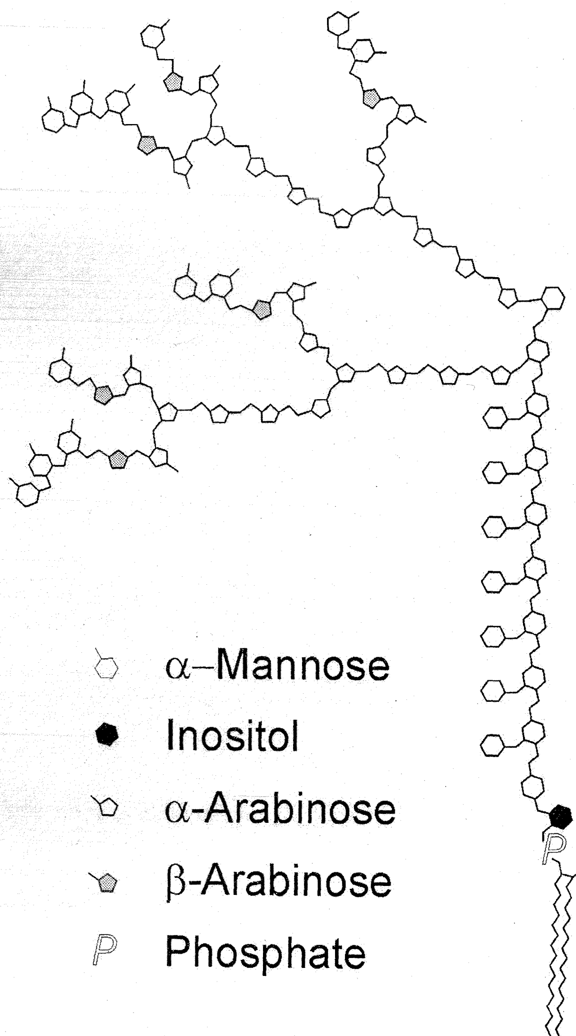


Fig 1.8 Structure of LAM

To understand the role of these lipids in protection against tuberculosis, it is important to clarify whether the repertoire of immunogenic lipids synthesized by *M. tuberculosis* is limited to a few molecules or rather comprises a wide spectrum of structurally distinct molecules. The identification of the most immunogenic lipids will facilitate selection of lipid-based diagnostic methods and lipid-based vaccines against tuberculosis. However, the pool of studied mycobacterial antigenic lipids is small compared with the diversity of the lipid components of the *M. tuberculosis* envelope. Research over the past five decades has implicated various compounds of the mycobacterial cell wall matrix in host response. These mycobacterial lipids include phenolic glycolipid, trehalose-6,6' dimycolate (cord factor), lipoarabinomannan, sulfatides, mycolic acids and phosphoinositol mannosides. While some of these molecules such as sulfolipid or cord factor have been implicated in host-pathogen interaction, a proof of their involvement in pathogenesis is far from conclusive. The present study is designed to isolate some of these mycobacterial lipids and to analyze the critical role of lipids in the pathogenesis as well as their potential approach in the laboratory diagnosis of tuberculosis.

1.4 OBJECTIVES OF THE STUDY:

The specific objectives of the study are

1. To isolate and characterize the major lipid antigens of *M. tuberculosis* – mycolic acid containing glycolipids (tuberculosis-associated glycolipid antigen, TBGL), cord factor (trehalose 6, 6' dimycolate), sulfolipid-I (2, 3, 6, 6' tetraacyl

α, ά D trehalose 2' sulfate) and lipoarabinomannan (LAM) from *M. tuberculosis* bacillus.

2. To evaluate the specific role of these mycobacterial antigens in the immunodiagnosis of tuberculosis by Enzyme-linked immunosorbent assay (ELISA) in patients with pulmonary and pleural tuberculosis.

3. To evaluate the role of cord factor and sulfolipid-I in the chemotaxis of polymorphonuclear leukocytes (neutrophils) during tuberculosis infection.

CHAPTER.2

REVIEW OF LITERATURE

Mycobacteria were among the earliest organisms investigated for their lipid content but such is the complexity of lipids that a true understanding of their structure and role in directing the host-pathogen interactions has not yet been obtained. They are probably the highest containers of lipids of all bacteria and the lipids are mainly present in their cell envelope. While researches extending back over the last 60 years have implicated some of these molecules such as sulfolipid and cord factor in the host-pathogen interactions, proof of their involvement in pathogenesis is far from conclusive. As many of these lipids within the envelope have been found to have immunogenic and antigenic properties, lot of interest have been generated among researchers to use these lipid antigens for the diagnosis of tuberculosis.

Though several investigators speculated the presence of high lipid content of the tubercle bacillus, it was the pioneering work of R J Andersen from 1920's to early 1940's that succeeded in delineating the major mycobacterial lipids and this laid the foundation for all subsequent work on mycobacterial lipids. He revealed that mycobacteria contained novel lipid components not found in any living organisms (Minnikin, 1982). He isolated lipoid fractions of moist living H37 strain of human tubercle bacillus by extracting with a mixture of alcohol and ether followed by extraction with chloroform at room temperature (Andersen, 1927). This lipoid fractions mainly contained wax, glycerides and phosphatides. Since then, several lipid fractions have been isolated from the tubercle bacillus

(Pangborn M C and McKinney J A, 1966; Lee Y C, 1966; Subramanyam D and Singhvi D R, 1965; Navalkar *et al*, 1965; Tanaka and Kitagawa, 1965; Azuma *et al* 1964; Lee and Ballou, 1964). The important reports explaining the isolation of major lipid antigens and evaluating their potential in diagnosis will be discussed in the following sections.

2.1. ISOLATION OF MYCOBACTERIAL LIPIDS:

In 1974, Reggiardo and Middlebrook isolated three serologically active glycolipids along with cardiolipin from *M Bovis* by methanol and chloroform extraction followed by silicic acid chromatography. They named them as glycolipid A, B and C (Reggiardo and Middlebrook, 1974a). Their serological study using Coomb's passive heamagglutination and inhibition methods showed that glycolipid B and C were reactive against BCG immunized rabbit sera and sera of tuberculosis as well as leprosy patients. Glycolipid A family was found to be reactive only against sera of tuberculosis patients (Reggiardo and Middlebrook, 1974b).

Toida *et al* extracted lipids from different strains of *M. tuberculosis* with chloroform: methanol (2:1) and separated the lipid fractions by thin layer chromatography using the solvent system- chloroform: methanol: acetone (90: 10:5). In the study, $H_{37}Rv$ strain showed four anthrone–positive spots, namely trehalose monomycolate, an unidentified glycolipid, trehalose dimycolate and Gl-Rv (a new glycolipid designated by them) and $H_{37}Ra$ showed only two spots

corresponding to trehalose monomycolate and trehalose dimycolate. He used the glycolipid patterns for strain differentiation but it was not used in ELISA for the immunodiagnosis of tuberculosis (Toida et al, 1989).

2.1.1 Isolation of tuberculosis associated glycolipid (TBGL) antigen

In 1997, Kawamura *et al* of the Fuji Research Laboratories, Kyowa Medex Co. Shizuoka, Japan, reported the development of a special TLC immunostaining that could detect specific antigens for antibodies in the serum of tuberculosis patients. The detected specific antigens included cord factor (trehalose dimycolate) and specific glycolipid fraction. These glycolipids were individually purified from *M. tuberculosis* $H_{37}Rv$ by column chromatography. The two purified fractions were mixed and the mixture was named as TBGL (Kawamura et al, 1997). Several studies were published about the diagnosis of tuberculosis using this antigen which will be discussed in section 2.2.1.

2.1.2 Isolation of cord factor:

The toxic glycolipid cord factor (trehalose 6, 6' dimycolate) was first identified by Bloch in 1950. In 1955, Noll and Bloch explained a method to isolate cord factor. They extracted the *M. tuberculosis* cell with ether and methanol (1:2) and subsequently with chloroform. The cord factor was then separated from this extract by magnesium silicate-celite chromatography followed by silica gel column chromatography. Though this method was tedious and time

consuming, it was used extensively by the researchers to isolate cord factor for the further characterization of cord factor. Later researchers used direct chloroform: methanol (2:1, 1:2, 4:1, 3:1 or 1:1) extraction followed by silica gel thin layer chromatography for the final separation. (Silva and Faccioli, 1988, Spargo *et al* 1991, Ozeki *et al*, 1997). But this method was also time-consuming and yielded small amounts of cord factor. Some other studies reported petroleum ether extraction and claimed that petroleum ether extracts of mycobacteria contain primarily TDM (>95% of total extract), with only relatively small quantities of free mycolic acid, glycerides, menaquinones and hydrocarbons (Silva *et al*, 1985; Indrigo *et al*, 2002, Indrigo *et al*, 2003).

2.1.3 Isolation of Sulfolipid -I:

Dubos and Middlebrook observed that the virulent cord-forming strains of *M. tuberculosis* invariably absorbed the cationic dye neutral red, while most attenuated strains of *M. tuberculosis* and saprophytic mycobacteria did not (Dubos and Middlebrook, 1948). In a search for the component of virulent mycobacteria that induced uptake of the dye, Middlebrook and coworkers isolated sulfolipids by hexane-0.1% decylamine extraction. Hexane solutions containing this sulfolipid extracted neutral red from an aqueous phase into the organic layer and the amount of dye fixed was proportional to the specific radioactivity of lipids labeled with ^{35}S. Similar extracts of avirulent or attenuated strains showed little neutral red activity and correspondingly little radioactive sulfur in the lipids extracted by this mild solvent (Middlebrook, 1959).

Gangadharam established a correlation between the levels of sulfolipid elaborated by different strains of *M. tuberculosis* (Gangadharam, 1963). In his study, he primarily extracted the cells with 0.1 % decylamine in n-hexane; the extract was treated with 2 N citric acid, hot ethanol etc and cooled to -20ºC and finally treated with sodium lactate. The supernatant was collected, and separated by thin layer chromatography. Since then, this method is followed by the researchers for the effective isolation of sulfolipid I. But it was later concluded that the sulfolipid recovery scheme employed by them often failed to separate and distinguish the sulfatides from contaminant phospholipids or perhaps from relatively polar carboxylic acids (Goren *et al*, 1974).

Goren identified SL-I as 2,3,6,6'-tetraacyl α, α' –D-trehalose 2'-sulfate(Goren, 1970). In 1971, Goren *et al* described its nature of acyl substituents but the specific locations of acyl substituents were assigned in 1976 (Goren *et al*, 1976).

2.1.4 Isolation of LAM

In 1986, Hunter *et al* purified lipoarabinomananan from *M. tuberculosis* in its native acylated state using anion-exchange and gel filtration chromatography after delipidating the cells. Later several researchers modified this method by including more extraction steps and phenol biphasic partition step. (Chatterjee et al, 1992a; Chatterjee et al, 1992b; Venisse et al, 1995). Slayden and Barry described an easier method excluding the chromatographic steps as well as including dialysis.

2.2. MYCOBACTERIAL LIPIDS IN DIAGNOSIS

The first study depicting the use of mycobacterial glycolipid antigen in ELISA for serodiagnosis of tuberculosis was reported by Reggiardo *et al* (Reggiardo *et al*, 1980). In 1974, Reggiardo and Middlebrook showed that the glycolipids C (described in section 2.1.1) could be used for the serodiagnosis of tuberculosis (Reggiardo and Middlebrook, 1974b). In their study, positive results were found in 95% of tuberculosis patients with advanced tuberculosis, 80% with newly diagnosed tuberculosis and 75 % with atypical mycobacterial diseases. 4% false positive results were also encountered in the study. Reggiardo and Vanquer studied and compared ELISA and heamagglutination test using these three serologically active glycolipids (Glycolipids A, B and C) for their use in tuberculosis diagnosis. Both the tests found to have equivalent specificity and sensitivity in detecting mycobacterial diseases. But some sera were positive by ELISA and negative by heamagglutination while some sera were negative by ELISA and positive by heamagglutination. So they concluded that the glycolipid molecules in these two methods are presented differently and different IgG antibodies are involved in the two reactions (Reggiardo and Vanquer, 1981). Later, Papa *et al* (1989) demonstrated the specificity of some of these glycolipids. In their study, immune sera raised in rabbits reacted exclusively with the corresponding antigen and with the crude extracts of *M. tuberculosis* complex, but not with crude extracts from 39 other mycobacterial species.

These findings prompted the researchers to verify whether these molecules had a potential for tuberculosis serodiagnosis. Therefore, in the 90s many studies using the ELISA technique were performed to test the utility of several glycolipids in tuberculosis diagnosis. Moreover, Niculescu *et al* (1995) demonstrated that the glycolipids are more adequate to be used as antigen than the whole BCG suspension and recommended anti-tuberculosis glycolipid antibody detection as a supporting test for direct smear examination. However, results obtained in these studies showed a lack of uniformity. This discrepancy can be attributed to the methodological variations in the ELISAs used. (Julian *et al*, 2001).

2.2.1. TBGL antigen in tuberculosis diagnosis:

One of the main glycolipid antigenic preparations evaluated for the diagnosis of tuberculosis was tuberculous glycolipid antigen or TBGL antigen developed by Kawamura *et al.* In their study, this antigen was applied to an enzyme immunoassay for the measurement of anti-tuberculosis IgG antibodies in serum and used it for the serodiagnosis of tuberculosis. This enzyme immunoassay showed a high serodiagnostic discriminating power of 90% sensitivity and 98% specificity (Kawamura et al, 1997). The Kyowa Medex Co. Ltd developed a kit based on this immunoassay and has put this invention to practical use as a commercially available product, "Determiner TBGL Antibody" in 2000. Kishimoto *et al* (1999) set the cut-off value for the kit at 4U/ml for diagnosis of tuberculosis and 2U/ml for screening patients with tuberculosis.

The first laboratory evaluation of this serodiagnostic kit developed by Kyowa Medex Co Ltd was reported in a Japanese journal by Toyoda *et al* in 1996. The important findings in the study were a) patients excreting large amounts of acid fast bacilli show high positivity rates b) The antibody detection is of no use in prognosis as antibody titer decreases only slightly even after two years of anti-tuberculosis chemotherapy and c) antibody titer is not increased by BCG vaccination. In this study, 34/39 patients (87.2%) with active pulmonary tuberculosis provided positive results. But, two out of four non-tuberculous mycobacteriosis (50 %) cases also showed high antibody titers and, all three AIDS patients with tuberculosis included in the study showed low antibody titers.

Maekura *et al* also evaluated the utility of the D-TBGL kit developed by Kyowa Medex Co., Ltd. In smear and culture negative patients, the sensitivity was 73.5% and in smear/culture positive patients the sensitivity was 84.7%. Antibody titers in sera from 318 patients with active pulmonary tuberculosis (216 positive for *Mycobacterium tuberculosis* in smear and/or culture tests and 102 smear and culture negative and clinically diagnosed), 58 patients with old tuberculosis, 177 patients with other respiratory diseases, 156 patients with non-respiratory diseases, and 454 healthy subjects were examined. When the cutoff point of anti-TBGL antibody titer was determined as 2.0 U/ml, the overall sensitivity for active tuberculosis patients was 81.1% and the overall specificity was 95.7% (Maekura *et al,* 2001).

Iinuma *et al* (2002) compared the potential of this kit with nucleic acid amplification and sputum culture in pulmonary patients. This was a multicenter

study comparing the assay with the other methods in 78 patients with active pulmonary tuberculosis and in 54 controls with non-tuberculous lung diseases. The sensitivity of the immunoassay kit (89.4 %) in sputum smear positive patients was comparable with that of the nucleic acid amplification (88.9 %). For TBGL antigen kit Iinuma *et al* obtained 79.5% sensitivity and 30% specificity in their study. In smear negative patients the sensitivity was 64.5 % only.

In another study, Maekura *et al* used Roche Amplicor mycobacterium test (Amplicor MTB, Roche diagnostics, Laval, Quebec, Canada) for nucleic acid amplification and TBGL serodiagnostic test kit (Kyowa Medex Co., Ltd, Tokyo, Japan) for anti-TBGL antibody detection for assessing their utility in establishing diagnosis of tuberculosis. Both the tests were individually not so useful for the rapid diagnosis of smear – negative active tuberculosis in this study. But in combination, they obtained a sensitivity of 75.3%. Hence, they advocated combined use of anti-TBGL antibody detection and nucleic acid amplification test for the improved diagnosis of tuberculosis (Maekura *et al,* 2003). The results obtained by Iinuma *et al* and maekura *et al* show the difference in the efficiency of the kit in different population.

Okuda *et al* (2004) compared the utility of three antibody detection kits for diagnosis of tuberculosis. The antigens studied were tuberculous glycolipid (TBGL), lipoarabinomannan and a protein antigen Antigen 60 (A 60). This study was conducted on the concept that the cell wall antigen composition of each patient isolate of tuberculous bacilli differs resulting in antibodies with different specificities. In this study, the percentage of patients positive in all three tests was

58.8% for smear positive active pulmonary tuberculosis cases. When the results of the three tests were combined, the sensitivities increased to 86% in smear and culture negative patients. The false positive rate of the three-test combination was 12.5% in healthy control groups. They advocated the combined use of these three antigens for more effective diagnosis.

In 2005, Nabeshima *et al* proved that the serological anti-TBGL antibody is not influenced by prior BCG vaccination. They studied the antibody titer before and after vaccination and observed that though 85 % of the subjects turned tuberculin skin test positive, the mean anti-TBGL antibody titer remained negative throughout the observation period. But the titer was observed elevated significantly when compared to pre-vaccination levels.

Mizusawa *et al* showed that there is a positive correlation between the amount of anti-TBGL antibodies and cavity formation. The patients with cavitary lesions showed significantly higher levels of anti-TBGL IgG and anti-TBGL IgA along with an increase in the number of white blood cells neutrophils, basophils and natural killer cells (Mizusawa *et al,* 2008).

Yanai *et al* (2006) evaluated the utility of anti TBGL antibody detection in hemodialysed patients who had previous history of treated tuberculosis. Positivity of anti-TBGL antibody was found to be significantly higher in patients with findings of old tuberculosis on the chest X-ray than those without findings. This results point out that these serological tests are positive more frequently in hemodialysis patients without any proof of active tuberculosis than in healthy subjects (2%) and careful interpretation is necessary for relevant results. At the

same time, Ashino *et al* (2005) proved that in elderly gastrectomised tuberculosis patients, only a low antibody response is evident against tuberculous glycolipid (TBGL) antigen.

Morimoto *et al* evaluated the use of this kit in the diagnosis of pleural tuberculosis by detecting the antibodies in pleural effusions. The results were compared with the results obtained for Mycodot assay for the detection of anti-LAM antibodies and adenosine deaminase assay. D-TBGL-Antibody and MycoDot each had low sensitivity (52.6 %) but high specificity (95.7% and 97.8% respectively) in the assay. When TBGL-Antibody detection is used in combination with Adenosine Deaminase, the sensitivity and specificity were both >90%. So they proposed this combination in tuberculosis diagnosis (Morimoto *et al,* 2006).

Tiwari *et al* reported a newer approach - a liposome agglutination test using glycolipid antigens in 2005. Glycolipids of *Mycobacterium tuberculosis* $H_{37}Rv$ antigens were first isolated and purified. After interchelation with liposome particles, these purified antigens were allowed to bind to the anti-glycolipid antibodies present in the sera of patients with tuberculosis. Positive reactions were evident by the formation of a blue agglutination. This diagnostic tool named as the TB Screen Test, was rapid than the other available products and was advocated by the authors to be used for the mass screening of a heavily afflicted population. It obtained 94% sensitivity and 98.3% specificity in the study (Tiwari et al, 2005). In 2007, Tiwari *et al* developed another liposome agglutination test for tuberculosis serodiagnosis and named it as TB/M card test. Affinity-purified

rabbit anti-glycolipid antibodies (IgG) were coupled to liposome particles (0.2-0.4 micrometer) in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride and N-hydroxysuccinamide to prepare the working reagent of the TB/M card test. Antibody-conjugated liposomes, when determined with the glycolipid antigens present in the specimens, formed a dark blue agglutination within 4 min. The test provided 97.4% clinical sensitivity and 96.9% specificity. But more studies in this area only can reveal the actual potential of these diagnostic methods.

2.2.2. Cord factor in tuberculosis diagnosis:

In 1973, Kato studied the antibody responses to cord factor in animal models as well as in humans and his study indicated lack of antibody response to cord factor in the serum of tuberculous patients presenting active or covalescent states or after the resection of lung foci (Kato, 1973a). But this observation was proved to be wrong when several researchers started using cord factor in immunoassays for the detection of anti-cord factor antibodies.

He *et al* were the first to study the utility of cord factor isolated from *M. tuberculosis* $H_{37}Rv$ strain in ELISA for the serodiagnosis of tuberculosis. Of the 99 cases of mycobacteriosis, 83 patients (83.8 %) had positive results (48/53 samples from patients (90.5 %) with bacilli in the sputum and 35/46 samples from patients (76%) with tuberculosis diagnosed clinically). The sera of five patients with lung cancer and the 100 controls all gave negative results. Thus, the sensitivity and specificity were 83.8% and 100 % respectively. Some of the

patients with non-tuberculous mycobacteriosis also gave positive results (He et al, 1991). But, the diseased control population in the study was very small for evaluating a serodiagnostic method.

Next year, Laszlo *et al* reported the potential of several synthetic cord factor analogues for tuberculosis serodiagnosis. They found out that all synthetic cord factor analogues except one with a short 8-carbon chain were better recognized by tuberculous sera with 93 % sensitivity and 93 % specificity. This is because either these artificial molecules are cross-reactants of similarly structured native glycolipids of *M. tuberculosis* or that they bear close resemblance to actual phagosome-lysosome-modified antigens than to native mycobacterial ones (Laszlo et al, 1992). Thus, this work showed the cross-reactivity of cord factor antibodies to other structurally similar molecules.

In 1993, Maekura *et al* reported the second study evaluating the utility of detecting IgG antibodies against cord factor isolated from $H_{37}Rv$ strain of *M. tuberculosis*. Serum specimens from 65 patients with active pulmonary tuberculosis, 58 patients with inactive pulmonary tuberculosis, 36 patients with diseases other than tuberculosis, and 66 healthy adults were examined. In patients with active and untreated pulmonary tuberculosis, this ELISA had a sensitivity of 81% and a specificity of 96%. They also showed that the antibody titers decline to the normal levels as a result of anti-tuberculous chemotherapy.

Kashima *et al* evaluated the utility of the detection of anti cord factor antibodies in intestinal tuberculosis for its differential diagnosis from other inflammatory bowel diseases like Crohn's disease, ulcerative colitis etc. IgG

antibodies against cord factor from 27 patients with intestinal tuberculosis, 16 patients with Crohn's disease (CD), and 27 patients with ulcerative colitis (UC) were tested by ELISA with cord factor purified from *Mycobacterium tuberculosis* $H_{37}Rv$ as the antigen. The study obtained a sensitivity of 85 % and 96% of ulcerative colitis patients did not show any anti-cord factor antibody elevation (Kashima et al, 1995).

Glycolipid fraction containing mainly cord factor, isolated from $H_{37}Rv$ strain of *M. tuberculosis* was used as antigen in ELISA by Wada *et al.* Overall positive rates of the ELISA tests in the patients with active pulmonary tuberculosis, those with non-tuberculous mycobacteriosis, and those with other diseases were 67.4%, 75.6%, and 6.5%, respectively. He proposed that the test could be used in the diagnosis of tuberculosis in smear negative and culture negative patients as they obtained 50% sensitivity in this group. They also found positive correlation between high positive rate in the assay and the bacillary load (Wada et al, 1997).

To clarify the exact antigenic epitope in cord factor recognized by a anti-cord factor IgG antibody, Fujiwara *et al* immunized rabbits with two kinds of cord factors isolated from *M. tuberculosis* or *M avium* and the reactivities of sera were tested against cord factors or component mycolic acid methyl esters (which differs according to the species of mycobacterium) by ELISA. The serum from rabbits immunized with *M. tuberculosis* cord factor was highly reactive against *M. tuberculosis* cord factor but less reactive against *M. avium* cord factor. An opposite reaction was shown by serum from rabbits immunized with *M. avium*

cord factor (Fujiwara et al, 1999). They also proved that the anti-cord factor IgG antibody recognizes hydrophobic moiety rather than carbohydrate moiety of cord factor i.e., they can recognize mycolic acid subclasses of cord factor. Lopez-Marin *et al* isolated trehalose-6, 6'-dimycolate from *M. fortuitum* and developed ELISA with higher specificity and sensitivity for the diagnosis of tuberculosis. The sensitivity and specificity obtained were 66.6%-74.1% and 95.2-99% respectively (Lopez-Marin et al, 2003).

In 2002, IgG, IgM and IgA antibody response towards cord factor was reported. This is the first study that explained the IgA response in tuberculosis patients. They found that IgG was comparatively more reactive, giving the highest absorbance values and highest sensitivities, whereas IgA was found to be more specific. IgG, IgA and IgM antibody to cord factor provided sensitivities of 60.34 %, 32.75% and 1.72 % respectively. The specificities were 75 %, 95.83 % and 98 % respectively (Julian et al, 2002).

In 2001, Sakai *et al* in their study, made an attempt to evaluate the detection of anti-cord factor IgG antibody for its utility in the diagnosis of ocular tuberculosis. Cord factor was prepared from *M. tuberculosis* $H_{37}Rv$ strain. Study population consisted of 15 patients with uveitis and retinal vasculitis, nine patients with presumed ocular tuberculosis, three patients with sarcoidosis, and three patients with Behçet's disease. All cases of clinically presumed ocular tuberculosis were positive, whereas all of the cases of sarcoidosis or Behçet's disease were negative for anticord factor antibodies (Sakai et al, 2001).

Fujita *et al* (2005a) reported the humoral immune responses of active TB patients against six mycobacterial lipid antigens including cord factor from *Mycobacterium bovis* BCG. The other antigens studied were trehalose 6, 6'-dimycolate from *Mycobacterium avium* complex, trehalose 6-monomycolate from *M. bovis* BCG and *M. avium* complex, triacyl (PL-2) and tetraacyl (PL-1) phosphatidylinositol dimannosides. A positive result in any one of the six tests was obtained in 91·5 % of all 924 hospitalized patients and 93·3 % of 210 patients at their first visit to the outpatient clinic included in the study. In the study, the IgG antibody response differed considerably from patient to patient and the antibody levels paralleled the bacterial burden. After anti-TB chemotherapy was initiated, IgG antibody levels decreased dramatically, paralleling the decrease in the amount of excretion of bacteria. He also reported a multiple antigen ELISAs using three lipid antigens including cord factor from BCG Tokyo 172 strain (Fujita et al, 2005b). But the results were not so promising. When any of the three tests was positive, sensitivity was only 71.0%. In 2006, Fujita *et al* explained a multiple antigen ELISA for detecting IgG antibodies against lipid antigens including cord factor along with monoacyl phosphatidylinositol dimannosides, trehalose monomycolate from *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) (TMM-T), and (4) trehalose monomycolate (TMM-M) and (5) GPL-core from MAC for the differential diagnosis of M*ycobacterium avium* infection from tuberculosis.

2.2.3. Sulpholipid in tuberculosis diagnosis:

A very few studies were reported about sulpholipid ELISA in tuberculosis diagnosis. First report was from Cruaud *et al* in 1989 evaluating the utility of SL-I and SL-IV in ELISA for tuberculosis diagnosis and found out that SL-IV is better than the other and can be used as a diagnostic tool. The sensitivity and the specificity of the test were respectively 59 and 100 %. In the study of Chanteaeu *et al*, SL-IV provided a sensitivity and specificity of 95 % and 36 % respectively. Study population included 191 active tuberculous patients, 29 healthy subjects, 102 healthy blood donors, 82 contacts of new TB patients, and 20 leprosy patients before treatment. The predictive values for a positive result, assuming a prevalence of 15 % among patients with respiratory symptoms, were of 30 % (Chanteau et al, 1992).

But later, Rojas-Espinosa *et al* (1999) ruled out the possibility of using SL-I as a tool for the differential diagnosis of tuberculosis. In their study, purified SL-I was used as antigen in an ELISA test and serum from 43 leprosy patients, 44 pulmonary tuberculosis patients and 38 healthy individuals were examined. They found out that there is no specific recognition of the lipid antigen by serum from both leprosy and tuberculosis patients. Some healthy individuals also were found to contain significant levels of antibodies to the SL-I antigen.

David *et al* in 1992 measured anti-SL IV IgG and IGM antibody titers by ELISA. There was an initial rise of specific IgM antibodies that was switched afterwards to IgG production in sub-clinical tuberculosis infection.

But Julian *et al* encouraged the use of SL-I antigen in TB diagnosis. In their study, using an enzyme-linked immunosorbent assay, they made a comparative study of the immunoglobulin G (IgG), IgM, and IgA antibody responses to four trehalose-containing glycolipids purified from *M. tuberculosis*: diacyltrehaloses, triacyltrehaloses, cord factor, and sulfolipid I (SL-I). Sera from 92 tuberculosis patients (taken before starting anti-tuberculosis treatment) and a wide group of control individuals (84 sera from healthy donors, including purified protein derivative-negative & positive, healed, and vaccinated individuals, and 52 sera from non-tuberculous pneumonia patients) from Spain, were studied. The results indicated a significantly elevated IgG and IgA antibody response in tuberculosis patients, compared with controls, with all the antigens used. According to them, SL-I was the best antigen studied, showing test sensitivities and specificities for IgG of 81 and 77.6%, respectively, and of 66 and 87.5% for IgA. Using this antigen and combining IgA and IgG antibody detection, high test specificity was achieved (93.7 %) with a sensitivity of 67.5 %. The SL-I IgG test was found to be the most efficient for detecting smear-negative TB cases in their study. They also found out that the sera from TB patients reacted more strongly to glycolipids than to the antigens in the commercially available tests (Julian *et al*, 2002).

2.2.4. LAM in the diagnosis of tuberculosis:

LAM was purified in its native acylated state in 1989 by Hunter *et al* to study its potential in the serodiagnosis of mycobacterial disease. Sada *et al* for the

first time, studied anti-LAM antibodies in 31 cases of culture-proven pulmonary tuberculosis, 17 cases of miliary tuberculosis, 18 extra-pulmonary tuberculosis, 85 patients with non-tuberculosis pulmonary diseases and 32 healthy controls. The respective positive outcomes were 81 %, 82 %, 50 %, 11 % and 3% (Sada et al, 1990).

The serological response towards LAM among tuberculosis patients seems to vary with geographical location. The commercially available anti-LAM kit, Mycodot™ has a positive rate of 90 % in Italy (Del Prete *et al*, 1998), 52% in Thailand (Ratanasuwan *et al*, 1997), 44 % in Ghana (Lawn *et al*, 1997), 20 % in Tanzania (Somi *et al*, 1999), 63 % in Republic of Guinea-Bissau (Antunes *et al*, 2002), 79 % in Japan (Tsubura *et al*, 1997), 55.9 %-71.9 % in China (Gao and Tian, 1999) and 19 % in Spain (Julian *et al*, 1997).

Lower sensitivities were reported in newly diagnosed tuberculosis (Julian *et al*, 1997), in children (Demkov *et al*, 2006) and in HIV patients (Boggian *et al*, 1996). In the study of Boggian *et al* only 9/65 AIDS patients with tuberculosis were positive in the Mycodot™ assay. This is explained by the fact that, in AIDS patients the humoral response of IgG_2 subclass, the main humoral response to LAM, is affected. In HIV-negative patients, the sensitivity ranged from 21.5 to 89% (Lawn *et al*, 1997; Ratanasuwan *et al*, 1997; Del Prete *et al*, 1998) but was between 7% and 40% in HIV-positive patients. Lower sensitivity in children is also due to the impairment in the production of IgG and IgA production in them (Demkov, 2006). The positive cases were observed only in children aged above

10. In newly diagnosed tuberculosis, the delay in the production of IgG antibodies is responsible for the low antibody titer.

False positive results in anti-LAM antibody detection have been reported in histoplasmosis patients and hemodialysed patients (Yanai *et al*, 2006, Eleftheriadis *et al*, 2005). This may be due to latent tuberculosis infection in these patients or due to cross-reaction of serum antibodies. It was also found that the mean level of the IgG, IgA and IgM antibodies against LAM was higher in mycobacterial infection other than tuberculosis when compared to tuberculosis patients (Demkov *et al*, 2006). But, Da Costa *et al* found no anti-LAM antibodies in 76 controls, even in area highly endemic for tuberculosis, despite a 76 % prevalence of anti-LAM antibodies among the 74 tuberculosis patients involved in the study (Da Costa et al, 1993).

Apart from detecting in sera, antibodies to LAM antigen was detected in pleural fluid (Yokoyama *et al*, 2005; Demkow *et al*, 2004), pericardial fluid (Demkow *et al*, 2004), bronchio-alveolar lavage (Kozlowska *et al*, 2007) cerebrospinal fluid (Demkow *et al*, 2004, Xue *et al*, 1999, Chandramuki *et al*, 1989, Park *et al*, 1993) and urine (Tessema *et al*, 2002) also. In the study of Yokoyama et al, the sensitivity of 50 % and specificity of 93.8 % were obtained in pleural fluids. But Demkow et al found no significant differences in the anti LAM IgG titer in pleural fluids and pericardial fluids of tuberculosis and non-tuberculosis group. In broncheoalveolar lavage, the sensitivity was 33 % and the specificity was 93 %. But the study population was very small with only nine

patients having active tuberculous pleurisy, 1 patient with chronic tuberculous empyema, and 16 patients with nontuberculous pleural effusions in this study.

In the studies involving cerebrospinal fluid, the sensitivities ranged between 42-85.2 % and specificity ranged between 95.9-100%. In the study of Chandramuki *et al*, a sensitivity of 61% was achieved by using immunoglobulin G titers to lipoarabinomannan in CSF. In this study, seventy-four patients with tuberculous meningitis (26 culture-positive) were compared with 26 patients with purulent meningitis, 69 patients with suspected but excluded tuberculous meningitis, and 29 patients with other neurological diseases. Park *et al* proved that the reactivity was more frequently positive in the CSF than in the sera, suggesting a local synthesis of IgG in the central nervous system. He conducted ELISA for IgG activity in CSF and sera in 27 patients with tuberculous meningitis by LAM antigen. 29 patients with aseptic meningitis and 49 patients with non-inflammatory neurological illnesses served as controls. The sensitivity and the specificity were 85.2% and 95.9% respectively. In the study of Xue et al, the positive rate of LAM-IgG in 73 patients with tuberculous meningitis was 51%, 36 control cases were all negative making the specificity 100%. In these studies authors recommended the use of these tests in combination with other diagnostic tests for increasing the effective diagnosis of tuberculosis.

LAM antigen detection assay was attempted in urine samples of tuberculosis patients by Hamasur *et al* (2001). All 15 patients with active TB included in the study showed intermediate to high levels of LAM in their urine. Only one sample showed an absorbance value below the chosen cut off value of

0.4. All but one of the urine samples from 26 healthy nursing workers in the work exhibited optical density value below 0.4 cut off. In 2001, direct capture ELISA for LAM was performed on urine samples from 200 tuberculosis (TB) patients and 800 non-TB patients routinely diagnosed among consecutive suspects in an Ethiopian TB centre by Tessema *et al* (2001). The sensitivity and specificity of the LAM-ELISA for TB patients versus Ethiopian non-TB patients were 74% and 86.9%, respectively; the positive and negative predictive values were 58.5% and 93.0%. A direct antigen-capture ELISA based on the detection of mycobacterial LAM in unprocessed urine was evaluated by Boehme *et al* (2005). Of 132 patients with confirmed pulmonary mycobacterial disease (positive sputum culture), 106 were positive using the LAM-ELISA (sensitivity 80.3%). Of the 231 patients with suspected pulmonary tuberculosis, 17 were both culture- and AFB-negative, but had typical radiographic signs of pulmonary mycobacterial infection and did not respond to antibiotic treatment. Of these 17 patients, 13 (76.5%) had positive LAM-ELISA test results. To define the specificity of the assay, urine samples from 103 healthy volunteers were also screened using LAM-ELISA. All but one had an optical density below the cut-off (specificity 99%).

In 1992, Sada *et al* explained a co-agglutination test using LAM antigen with a specificity of 100% and sensitivity of 88% in smear positive active tuberculosis and 67% in smear negative active tuberculosis and 57 % in TB/AIDS co-infected patients. But later this result was questioned by Arya (1993). The utility of this antigen in our population is proved only after a thorough evaluation

as this antigen showed widely varying sensitivity and specificity in different populations

2.3. MYCOBACTERIAL LIPIDS- CORD FACTOR AND SULFOLIPIDS IN TUBERCULOSIS INFECTION:

Within the domain of mycobacterial lipids, persistent efforts have been dedicated to an avid search for substances that might be co-relatable with pathogenicity. The search led to the discovery of the very toxic glycolipid, designated "cord factor" whereas in a parallel effort Middlebrook *et al* discovered the sulfolipids of virulent *M. tuberculosis var. Hominis.*

2.3.1 Cord factor in pathogenesis of tuberculosis:

A study conducted 61 years ago by Middlebrook and collaborators recognized that the curious ability of the tubercle bacillus to grow in culture as serpentine cords was relatively restricted to virulent strains (Middlebrook *et al*, 1947). Bloch (1950) observed that virulent *M. tuberculosis* strains subjected to surface lipid extraction by petroleum ether treatment became avirulent and unable to form cords, but they retained their viability. This petroleum ether fraction was termed as cord factor. Subsequently, it was demonstrated that cord factor was trehalose 6, 6' dimycolate (TDM) (Noll *et al*, 1956). Four decades of research on TDM have uncovered a number of biological activities involved in the pathogenesis of mycobacterial disease. It is found to be the most toxic, most granulomagenic, and most abundant lipid extractable from the surface of *M.*

tuberculosis (Yano I, 1998). Cord factor from different strains of mycobacteria like *M. tuberculosis*, *M. kansasii*, and *M. bovis* in very less amounts (less than 5μg) amounts were shown to produce lung granuloma in mice (Bekierkunst 1968; Bekierkunst *et al*, 1969). Cord factor is also shown to induce macrophage activation (Yarkoni *et al*, 1977), induce thymic atrophy via apoptosis (Ozeki *et al*, 1997), inhibition of fusion between phospholipid vesicles (Spargo *et al*, 1991) and has anti-tumour (Bekierkunst *et al*, 1971; Bekierkunst *et al*, 1974) and adjuvant activity (Saito *et al*, 1976). Either active immunization with trehalose-6, 6'-dimycolate (cord factor)-methylated bovine serum albumin complex or passive transfer of rabbit anti-cord factor serum induced an enhanced resistance against infection with virulent human *Mycobacterium tuberculosis* in mice (Kato, 1972; Kato, 1973). The action of trehalose-6, 6'-dimycolate (cord factor) of *Mycobacterium tuberculosis* to induce inhibition of mitochondrial oxidative phosphorylation and to stimulate adenosine triphosphatase activity was neutralized by its specific antibody. The activity was restored after dissociation of cord factor from the immune complex. (Kato and Goren, 1974).

TDM contributes to macrophage activation and a cascade of events required for initiation of the infection. As neutrophils are the first immune cells recruited to the infection site soon after infection, the interaction of the mycobacterial components with the neutrophils is crucial in tuberculosis infection. Cord factor at specific concentration is found to attract neutrophils and macrophages in mice (Ofek and Bekierkunst, 1976). Mice treated with viable *M.*

tuberculosis with no glycolipid trehalose dimycolate (TDM) on the outer cell wall (delipidated *M. tuberculosis*) by intraperitoneal or intratracheal inoculation presented an intense recruitment of polymorphonuclear cells into the peritoneal cavity and an acute inflammatory reaction in the lungs, respectively (Lima et al, 2001). The subcutaneous, intradermal and pulmonary inflammatory lesions induced in mice by viable *Mycobacterium bovis* (BCG) with no glycolipid cord factor on the outer cell wall (delipidated BCG, dBCG) was of an acute nature with cells making up the inflammatory infiltrate exhibited polymorphonuclear-like (PMNs) morphologic characteristics. There was a decrease in delayed hypersensitivity response, and the lesion was resolved around the 16th day after the inoculation (Silva *et al*, 1985). From these data, it is understood that the cord factor is involved in attracting neutrophils to infection focus. However, the exact potential of cord factor in recruiting neutrophils to infection site is not yet clear. And also, the enhancing/inhibiting factors involved in the recruitment of neutrophils in the tuberculosis patients is an area of special concern. Hence, these areas will be addressed in this work.

2.3.2. Sulfolipid in pathogenensis of tuberculosis:

This family of molecules is composed of sulfated trehalose esters acylated with three to four acyl groups consisting of one short saturated fatty acid (palmitic acid or stearic acid) and different combinations of long-chain multiple methyl-branched fatty acids (the phthioceranic and hydroxyphthioceranic acids). Consistent with their suspected role in virulence, sulfolipids are found only in the

human pathogen *M. tuberculosis* (Goren, 1990). Interestingly, sulfolipids are present in the virulent laboratory strain *M. tuberculosis* $H_{37}Rv$ but absent from the avirulent strain *M. tuberculosis* $H_{37}Ra$ (Middlebrook *et al*, 1959).

In vitro, SL-I induced swelling and disruption of mitochondrial membranes and strongly inhibited mitochondrial oxidative phosphorylation (Kato and Goren, 1974). Goren and collaborators (Goren *et al*, 1976) showed that *M. tuberculosis* sulfolipids are capable of preventing phagosome-lysosome fusion in cultured macrophages and able to modulate the oxidative response and the cytokine secretion of human monocytes and neutrophils (Brozna et al, 1991; Pabst et al, 1988; Zhang et al, 1991; Zhang et al, 1988).

Sirakova et al (2001) constructed an isogenic sulfolipid-deficient mutant of M. tuberculosis $H_{37}Rv$. This mutant is unable to produce hepta- and octamethyl phthioceranic acids, the major acyl constituents of sulfolipids, and is thus unable to produce sulfolipids. Studies on this mutant provided evidences that show that sulfolipid is not responsible for cationic neutral red uptake (Andreu *et al*, 2004) and sulfolipid deficiency does not significantly affect the replication, persistence, and pathogenicity of *M. tuberculosis* $H_{37}Rv$ in mice and guinea pigs or in cultured macrophages (Rousseau *et al*, 2003).

The effect of sulfolipid on neutrophils and initiating mycobacterial infection has also been studied earlier. It is shown that sulfolipid I is taken up in significant amounts by human neutrophils and in lesser amounts by monocytes

and lymphocytes. Superoxide (O2-) production by neutrophils is also significantly increased by sulfolipid I (Zhang *et al*, 1988) and this needs extracellular calcium. As sulfolipid I is directly involved in regulating the action of neutrophils, it may also have a role in recruiting them to the infection, which will be studied by chemotaxis assay *in vitro*.

CHAPTER.3

MATERIALS AND METHODS

3.1 ISOLATION OF LIPID ANTIGENS FROM THE CELL WALL OF *MYCOBACTERIUM TUBERCULOSIS*

The cell wall lipid antigens isolated from *M. tuberculosis* in this study were total mycolic acid containing glycolipids designated as tuberculosis associated glycolipid (TBGL) antigen, cord factor (trehalose-6,6'-dimycolate), sulfolipid-I (2,3,6,6' tetraacyl α, α' trehalose 2' sulfate) and lipoarabinomannan. These antigens were selected for the study because of following reasons. Mycolic acid containing tuberculous glycolipid antigen contains more than one component in it and hence expected to elicit more than one type of antibody response in tuberculosis patients. As the primary aim of the study was to develop a diagnostic method for tuberculosis, these antibodies with differing specificities were expected to be more efficient and useful in screening tuberculosis patients.

Cord factor is selected for the study as it is present in majority of the mycobacterium species and thereby assumed to present a prominent immune response. Sulpholipid - I is the major sulpholipid, which is present only in the virulent strains of *M. tuberculosis*. The specificity of a diagnostic test relies upon the specificity of the antigen used. A specific immune response to tuberculosis infection is therefore expected for this antigen.

Lipoarabinomannan is a well-studied lipopolysaccharide found in the cell envelope of this pathogen. The antibody response to individual antigens was shown to be different in different population. Hence, evaluating their utility in the population under consideration is essential before establishing these tests in a

routine basis. The above-mentioned antigen was not studied earlier in our population for using as diagnostic adjunct and hence this antigen was also selected for the study.

3.1.1 Isolation of total mycolic acid containing tuberculous glycolipids or tuberculosis associated glycolipid (TBGL)

Tuberculous glycolipid antigen (TBGL) was isolated from *M. tuberculosis* $H_{37}R_a$ or $H_{37}R_v$ strain obtained from Tuberculosis Research Centre, Chennai.

- Culture medium (250-300 ml) in 1000 ml Roux culture bottle (with off-neck) was inoculated with 1 ml suspension containing viable *M. tuberculosis* bacilli from 10-14 days old 'seed-culture' on Lowenstein-Jensen medium. Following an optimum growth (which was attained after 4-5 months incubation at 37°C), the Roux bottles containing *M. tuberculosis* culture were autoclaved.

- The mycobacterial cells were collected by filtering through Whatman No:1 filter paper. The cells were washed twice with distilled water and again the cells were collected through filtration.

- The glycolipids were repeatedly extracted from the cells using chloroform: methanol (2:1 vol/vol) with constant stirring at 55° C.

- The organic extract was collected, concentrated by evaporating the chloroform and 500 ml acetone was added to it. The mixture was kept at –20°C for 12-16 hours to allow complete flocculation of the lipids.

- The precipitate was then centrifuged at 17,000g at 4°C for 30 minutes to recover the mycolate containing glycolipids.

- The precipitate was then air-dried, quantified and re-suspended in 1-2 ml of chloroform: methanol (3:1 vol/vol) for storage.

3.1.2 Isolation of cord factor (Trehalose 6, 6' dimycolate)

- After 4-5 months of growth in the Sauton's media, *M. tuberculosis* cells were extracted with chloroform: methanol (2:1) at 50° C and centrifuged at 15000 g for 30 minutes. The pellet was discarded and the supernatant was concentrated by allowing the chloroform to evaporate.
- Cord factor was isolated from the supernatant by Silica gel column chromatography, Silica gel flash column chromatography (for amounts more than 10 mg) or Silica gel thin layer chromatography (for smaller amounts). For Silica gel column chromatography, Silica gel (100-200 mesh) was washed twice with chloroform, dried and heated to 150°C in a hot air oven for activation. The silica was then mixed with chloroform and applied onto a chromatography column (18mm diameter) at a height of 6.5 cm. But separation of cord factor by column chromatography took 2 days to complete and hence flash column chromatography was adapted for further studies. For flash column chromatography, Silica gel (200-400 mesh) was washed two times with chloroform to remove contaminants. After drying, it was activated by heating at 120°C for 1 hour. The column height was always approximately 6.5 cm.

- The extracted glycolipid was dissolved in chloroform and applied onto the column.
- Different fractions were eluted in a step-gradient fashion with chloroform and chloroform containing 5%, 10%, 15%, 20% and 30% methanol. The top-pressure was applied using an aqua pump.
- The various fractions were then analyzed by thin layer chromatography on pre-coated Silica gel plates (Merck, Germany). The solvent system used was chloroform: methanol: ammonium hydroxide (80:20:2).
- The plates were dried and stained using Iodine vapors. As it is a temporary stain, the same lipid material could be scraped out for further studies.
- In order to assess the purity of cord factor isolated using the above methods, a reference cord factor obtained from Colorado State University was also run simultaneously on TLC plates.

Thin layer chromatography was also used for small scale preparation of cord factor (less than 20 mg). For the separation of cord factor on thin layers of silica gel, the solvent system chloroform: methanol: ammonium hydroxide (80:20:2) was used.

3.1.3 Isolation of sulfolipid - I

Sulfolipid – I was isolated from the $H_{37}Rv$ strain of *M. tuberculosis*. The method employed was as described by Gangadharam *et al* (1963) with appropriate modification.

- After 4 weeks of growth in Sauton's medium, the cells were harvested by filtration after autoclaving the cells and the cells were allowed to dry at 37°C.
- To this dried cells, 0.1 % of decylamine in n-hexane (4ml/g of cells) was added and shaken for 20 minutes. Then it was centrifuged, the supernatant was collected and dried in a stream of nitrogen. The pellet was re-extracted three more times with 0.1 % of decylamine in n-hexane and the above steps were repeated.
- The dried extract was re-dissolved in pure hexane and 20 ml of 2 N citric acid was added to it. It was shaken vigorously and centrifuged at 2000rpm for 10 minutes. The hexane layer was collected and the process was repeated. Total hexane soluble fractions were pooled and dried in a stream of nitrogen.
- This crude extract was dissolved in 50 ml of hot ethanol and shaken vigorously for 2 minutes. It was then cooled to -20°C and maintained at same temperature for 3 hours with occasional shaking. Then it was centrifuged at 2000 rpm for 30 minutes at 4°C.
- Supernatant was collected, dried and run on Silica gel TLC plates. Then, the separated sulfolipid-I was scraped out from the plates, re-chromtographed, eluted using n-hexane, dried and quantitated.

3.1.4 Isolation of Lipoarabinomannan (LAM)

- The *M. tuberculosis* cells were harvested from Sauton's medium and cells were extracted with 10 ml of chloroform: methanol: water (10:10:3) for 15 min at 55°C. Cellular materials were pelleted by centrifugation at 750 g for 30 min.
- The cellular pellet was then extracted two times with 5ml phenol and 5 ml of water for 30 min at 70°C with constant stirring. The phenol: water extract was cooled and centrifuged for 30 min at 750 g.
- The aqueous layer containing LAM was separated from the phenol phase and dialyzed against distilled water with a membrane having a 2500 molecular weight cut off until all the phenol is exchanged.
- LAM was quantified by phenol-sulfuric acid assay (Slayden and Barry, 2001). 200 µl of sample was added with 1 ml sulphuric acid and 200µl of 5% phenol and allowed to react for 10 minutes. Dextran was used to generate a linear calibration curve from 10µg to 50 µg. Absorbance was read at 490 nm.

3.2 DETECTION OF SECRETED LIPIDS

M. tuberculosis contains an abundance of lipids in their cell wall. So there could be a possibility of these lipids being secreted to the surrounding medium. In such a case, those secreted lipids will be an excellent candidate to be used in the serodiagnosis of tuberculosis. In an attempt to check the presence of any secretory

lipids in the culture medium, after 8-10 week of growth of $H_{37}Rv$ strain of *M. tuberculosis* in Sauton's medium, the cells were filtered off using Whatmann No: 1 filter paper. The culture medium was mixed with double volume of chloroform – methanol (2:1) for the extraction of glycolipids. The organic phase was collected and mixed with cold acetone kept at -20°C overnight. This mixture was centrifuged at 17000 g at 4°C for 20 minutes. The pellets were washed twice with acetone and thin layer chromatography was done to detect lipids in the medium. No positive staining pattern was observed by iodine vapour staining method which can be either due to the absence or insufficient quantity of secreted lipids in the medium.

3.3 CHARACTERIZATION OF LIPID ANTIGENS

All lipid antigens were characterized mainly for their immunochemical properties. TBGL antigen, cord factor and sulpholipid-I were analysed by thin layer chromatograpy and lipoarabinomannan was analysed by SDS-PAGE to view the different components in the preparations and to assess their purity. The purified antigens were then used in ELISA for the diagnosis of pulmonary tuberculosis and pleural tuberculosis. Chemoattractant nature of cord factor and sulfolipids for polymorphonuclear leukocytes were also investigated. These are explained in detail in the following sections.

3.3.1 Analysis of the purity of lipid antigen preparations

3.3.1.1 Analysis of TBGL antigen by thin layer chromatography:

- The air-dried TBGL antigen was dissolved in n-hexane (2mg/ml) and was spotted on thin layer chromatographic plates (Merck, Germany) and allowed to run for 3 hours using the solvent system chloroform: methanol: water (90:10:1).
- Then, the plates were air-dried and stained using iodine vapors.

Three different fractions were observed and their corresponding Rf values (distance traveled by the solute/distance traveled by the solvent) were calculated. The average Rf values of the three fractions were 0.30, 0.46 and 0.63. By comparing the Rf values of the known mycobacterial fractions in the literature (Baba *et al*, 1997), they were identified as cardiolipin, trehalose monomycolate, and cord factor (trehalose dimycolate) respectively.

3.3.1.2 Analysis of cord factor antigen by thin layer chromatography:

- In order to assess the purity of cord factor isolated in our laboratory, a reference cord factor sample procured from Colorado State University, USA was also run along with the isolated cord factor on silica gel TLC plates using the solvent system chloroform: methanol: ammonium hydroxide (80:20:2).
- The chromatographic plates were dried and stained with iodine vapour.

3.3.1.3 Analysis of SL-I antigen by thin layer chromatography:

- The sulfolipid-I antigen scraped out from the thin layer plates was allowed to dissolve in n-hexane.
- It was re-chromatographed on a silica gel thin layer plate using the solvent system chloroform: methanol: acetic acid: water (95:1:5:0.3).
- The plates were stained with iodine vapour and observed for the single band of isolated sulfolipid-I.

3.3.1.4 Analysis of LAM antigen by SDS-PAGE:

- The extracted LAM was analyzed by SDS-PAGE with 12.5% gel along with a reference sample procured from the Colorado state university, USA.
- The gel was fixed with 50% methanol and 10% acetic acid, washed with 10% methanol and 5% acetic acid, treated with potassium dichromate solution with shaking and washed with distilled water until the gel became colorless.
- The gel was stained with silver nitrate staining solution (0.5mg/ml) and developed by sodium carbonate solution containing 200µl of formaldehyde.

3.3.2 Analysis of the utility of lipid antigens for the diagnosis of pulmonary tuberculosis by serum indirect ELISAs.

3.3.2.1 Study population and selection criteria:

For this study, 102 patients with pulmonary tuberculosis (admitted to the Division of Respiratory Medicine, Pulayanarkotta) were included. In order to assess the specificity of the assay, patients with respiratory ailments other than tuberculosis like bronchial carcinoma and non-tuberculosis pneumonia (n=61) (admitted either to Pulayanarkotta Hospital or to SCTIMST) were taken as negative control. Twenty healthy blood donors (from the Blood bank, SCTIMST) were considered as healthy controls. Clinical details, sputum examination and the response of the patients to the given anti-tuberculosis therapy of all the patients were considered for the positive selection of patients in the pulmonary tuberculosis and "diseased" control group (Table: 3.1). 5 ml blood was collected from all the three groups, sera separated and aliquots were stored at -20° C until use.

Table 3.1: Demographic and clinical data of pulmonary tuberculous patients and controls

Patient group	No: of cases	Sex (men: women)	Age (yr) Mean ± SD
Pulmonary TB patients	102	84:18	50.049 ± 13.897
Smear/culture positive	40	36:4	45.175 ± 13.928
Smear/culture negative	62	48:14	53.194 ± 13.043
Diseased control	41	33:8	47.439 ± 12.62
Healthy control	20	20:0	33.050 ± 7.529

3.3.2.2 Estimation of anti-TBGL antibody in sera by ELISA

- 1000ng TBGL antigen in 100 µl of n- hexane was coated in the wells of ELISA microtiter plates (Dynatech laboratories; Alexandria Va).
- After 2 hours of incubation, the plates were washed with phosphate buffered Saline (pH-7.4). Tween 20 that was commonly used in the wash buffer was excluded from the washing solution as this was proved to remove the lipids from the microtiter plates (Julian *et al*, 2001).
- The plates were then blocked with 200 µl of Bovine serum albumin (1 g %) in phosphate buffered saline for 1 hour.
- The patient's sera was diluted from 1:100 to 1: 12800 in BSA in PBS and incubated overnight at 4°C.

- Next day, the plates were repeatedly washed with PBS and anti-IgG alkaline phosphatase conjugate (100µl, 1: 1000 dilution) was added and incubated for 2 hours.
- Then the substrate, Para-nitro phenyl phosphate (1 mg/ml in Diethanolamine buffer) was added and the color reaction was stopped after 15 minutes by the addition of 25µl of 3N NaOH.
- The absorbances were measured at 415 nm.

Based on the standardization ELISA, a cut-off value was selected at 0.567 (Mean absorbance value of the overall control sera + 2 Standard deviation) at 1:3200 dilution as described earlier (Ahmad *et al*, 1995).

3.3.2.3 Estimation of anti-cord factor antibody in serum samples by indirect ELISA

IgG antibody titer against cord factor antigen was determined by an indirect ELISA as described earlier (Julian *et al,* 2001).

- Following incubation (2 hr) with antigen (1000ng/well), the polystyrene microtiter ELISA plates (Dynatech laboratories; Alexandria Va) were washed with PBS and quenched with BSA in PBS (1g%, 1 hr).
- 100µl of diluted patient serum was added to each well and incubated overnight at 4˚C.
- Then, 100µl of anti-human IgG- Alkaline phosphatase was added (1 hour, 1:1000 dilution)

- The colour reaction was developed by the addition of para-nitrophenyl phosphate (1mg/ml in DEA buffer).
- The reaction was stopped by 3 N NaOH and the absorbance was measured at 415 nanometer using an ELISA reader (Bio-Rad Model 550, USA). The cut-off was selected at 0.565 at 1:1600 dilution, based on the standardization ELISA.

3.3.2.4 Estimation of anti-sulfolipid-I antibody in serum samples by indirect ELISA

- 1000ng SL-I antigen in n-hexane (100 μl) was coated in the wells of ELISA microtiter plates. After 2 hours of incubation, the plates were thoroughly washed with phosphate buffered solution.
- The plates were then blocked with 200 μl of BSA (1 g %) in phosphate buffered saline for 1 hour.
- Sera were diluted to 1:1600 in BSA in PBS and incubated overnight at 4°C.
- The plates were then washed with PBS, anti-IgG alkaline phosphatase conjugate (100μl, 1: 1000 dilution) was added and incubated for 2 hours.
- Then, the substrate, Paranitro phenyl phosphate was added and the color reaction was stopped after 15 minutes by the addition of 25μl of 3N NaOH.
- The absorbances were measured at 415 nm.

A cut-off point was selected at 0.51 units (mean absorbance of the diseased control+ 2 x SD) at 1:1600 dilution.

3.3.2.5 Estimation of anti-LAM antibody in serum samples by indirect ELISA

- 2 μg LAM in 100 μl carbonate - bicarbonate buffer was coated in polystyrene microtiter plates (2 hours).
- After three washes with phosphate-buffered saline containing 2% Tween-20, the plates were blocked for 1 hour at room temperature with 200 μl of 1% Bovine serum albumin in PBS.
- The 100 μl of the test sera diluted to 1:3200 in 1% BSA was incubated overnight at 4°C.
- After thorough washing anti-human IgG-alkaline phosphatase conjugate was added (100μl, 1:1000).
- After 2 hours, the wells were again washed and the substrate Para nitro phenyl phosphate was added (1mg/ml in DEA buffer) and allowed to react for 15 minutes.
- The reaction was stopped by 3N NaOH (25μl) and the absorbance was read at 415 nm.

Based on the standardization ELISA a cut off value was selected at 0.562 units at 1:3200 dilution.

3.3.2.6 Statistical analysis

Statistical analyses were performed using conventional methods. Student's t test was performed and 'P' value was measured using Microsoft excel. The sensitivity, specificity, positive predictive value, negative predictive value, positive diagnostic likelihood ratio and negative likely hood ratio of each assay were calculated by the following formulae.

$$\text{Sensitivity} = \frac{\text{True positives}}{\text{True positives} + \text{False negatives}}$$

$$\text{Specificity} = \frac{\text{True negatives}}{\text{False positives} + \text{True negatives}}$$

$$\text{Positive predictive value} = \frac{\text{True positives}}{\text{True positives} + \text{False positives}}$$

$$\text{Negative predictive value} = \frac{\text{True negatives}}{\text{False negatives} + \text{True negatives}}$$

Positive diagnostic likelihood ratio =

$$\frac{\text{True positives/ (True positives + False negatives)}}{\text{False positives/ (False positives + True negatives)}}$$

Negative diagnostic likelihood ratio =

$$\frac{\text{False negatives/ (True positives + False negatives)}}{\text{True negatives/ (False positives + True negative)}}$$

3.3.3 Analysis of the utility of lipid antigens for the diagnosis of pleural tuberculosis by pleural fluid indirect ELISAs

3.3.3.1 Study population and selection criteria:

In this study, 140 patients with clinical and radiological evidences of pleural effusion were selected over a period of two years (October 2004 - December 2006). The patients were admitted to the Hospital for Tuberculosis and Chest diseases, Pulayanarkotta; Thiruvananthapuram (n =129) and at Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram (n =11). There were 93 male and 47 female patients and age of the patients ranged from 20 to 82 years. Relevant clinical and radiological features were recorded from the case records of these patients. The most common clinical symptoms were productive cough, breathlessness, chest pain and pyrexia of more than 4

weeks duration. Constitutional symptoms such as loss of appetite, reduction in body weight and general malaise were also recorded in majority of these patients.

At the time of admission, 106/140 patients showed positive intra-dermal tuberculin test (>12 mm) and elevated erythrocyte sedimentation rates (45-120 mm). The plain skiagrams of the thorax were suggestive of pleural effusion of varying degree and the underlying lung lesions in the skiagrams were considerably masked due to the effusion. In 114 patients, the pleural effusion was unilateral and 26 patients had bilateral effusions. Radiological features of the thorax alone could not define the precise aetiology of pleural effusion necessitating further analysis of pleural fluid in the 140 patients.

10 ml pleural fluid was collected from each patient during thoracocentesis and they were simultaneously subjected to cytological, bacteriological and immunological investigations. For cytological studies, a Cytospin (Wescor, model No: 7620 cytocentrifuge; Canada) was used. Four smears from each sample were fixed in cold methanol. For ELISA, the pleural fluid specimens were centrifuged at 2000 rpm for 10 minutes and the supernatants were stored at –20°C until used.

Pleural fluids could be broadly classified into 2 groups based on the cytologic features in hematoxylin and eosin stained smears. A) Sixty-nine pleural fluids were inflammatory in nature, predominantly composed of lymphocytes. Five of them showed an admixture of macrophages with lymphocytes. B) Seventy-one cytospin smears showed the presence of malignant cells.

Pleural fluid and sputum specimens were subjected to conventional bacteriologic methods (ZN staining and cultures on Lowenstein–Jensen medium).

M. tuberculosis was not demonstrated in any one of the 71 patients with malignant pleural effusion. *M. tuberculosis* was isolated by cultures in 11 pleural specimens of which 3 specimens were AFB positive in ZN-stained smears of pleural fluids.

Based on the results of the above, as well as radiological and clinical findings, we classified the patients into 3 groups: a) Seventy-one pleural fluid specimens showed the presence of malignant cells in the pleural fluid and bacteriologic studies in them did not demonstrate *M. tuberculosis*. They were classified as malignant pleural effusions ("disease" control) in conjunction with their clinical features. b) The bacteriologic studies revealed the presence of *M. tuberculosis* in 11 pleural fluid specimens, and hence, they were regarded as "confirmed" patients with pleural tuberculosis c) The bacteriologic cultures in the 58 pleural fluid specimens, on 3 occasions, did not yield *M. tuberculosis* or any other pyogenic bacteria. However, the radiological and clinical features in these patients were suggestive of tuberculosis, and these patients were given a course of ATT. They showed optimal clinical recovery. Hence, these 58 patients were classified as "probable" patients with PTB. The cases, which were suspicious (clinically) to be having a coexistence of TB and malignancy, were not included in this study. Pleural effusions doubted of other inflammatory causes were also not included in this study because of lack of proper clinical evidence.

3.3.3.2 Estimation of anti-TBGL antibody in pleural fluids by indirect ELISA:

IgG antibody titer was determined in all pleural fluid samples as described previously for that of serum samples.

- The purified TBGL antigen (1000ng in 100µl *n*-hexane/ well for 2 hours) was coated on the wells of ELISA plates.
- The plates were quenched with 1 g % bovine serum albumin (BSA) in PBS.
- After emptying the plates, the pleural fluid samples from patients with malignant pleural effusion and tuberculous pleural effusion were diluted in 1% BSA in PBS and 100 µl (1:3200 dilution) was added to each wells and incubated overnight at 4°C.
- The plates were then washed thoroughly and incubated for 2 hours with 100µl of anti-human IgG-alkaline phosphatase conjugate (1:1000).
- The colour reaction was developed by the addition of a substrate containing para-nitrophenyl phosphate (1mg/ml in DEA buffer) and the plates were incubated for 15 minutes.
- 25 µl of 3N sodium hydroxide was added to the wells in the microtiter plate to stop the reaction.

Whenever ELISA was performed in a batch of pleural fluids, we used a positive control from a culture-proven pleural fluid from a patient with pleural tuberculosis. The absorbance was read at 415 nm. The absorbance in the pleural

fluids of tuberculous and non-tuberculous groups was recorded. In order to eliminate bias and inter-observer variation, the assay was initially done without the knowledge of clinical data. The reproducibility of the assay was evaluated by repeating the assay using the same samples on two different occasions.

3.3.3.3 Estimation of anti-cord factor antibody titer in pleural fluids by indirect ELISA:

- Anti-cord factor antibody titer was determined in all the pleural fluids using the protocol described for determining the titer in serum samples (section 3.3.2.3). A cut-off value was selected at 0.619 units (mean absorbance of the overall control group + 2 x SD) at 1:1600 dilution based on the data provided by the standardization ELISA.

3.3.3.4 Estimation of anti-SL-I antibody in pleural fluids by indirect ELISA:

- Anti-SL-I antibody titer was determined in all the pleural fluids using the protocol described for determining the antibody titer in serum samples (section 3.3.2.4). A cut-off value was selected at 0.598 units (mean absorbance of the overall control group + 2 x SD) at 1:1600 dilution based on the results of standardization ELISA.

3.3.3.5 Estimation of anti-LAM antibody in pleural fluids by indirect ELISA:

- Anti-LAM antibody titer was determined in all the pleural fluids using the protocol described for determining the antibody titer in serum samples (section 3.3.2.5). A cut-off value was selected at 0.576 units (mean absorbance of the overall control group + 2 x SD) at 1:3200 dilution based on the data provided by the standardization ELISA.

3.3.3.6 Comparison of antibody titer in pleural fluids and sera of patients with tuberculous pleural effusion

In order to find out whether detecting antibodies in pleural fluids of the patients with pleural tuberculosis is of any significant value in the diagnosis, serum samples and pleural fluid samples were collected from 14 patients with tuberculous pleural effusion and six patients with malignant pleural effusion. Indirect ELISA for the detection of anti-TBGL antibody was performed in both the samples of all the patients as described in section 3.3.2.2. and 3.3.3.2 and the results were compared.

3.3.3.7 Modified Indirect ELISA for the estimation of TBGL antigen in pleural fluid specimens:

3.3.3.7.1 Antibody to TBGL antigen:

- To raise immune sera, two albino rabbits were inoculated with TBGL antigen [2 mg in 1ml phosphate-buffered-saline (PBS) and 1 ml incomplete Freund's adjuvant] in two intra-muscular sites.
- The immunization was repeated on the 14th, 21st, 28th and 35th day and the antibody titers to TBGL antigen in the immune sera were sequentially assessed by indirect ELISA (Engvall and Perlmann, 1972). An end-point anti-TBGL antibody titer (1:24,800) was attained following the fifth immunization.
- Gammaglobulin fraction in the immune sera was prepared by the addition of 33 % ammonium sulphate followed by continuous dialysis against 0.05 M PBS (pH 7.4) and protein concentration was calculated by Lowry's method (Lowry *et al*, 1951).

3.3.3.7.2 ELISA for the estimation of glycolipid antigens of M. tuberculosis (TBGL):

ELISA was initially standardized with descending concentrations of TBGL antigen (500 ng to 15.6 ng in 100 µl n-hexane/well).

- Following incubation (2 hrs) with TBGL antigen, the polystyrene microtiter plates (Dynatech laboratories; Alexandria Va) were washed and quenched with BSA in PBS (1g%, 1 hr).

- 100μl of rabbit anti-TBGL antibody (1: 20,000 dilution) was added and incubated overnight at 4°C.
- Then, 100μl of anti-rabbit IgG-biotin conjugate (1:5000, 2 hrs) followed by Streptavidin- Alkaline phosphatase (1:1600, 1hr) was added and the colour reaction was developed by the addition of para-nitrophenyl phosphate (1mg/ml in DEA buffer).
- After 15 minutes, 25 μl of 3N sodium hydroxide was added to the wells in the microtiter plate to stop the reaction.
- The absorbance was measured at 415 nanometer using ELISA reader (Bio-Rad, USA).
- The absorbances were plotted against antigen concentrations and a linear graph was obtained.
- Pleural effusions from patients with PTB and control groups were serially diluted, similarly assayed and the antigen concentrations were directly measured from the linear standard graph.

To ascertain the reproducibility as well as inter-observer variation, the assay was repeated with the same samples.

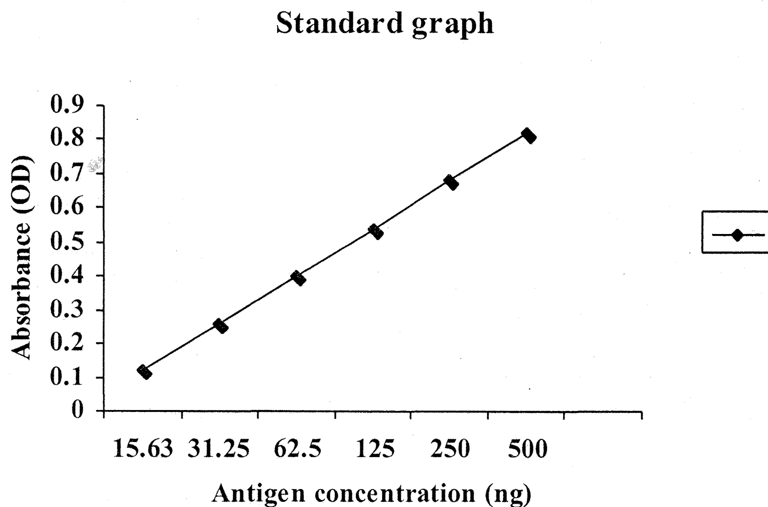


Fig 3.1 Standard linear graph obtained by plotting absorbance (optical density) against TBGL antigen concentration

Statistical analyses were done for all the assays as described in section 3.3.2.6.

3.3.4 Chemotactic response of polymorphonuclear cells towards cord factor and sulfolipid-I antigens of *M. tuberculosis*

3.3.4.1 Polymorphomuclear leukocyte isolation:

10 ml venous blood was collected from healthy human donors and Polymorphonuclear leukocytes (PMNs) were isolated from the blood as described below.

- Blood was collected in a tube containing 2.8 ml Acid Citrate Dextrose (ACD) and 8.5 ml dextran (6g% in PBS) and it was allowed to stand for 2

hours at room temperature for sedimentation of red blood cells (RBC) to take place.

- Then, PMNs were separated by density gradient centrifugation with Ficoll-Hypaque solution. Leukocyte rich plasma was layered over the Ficoll-hypaque solution and centrifuged at 1500 rpm at 4°C.
- The separated PMNs were washed thrice in Hank's balanced salt solution (HBSS) solution and the RBCs if present were lysed by treating it with 2 ml cold distilled water (1 minute) and 660 µl of ice-cold 0.6 M NaCl. The PMNs thus obtained was washed thrice with fresh HBSS solution and re-suspended in 1ml of fresh HBSS solution.
- The yield was assessed by a Neubar's counting chamber and the viability was assessed by trypan blue exclusion technique. Cell viability was always found to be 97 –98 %.
- Finally, the cells were resuspended in 1ml RPMI medium in HBSS containing10% fetal calf serum (FCS).

3.3.4.2 Preparation of cord factor emulsion:

- Cord factor and sulfolipid antigens at different concentrations (5-50µg/ml) in n-hexane were added to a 5-ml sterile beaker (which was finally used as the lower compartment of the chemotaxis chamber) and allowed to dry. Then, 3 ml of a solution containing RPMI medium (0.208g/20 ml) in HBSS (18 ml) and Tween 80 (0.4µl) was added and thoroughly mixed

with the lipid antigens. The final concentration of Tween 80 in the solution was 0.002%.

- Finally, fetal calf serum (FCS, 2 ml) was added and mixed properly.

3.3.4.3 *In vitro* Chemotaxis assay:

A modified form of Boyden's chamber was used to study *in vitro* chemotactic response of neutrophils towards the lipid antigens. The upper compartment was developed from an eppendorf rack and the lower compartment used was a 5-ml glass beaker (on which the emulsions have been prepared). A membrane filter of 3-µm pore size (Millipore, India) was then attached onto the edges of the upper compartment so that the cells were allowed to migrate to the lower compartment only through this membrane (fig: 3.2).

- In all experiments, the upper compartment was added with 5×10^5 neutrophils/150 µl of the solution containing RPMI medium in HBSS and 10% fetal calf serum. The lower chamber was added with different concentration of cord factor (from 5 µg –50 µg/ml) in HBSS containing FCS, RPMI medium and 0.002% Tween 80. In yet another set, SL-I at different concentrations from 5 µg – 50 µg/ml was taken. As a positive control, in one lower compartment, a known, chemoattractant casein (500 µg/ml) was added and as a negative control, a set with no chemoattract was also taken.

- The chambers were then incubated at 37°C for 2 hours. Cells were allowed to migrate into the Millipore membrane separating the two compartments of the chamber.
- After 2 hours, the filters were carefully taken out, wiped and immediately fixed in 100% ethanol in cold.

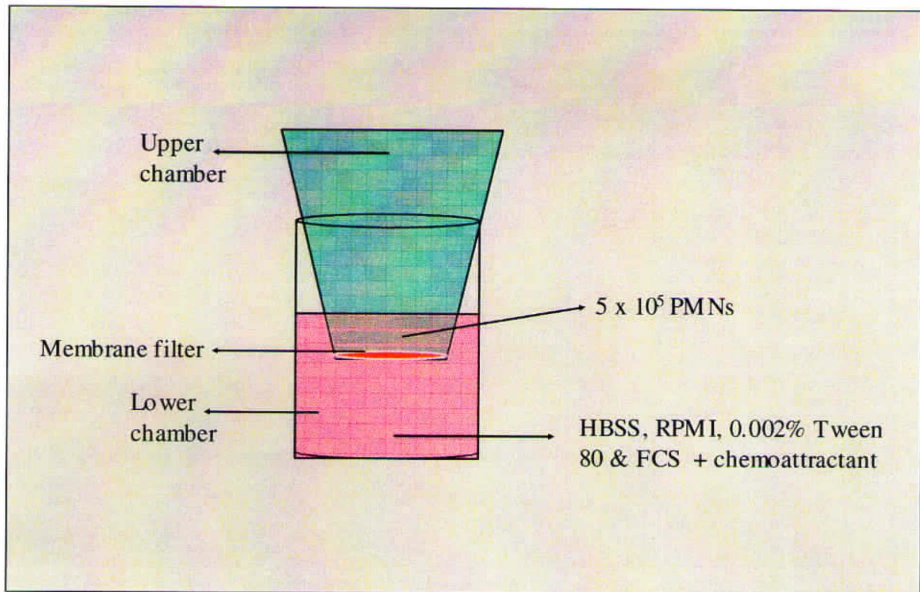


Fig 3.2. Diagram of the chemotaxis chamber used in the study.

3.3.4.4 Staining of filters:

Fixed filters were then treated as follows:

90 % ethanol 1-3 dip

70% ethanol 1-3 dip

50% ethanol 1-3 dip

Distilled water	I minute
Hematoxylin stain	0.5 sec – 1 minute
Distilled water	1 minute
Tap water	10 minutes
70% propanol	2 minutes
95 % propanol	3 minutes
100 % propanol	5 minutes
Xylene	10 minutes (for making the membrane filter transparent)

The filters were then fixed to microscopic slides with a drop of DPX mountant and viewed under a microscope. Cell migration was assesses by lower surface count method. The number of cells that reached the lower surface of the filter after 2 hours time interval is counted. The migrated cells in 10 different microscopic fields were counted with a 10x ocular and a 40 X objective and the average number of cells per microscopic field were calculated.

3.3.4.5 Inhibition of PMN migration in the presence of serum/pleural fluid samples from tuberculosis patients:

In order to find out whether any factor in the pleural fluid/serum samples of tuberculosis patients affects the chemotactic response of polymorphonuclear nucleocytes to cord factor/SL-I, 100 µl and 400 µl of pleural fluid/serum samples from tuberculosis patients were added along with the cord factor/SL-I/Casein in the lower compartment in HBSS solution containing 0.002% Tween 80, RPMI medium and 10 % fetal calf serum. As a negative control, 100 µl and 400 µl of

pleural fluid/serum samples from lung cancer patients were added to the lower compartment. A blank containing no chemoattractant and a control containing no pleural fluid were also set up. A cell suspension containing $5x10^5$ cells was added to the upper compartment of the chamber and the chamber was incubated at 37°C for 2 hours. The 3 µm pore sized membrane filter between the two compartments were taken out and treated as described earlier in section 3.3.4.4. The number of migrating PMNs in 10 different microscopic fields was counted with a 10x ocular and a 40 X objective and the average number of cells per microscopic field were calculated.

CHAPTER.4

RESULTS & DISCUSSION

4.1 ISOLATION OF LIPID ANTIGENS FROM THE CELL-WALL OF *MYCOBACTERIUM TUBERCULOSIS*

4.1.1 Isolation of tuberculosis associated glycolipid (TBGL) antigen:

In this study, major mycolic acid containing lipids (tuberculosis associated glycolipid, TBGL) were extracted by chloroform: methanol extraction. Acetone-insoluble fraction was then separated from the extract and analyzed by thin-layer chromatography. The chromatogram showed mainly three bands (fig: 4.1). The average Rf values of the three fractions were 0.30, 0.46 and 0.63. By analyzing the Rf values of the different components on similar preparations, they were identified as cardiolipin, trehalose monomycolate and cord factor (trehalose dimycolate) respectively (Baba *et al*, 1997).

4.1.2 Isolation of Cord factor antigen:

Isolation of cord factor antigen in adequate quantities was cumbersome by normal column chromatography. We obtained the procedure used in the laboratory of the Colorado State University and according to that; certain modifications were introduced in the extraction protocol. The extraction was done at 55° C and this yielded more amount of the antigen. The separation of antigen was easy in thin layer chromatography though it yielded very less quantity of

purified antigen. For large scale preparation, column chromatography was employed but it took very long time (2 days) for eluting out the cord factor fraction. The application of pressure by introducing flash column chromatography increased the yield as well as reduced the time needed for the separation. The antigen obtained gave a single band corresponding to the reference cord factor sample obtained from the Colorado State University (fig: 4.2).

4.1.3 Isolation of Sulfolipid-I antigen:

Sulfolipid antigen was prepared as described by Gangadharam *et al* (1963) with some modifications. The method yielded substantial amount of purified antigen and when re-chromatographed, the purified antigen yielded single band on thin layer chromatography showing absolute purity (Fig 4.3).

4.1.4 Isolation of Lipoarabinomannan (LAM) antigen:

LAM antigen was prepared as described by Slayden and Barry (2001). The purified LAM showed a diffused band on SDS-PAGE corresponding to the band obtained for the reference LAM from Colorado State University (Fig: 4.4).

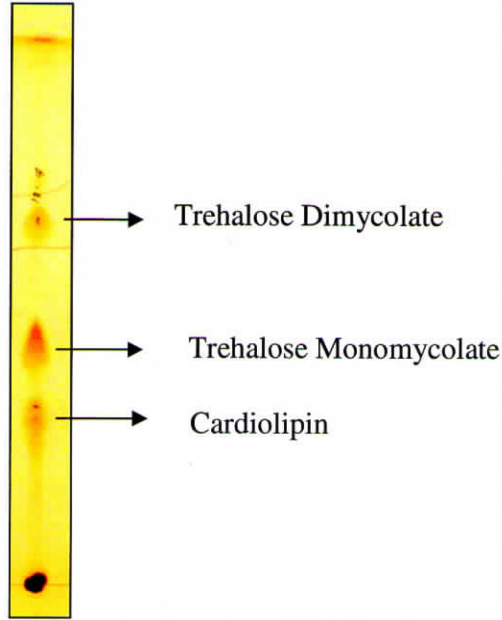


Fig: 4.1 Thin-layer chromatogram showing different components of TBGL antigen

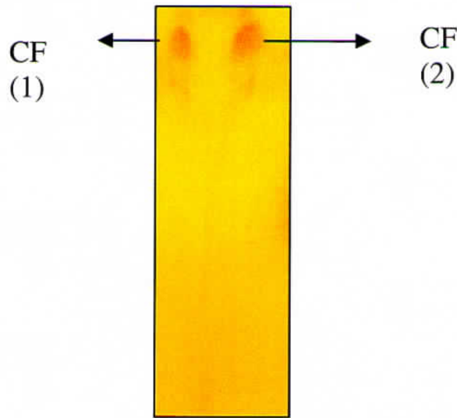


Fig: 4.2 Thin layer chromatogram showing cord factor
lane 1: Cord factor isolated from *M tuberculosis* $H_{37}Rv$,
Lane 2: Cord factor procured from CSU, USA

→ SL-I

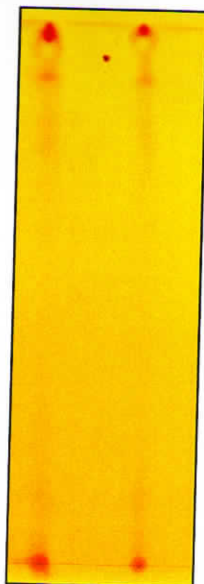


Fig: 4.3 Thin layer chromatogram showing single band of purified SL-I

Fig: 4.3 Thin layer chromatogram showing single band of purified SL-I

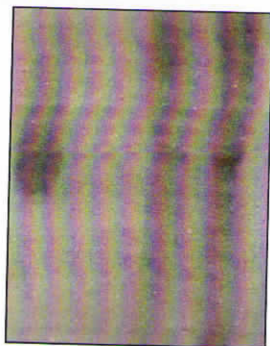


Fig: 4.4 Poly acrylamide gel showing Lipoarabinomannan Lane 1: reference lipoarabinomannan from Colorado State University, USA, lane 2&3: Lipoarabinomannan isolated from $H_{37}Ra$ strain of *M tuberculsosis*

4.2 SERUM INDIRECT ELISAs USING MYCOBACTERIAL LIPID ANTIGENS FOR THE DIAGNOSIS OF PULMONARY TUBERCULOSIS

In tuberculosis patients, the mean values of the absorbance of IgG antibodies to each of the four glycolipid antigens tested were significantly elevated (P < 0.001) above those of the overall control sera samples. For all the four assays, higher test sensitivities were obtained in smear/culture positive tuberculosis patients when compared to smear/culture negative tuberculosis patients. No significant differences were observed between the mean values of the healthy controls and diseased controls within each test.

4.2.1 Result of indirect ELISA for detection of anti - TBGL antibody in sera:

Standardization ELISA with TBGL antigen showed maximum discrimination between controls and tuberculosis patients at 1:3200 dilution and hence this dilution was used in the study. In tuberculosis patients, the mean absorbance value of IgG antibodies to TBGL antigen were significantly elevated (P<0.001) than the healthy control and diseased control sera. The absorbance value ranged between 0.30-0.797 units for smear/culture positive tuberculosis sera and 0.241-0.744 units for smear negative tuberculosis sera. Diseased control group showed a slight elevation in the mean antibody titer than the healthy control group but the difference was not significant (P>0.05). In healthy and diseased control group, the absorbance ranged between 0.21-0.386 units and 0.216-0.575

units respectively. The normal range of the antibody titer among tuberculosis patients was determined to be lower than the 2 SDs above the mean of the absorbances in the overall control group. Hence, cut-off value was selected at 0.567 (Mean absorbance value of the overall control sera + 2 Standard deviation) as described by Ahmad *et al* (1995). The absorbance values above this cut-off were considered positive.

The mean absorbance values of the anti-TBGL antibody titer in each group are summarized in Table: 4.1. The overall sensitivity of the anti-TBGL antibody titer detection in tuberculosis serodiagnosis was 83.33 % (85/102) and the specificity was 93.44 %. In smear/culture negative tuberculosis patients, the sensitivity was 79 %. The positive predictive value and the negative predictive values of the assay were 95.5 % and 77 % respectively. The positive diagnostic likelihood ratio and negative diagnostic likelihood ratios were 12.72 and 0.2044 respectively.

TABLE : 4.1 Results of the indirect ELISA for the determination of IgG antibody titer to TBGL antigen in sera samples

Patient group and diagnostic results	**No: of cases**	**% positivity to TBGL antigen**		**Mean antibody titer (units)**
		n	**%**	
Pulmonary TB patients	102	85	83.33	0.605 ± 0.157
Smear/culture positive	40	36	90	0.625 ± 0.148
Smear/culture negative	62	49	79	0.593 ± 0.070
Diseased control	41	4	9.8	0.378 ± 0.119
Healthy control	20	0	0	0.287 ± 0.056

Different approaches in using *M. tuberculosis* glycolipid antigens for the serodiagnosis of tuberculosis have been investigated earlier. In majority of these studies, a commercially available kit known as Determiner-TBGL developed by Kyowa Medex Co Ltd was used (Kawamura et al, 1997; Maekura *et al,* 2001; Iinuma *et al,* 2002; Okuda *et al,* 2004). The antigenic preparation in this kit consisted of trehalose dimycolate and minor hydrophilic glycolipids such as trehalose monomycolate, diacyl trehalose, phenolic glycolipid, 2,3,6,6' tetraacyl-trehalose-2-sulfate and 2,3,6 triacyl trehalose (Maekura et al, 2001). In the evaluation studies, this kit provided sensitivity and specificity of 56.8 - 87.2% and 30% - 73.5 % respectively. To improve the sensitivity, the kit was also advocated to use along with nucleic acid amplification method (Maekura et al, 2003).

In the present study, total mycolic acid containing glycolipids (also designated as TBGL for tuberculosis- associated glycolipid antigen) were used in the ELISA method. This antigenic preparation is different from what mentioned in the above studies. This TBGL preparation contained mainly trehalose dimycolate, trehalose monomycolate and cardiolipin as evident from the thin layer chromatography results (fig: 4.1). When compared to the antigen isolation strategies for the Determiner TBGL kit which employed TLC, TLC immunostaining and column chromatography, isolation of this antigen is easy and the antigen could be stored for a long time at 4°C even after coating in the ELISA plates. The sensitivity of this assay was comparable with that of commercial TBGL kit and the antigen yielded higher specificity (93.44 %) also. In a country like India where tuberculosis is endemic the assay provided a PPV of 95.5 %.

Moreover, the assay is cost-effective and can be used in laboratories of developing countries where the people cannot afford other expensive mycobacterium detecting methods.

4.2.2 Result of indirect ELISA for the detection of anti-cord factor antibody in sera

Standardization ELISA showed maximum discrimination between the control group and the tuberculosis group at 1:1600 dilution. Mean of the absorbance of the overall control group + 2 SDs (= 0.565) was selected as the cut-off value.

The anti-cord factor antibody was found to be elevated in most of the tuberculosis patients ($P < 0.001$), but a positive result was established only in 34 patients with smear/culture positive and 44 patients with smear/culture negative tuberculosis. The antibody titer against cord factor antigen in tuberculosis patients was lower than that observed for TBGL antigen at 1: 3200 dilution. In tuberculosis patients, the antibody titer ranged between 0.202-0.801 at 1:1600 dilution and in control group, the titer ranged between 0.117-0.534. The result is summarized in table 4.2. The main attraction of the test was that it yielded 100 % specificity in the population studied. The sensitivities in smear/culture negative and smear/culture positive patients were 70.97 % and 85 % respectively. The PPV, NPV, positive diagnostic likelihood ratio and negative diagnostic likelihood ratios were 100 %, 71.76 %, α (infinity) and 0.2353 respectively.

TABLE : 4.2 Results of the indirect ELISA for the determination of IgG antibody titer to cord factor (TDM) antigen in sera samples

Patient group and diagnostic results	No: of cases	% Positivity to CF		Mean antibody titer (units)
		n	%	
Pulmonary TB patients	102	78	76.47	0.667 ± 0.166
Smear/culture positive	40	34	85	0.753 ± 0.092
Smear/culture negative	62	44	70.97	0.611 ± 0.106
Diseased control	41	0	0	0.403 ± 0.096
Healthy control	20	0	0	0.318 ± 0.064

Several studies have been reported about indirect ELISAs using cord factor antigen and in these studies the sensitivity and specificity ranged between 60.34 – 81 % and 75 -100 % respectively. Maekura *et al* (1993) in Japan reported the highest sensitivity. The efficiency of synthetic cord factor analogues was also studied (Laszlo *et al*, 1992). Fujiwara *et al* showed that the antibody response against cord factor was highly specific. In their study, serum from rabbit immunized with *M. tuberculosis* cord factor was highly reactive against *M. tuberculosis* cord factor and less reactive against *M. avium* cord factor (Fujiwara et al, 1999). But on the contrary, Lopez-Marin *et al* obtained specificity up to 99% in their study using cord factor from *M. fortuitum* (Lopez-Marin et al, 2003).

In the present study, the highest specificity (100 %) among the entire four antigen studied was exhibited by cord factor antigen. It did not cross-react with any other antigens present in the control group. The specificity of an antigen mainly depends upon its purity and species-specificity. The cord factor purified from $H_{37}Rv$ strain of *M. tuberculosis* in this study was pure as it gave only one

spot on thin layer chromatogram and this may explain the high specificity exhibited by the antigen.

4.2.3 Result of indirect ELISA for the detection of anti-IgG Sulfolipid –I antibodies in sera

Anti-SL-I antibody was also seen elevated in most of the tuberculosis patients. The cut-off value was selected at 0.51 (Mean of the absorbance of the overall control group + 2 SD) at 1:1600 dilution. Based on this criterion, 76/102 patients with pulmonary tuberculosis were found to be positive in which 35 were patients with smear/culture positive and 41 were patients with smear/culture negative active tuberculosis. In diseased control group, 40/41 subjects showed a negative result in the assay. All the healthy control cases were also negative. Thus the sensitivity and specificity of the assay are 74.51% and 98.36 % respectively.

TABLE : 4.3 Results of the indirect ELISA for the determination of IgG antibody titer to sulfolipid-I antigen in sera samples

Patient group and diagnostic results	**No: of cases**	**% Positivity to SL-I antigen**		**Mean antibody titer (units)**
		n	**%**	
Pulmonary TB patients	102	76	74.51	0.638 ± 0.164
Smear/culture positive	40	35	87.5	0.699 ± 0.112
Smear/culture negative	62	41	66.13	0.598 ± 0.114
Diseased control	41	1	2.43	0.382 ± 0.087
Healthy control	20	0	0	0.267 ± 0.062

The positive predictive value and the negative predictive value were found to be 93 % and 68.3 %. The positive diagnostic likelihood ratio and negative diagnostic likelihood ratios were 45.43 and 0.259 respectively.

Despite SL-I being an exclusive antigen to virulent *M. tuberculosis*, only three previous serological evaluations of IgG antibodies to SL-I are reported. The study reported by Cruaud *et al* and Rojar Espinosa *et al* showed a poor sensitivity up to 50 % and specificity up to 100 % (Cruaud *et al* 1989; Rojar Espinosa *et al,* 1999). But Julian *et al* found, as in this study, the sensitivity of the ELISA was 81 % and the specificity was 77.6 % in their population (Julian *et al*, 2002). In our study, even though the specificity was enhanced up to 98.36 % with only one false positive result, the diagnostic likelihood ratios were not as attractive as that of cord factor antigen or TBGL antigen for using this antigen in a routine basis.

4.2.4 Result of indirect ELISA for the detection of LAM antibody in sera:

For the indirect ELISA for the estimation of anti-LAM antibodies, the cut off value was set at 0.562 (mean + 2 SD) at 1:3200 dilution based on standardization ELISA. Sera from 83/102 pulmonary tuberculosis patients showed positive result. In them, 37 patients were smear/culture positive and 46 were smear/culture negative. The antibody level in the tuberculosis patients against LAM antigen was higher than that against cord factor and sulfolipid-I at 1:3200 dilution. The sera from 6 patients in the diseased control group and 1 healthy control also gave positive results in the assay. Thus the sensitivity and specificity of the assay were 81.37% and 88.52 % respectively. Positive predictive

value and negative predictive value were 92.2 % and 73.97 % respectively. The positive diagnostic likelihood ratio and negative diagnostic likelihood ratios were 7.088 and 0.21 respectively.

TABLE : 4.4 Results of the indirect ELISA for the determination of IgG antibody titer to LAM antigen in sera samples

Patient group and diagnostic results	No: of cases	% Positivity to LAM antigen		Mean antibody titer (units)
		n	%	
Pulmonary TB patients	102	83	81.37	0.565 ± 0.192
Smear/culture positive	40	37	92.5	0.659 ± 0.088
Smear/culture negative	62	46	74.19	0.584 ± 0.164
Diseased control	41	7	17.07	0.383 ± 0.107
Healthy control	20	0	0	0.308 ± 0.076

LAM antigen is a well-studied antigen and this antigen was shown to evoke different serological responses in different geographical locations. In different studies, the commercially available anti-LAM kit Mycodot^{TM} provided sensitivity and specificity ranging between 19-90 % and 84-100 % respectively. In this study, the sensitivity was 81.37 % and specificity was 88.52 %. The low specificity of the LAM isolated in our laboratory may be due to cross-reaction with antigens expressed in malignancy. False positive results for this antigen in fungal diseases were reported earlier (Wheat *et al*, 1986; Sada et al, 1990) but in this study, 4 patients with lung carcinoma also showed positive results, like in the

results obtained by Sada *et al,* suggesting similarity of the antigenic determinants with tumor antigens as well.

4.2.5 Comparison of the diagnostic modalities in the diagnosis of pulmonary tuberculosis:

It is now well accepted that the TB patients do not produce antibodies against all antigenic substances in the cell walls of the tuberculous bacilli and the specificities of the antibodies differ among patients. (Chaikumpar *et al*, 1997; Fujiwara, 1997; Demkov *et al*, 2006a). The cell wall antigen compositions of the tuberculous bacilli also differ among clinical isolates. The diverse antibody response to *M. tuberculosis* may be governed by HLA types (Arend *et al*, 2000). Variability in the sensitivity of these assays also depends on the investigator and the survey participants (Okuda *et al*, 2004). Thus, assessing utility of these antigens in the serodiagnosis of tuberculosis in diverse population becomes essential before introducing it into routine practice. In this study, the best useful test was found to be the detection of IgG antibodies to TBGL antigen. It provided the highest sensitivity of 83.33% in the study together with a good specificity (93.44 %). This is probably the first report that explains the utility of tuberculous glycolipid antigen in Indian population.

Test used	Efficiency of the test					
	sensitivity	**specificity**	**PPV**	**NPV**	**Positive DLR**	**Negative DLR**
TBGL/IgG	**83.33**	93.44	95.55	77	12.72	**0.2044**
Cord factor/IgG	76.47	**100**	**100**	71.76	α	0.2353
SL-I/IgG	74.51	98.36	93	68.3	45.43	0.259
LAM/IgG	81.37	88.52	92.2	73.97	7.088	0.21043

Table 4.5 Data showing the sensitivities, specificities, PPVs, NPVs, positive and negative DLRs of the antibody based assays for the diagnosis of pulmonary tuberculosis

The positive and negative likelihood ratios are valuable tools for comparing the accuracy of diagnostic tests to the 'gold standard' though it has not yet been included even in peer-reviewed journal (Puhan *et al*, 2005). A useful test will have higher positive diagnostic likelihood ratios and negative diagnostic likelihood ratios will be close to zero. Unlike PPV and NPV; they are not dependent upon the prevalence of the disease also. In this study, the anti-TBGL antibody detection yielded high positive diagnostic likelihood ratio and lowest negative likelihood ratio. Positive predictive value was also found to be very high. Hence, among the four diagnostic methods, detection of anti-TBGL antibody was found to be the most suitable method for screening tuberculosis patients in a population particularly where tuberculosis prevalence is high.

The anti-cord factor IgG test can be considered as a confirmatory test as it was 100 % specific, even though it failed to pick up maximum number of positive cases. But the detection of IgG antibodies against SL-I and LAM were less useful as it is evident from the data provided in the table.4.5.

4.3 PLEURAL FLUID INDIRECT ELISAs USING MYCOBACTERIAL LIPID ANTIGENS FOR THE DIAGNOSIS OF PLEURAL TUBERCULOSIS:

The antibody titer against mycobacterial lipid antigens in the pleural fluids of most of the patients with pleural tuberculosis was seen elevated significantly than that of patients with malignant pleural effusion. Cut-off point was selected at mean absorbance value of the malignant pleural effusion + 2 standard deviations,

as discrimination of patients with tuberculous pleural effusion from patients with malignant pleural effusion was possible by this criterion. Sensitivities of all the assays studied were higher in smear/culture positive patients than in smear/culture negative pleural tuberculosis patients.

4.3.1 Result of the detection of antibody titer of TBGL antigen in pleural fluids:

Based on the preliminary standardization ELISA, a cut-off value was set at 0.534 at 1:3200 dilution for scoring a test positive for tuberculosis etiology. Of the 11 'confirmed' cases of pleural tuberculosis, 10 were found to be positive. In the smear/culture positive patients, the antibody titer in the pleural effusion ranged between 0.486-0.763. In 'probable' pleural tuberculosis patients, the antibody titer ranged between 0.398 – 0.761 and 35/58 of them were positive by this assay. The mean absorbance values are expressed in table: 4.6. Nine out of 71 patients with malignant pleural effusion also gave positive results. The sensitivity and specificity of the test was found to be 65.21 % and 87.3% respectively. In smear/culture negative patients and smear/culture positive patients, the sensitivities were 60.34 % and 91% respectively. The positive predictive value and negative predictive value of the assay were 83.33 % and 72.1 % respectively

Table 4.6 Results of the indirect ELISA for the determination of IgG antibody titer to TBGL antigen in pleural fluid samples

Patient group and diagnostic results	No: of cases	% Positivity to TBGL antigen		Mean antibody titer (units)
		n	%	
Patients with tuberculosis pleural effusion	69	45	65.21	0.604±0.122
Smear/culture positive	11	10	91	0.691±0.120
Smear/culture negative	58	35	60.34	0.588±0.117
Patients with malignant pleural effusion	71	9	12.68	0.368±0.104

The only study evaluating commercial glycolipid antibody kit 'Determiner TBGL' in pleural fluid was reported by Morimoto *et al.* In their study using Determiner-TBGL kit, the sensitivity and specificity were 52.6 % and 95.7 % respectively. He recommended the use of this kit along with ADA for the diagnosis of pleural tuberculosis, as the sensitivity and specificity increased up to 90 % in combination (Morimoto *et al,* 2006). In the case of TBGL antigen isolated in our laboratory, nine false positive results were reported and thus, the specificity was found to be 87.32 %, which is comparable with that of Determiner TBGL kit. The false positive result may arouse due to two reasons. First, the antigen might have cross-reacted with the antigens present in malignant pleural effusion. Second, though we have taken efforts to exclude patients with both malignancy and tuberculosis based on the clinical details and their response to tuberculosis, we may not completely rule out undiagnosed dual disease in these

patients as both pleural fluid specimens were collected from the same hospital and as tuberculosis is endemic in our population.

4.3.2 Antibody titer in pleural fluids and sera of patients with tuberculous pleural effusion

The idea of detecting antibodies in pleural fluids for the diagnosis of pleural tuberculosis has been questioned by Levy *et al* as they obtained no difference in the antibody levels in sera and pleural fluid samples of pleural tuberculosis patients. They proposed that the antibody diffuses from serum to pleural fluid and there is no local production of antibodies in the pleural space (Levy *et al*, 1990). But contradicting this result, some other researchers reported increased antibody levels in pleural fluids than in serum in some pleural tuberculosis patients (Chierakul *et al*, 2001, Van Vooren *et al*, 1990).

Hence, to check whether detecting antibodies in pleural fluids rather than in serum is of any diagnostic importance, sera and pleural fluids were collected from 21 patients with pleural tuberculosis. Of these, 14 patients were diagnosed with tuberculous pleural effusion and 7 patients with malignant pleural effusion. Only one of the pleural fluid exhibited acid-fast bacilli in it by Zeihl - Neelsen staining.

Of the 14 pleural tuberculosis patients studied, 6 showed higher antibody titer in pleural fluid samples than in sera samples. In 4 patients, the antibody titer was more or less similar in both pleural fluid and sera samples. In the rest 4 patients, the IgG antibody against TBGL antigen was higher in sera samples than pleural fluid samples. In all the 7 patients with malignant pleural effusion, the IgG

antibody against TBGL antigen was significantly less in both pleural fluids and sera but comparatively, lower titers were seen in the pleural fluids.

From the data obtained from this study, it is concluded that the antibody titer in pleural fluids varies between patients. Hence, in those patients in whom the result of serum antibody titer is inconclusive, the result of pleural fluid ELISA may be beneficial for a conclusive diagnosis.

4.3.3 Antibody titer of cord factor antigen in pleural fluids:

Table 4.7 shows the results of anti-cord factor ELISA in pleural fluids from patients with tuberculous and malignant pleural effusion. The level of antibody in pleural fluids of patients with pleural tuberculosis was significantly higher ($P<0.001$) than those of patients with malignant pleural effusion. The IgG antibody titer to cord factor ranged between 0.394-0.913 units in smear/culture positive and between 0.316-0.876 units in smear/culture negative patients. In patients with malignant pleural effusion, the antibody titer ranged between 0.226-0.671. The cut-off was selected at 0.619 (mean of the absorbances in the control + 2 SD) at 1: 1600 dilution based on standardization ELISA. The sensitivity, specificity, PPV, NPV, positive likelihood ratio and negative likelihood ratio were 59.42 %, 88.73 %, 83.67 %, 69.23 %, 5.273 and 0.457 respectively.

Table 4.7 Results of the indirect ELISA for the determination of IgG antibody titer to cord factor antigen in pleural fluid samples

Patient group and diagnostic results	**No: of cases**	**% Positivity to cord factor antigen**		**Mean antibody titer (units)**
		n	%	
Patients with tuberculosis pleural effusion	69	41	59.42	0.648±0.122
Smear/culture positive	11	10	91	0.722±0.133
Smear/culture negative	58	31	53.44	0.634±0.116
Patients with malignant pleural effusion	71	8	11.27	0.440±0.090

So far, no study has been reported about cord factor antigen being used in the diagnosis of pleural tuberculosis by assessing the antibody titer in the pleural fluid samples. Hence, attempt was taken to reveal the ability of the cord factor in diagnosing pleural tuberculosis. In this study, though the antigen could not provide cent percent specificity as showed in sera samples, the specificity of the assay was satisfactory. But the sensitivity was only 59.42 %.

4.3.4 Antibody titer of sulfolipid-I antigen in pleural fluids:

Based on the results of standardization ELISA, a cut-off was selected at 0.598 in 1:1600 dilution for the assay. The results are shown in the table 4.8. In smear/culture positive patients, 10/11 cases provided positive result yielding sensitivity of 91 %, but in smear/culture negative cases, only 26/58 patients (44.83 %) gave positive results. 5/71 patients with malignant effusion also

showed positive results in this assay. The antibody titer ranged between 0.31 - 0.794 in patients with tuberculous pleural effusion and 0.216 - 0.621 in patients with malignant pleural effusion. The sensitivity, specificity, PPV, NPV, positive DLR and negative DLR are given in the table 4.11.

Table 4.8 Results of the indirect ELISA for the determination of IgG antibody titer to sulfolipid I antigen in pleural fluid samples

Patient group and diagnostic results	**No: of cases**	**% Positivity to SL-I**		**Mean antibody titer (units)**
		n	%	
Patients with tuberculosis pleural effusion	69	36	52.17	0.626±0.121
Smear/culture positive	11	10	91	0.687±0.146
Smear/culture negative	58	26	44.83	0.614±0.113
Patients with malignant pleural effusion	71	5	7.04	0.432±0.083

No study was reported about the IgG antibody to this antigen earlier, therefore effort was taken to study its diagnostic potential. The results are compared with other assays but found to be less useful in our population.

4.3.5 Antibody titer of LAM antigen in pleural fluids:

The antibody titer in the pleural fluids of patients with pleural tuberculosis was elevated significantly than that of patients with malignant pleural effusion (P<0.001). In patients with tuberculosis the antibody titer ranged between 0.243-0.822 and in patients with malignant pleural effusion, the antibody titer ranged

between 0.115-0.606. A cut-off was selected at 0.576 in 1:3200 dilution. The results are shown in the table.4.9. The sensitivity, specificity, PPV, NPV, positive DLR and negative DLR are given in the table 4.11.

Table 4.9 Results of the indirect ELISA for the determination of IgG antibody titer to LAM antigen in pleural fluid samples

Patient group and diagnostic results	**No: of cases**	**% Positivity to TBGL antigen**		**Mean antibody titer (units)**
		n	%	
Patients with tuberculosis pleural effusion	69	46	66.67	0.603±0.124
Smear/culture positive	11	10	91	0.6965±0.122
Smear/culture negative	58	36	62.07	0.586±0.117
Patients with malignant pleural effusion	71	13	18.30	0.415±0.074

Two reports have been published about the IgG detection of LAM antigen. In the study of Yokoyama *et al* in 2005, nine patients with tuberculous pleurisy, one patient with chronic tuberculous emphyma and sixteen patients with non-tuberculous pleural effusion were studied for the presence of antibodies to LAM antigen in pleural fluids using Mycodot kit. Positive results were obtained in 5/10 (Sensitivity 50 %) patients with tuberculous aetiology and 15/16 (Specificity 93.8 %) patients with non-tuberculous aetiology (Yokoyama *et al,* 2005). In 2004, Demkov *et al* evaluated IgG, IgA and IgM mediated humoral immune response against LAM antigen in pleural fluid specimens. But they failed to produce a

significant difference of antibody titer in the pleural fluids of tuberculous and non-tuberculous group (Demkov *et al*, 2004). In our study, the sensitivity was increased to 66.67 % but only at the expense of a decrease in specificity (81.69 %). Hence, it cannot be very useful when compared to the other assays for IgG antibody detection against cord factor, sulfolipid or TBGL antigen.

4.3.6 Determination of TBGL antigen in pleural fluids:

In all the 71 pleural fluid specimens from the disease control, TBGL antigen concentration ranged between 0.3 and 1.34 μg/ml in the modified indirect ELISA. A test was considered positive if the antigen concentration was greater than 1.44 μg/ml [i.e., the mean value (0.71 μg/ml) of disease control + 2 SDs]. Using this criterion, in the 11 confirmed cases of pleural tuberculosis, TBGL antigen concentration ranged between 1.75 μg/ml and 3.8 μg/ml. In 48/58 patients with probable tuberculous pleural effusion, the TBGL concentration ranged between 1.6 and 3.6 μg/ml. The difference between the TBGL concentrations in the pleural fluid of confirmed and probable groups of pleural tuberculosis is statistically insignificant ($P>0.05$). In the remaining 10 patients with probable pleural tuberculosis, TBGL antigen concentration ranged from 0.73 to 1.4 μg/ml.

Table 4.10 Result of TBGL antigen detection assay in the pleural fluid samples

Patient group and diagnostic results	**No: of cases**	**% Positivity**		**Mean TBGL antgen titer (µg/ml)**
		n	%	
Patients with tuberculosis pleural effusion	69	59	85.5	1.8 ± 0.533
Smear/culture positive	11	11	100	2.5 ± 0.584
Smear/culture negative	58	48	82.75	1.57 ± 0.512
Patients with malignant pleural effusion	71	0	0	0.71±0.365

A novel method was used to study the concentration of antigen in pleural fluid samples. Estimation of TBGL antigen carried a sensitivity of 100% and 82.8% in confirmed and probable pleural tuberculosis patients, with an overall sensitivity of 85.5 %. More importantly, the assay did not yield false-positive results in any of the 71 patients in the disease control group. In an earlier study, Wadee *et al.* (1990) reported a specificity of 96.7% for the detection of mycobacterial sonicate antigen. Another study (Dhand *et al.*, 1988) reported a low specificity of 38%. They recorded high false-positive results in patients with malignant pleural effusion and attributed it to the antigenic similarities between malignant cells and Bacillus Calmette-Guérin (BCG) antigen.

To enhance specificity, we used an in-house rabbit anti-TBGL antibody and found that this antibody specifically bound to the glycolipid of *M.*

tuberculosis in pleural fluid but not with any components of malignant cells. Our findings suggest that ELISA-based estimation of TBGL antigen can distinguish between tuberculous and non-tuberculous pleural effusion, and holds promise in the diagnosis of pleural tuberculosis for the following reasons:

a) The technical aspects described in the assay are user-friendly and best suited to laboratories in developing countries where there are constraints in laboratory resources and technical expertise.

b) The assay carries a high specificity.

c) The assay is reproducible and permits storage of pleural samples by not altering the results.

However, before the assay, the pleural fluid specimens should be homogenously mixed in n-hexane to get consistent and reproducible results.

4.3.7 Comparison of the diagnostic modalities in the diagnosis of pleural tuberculosis

Several studies have been reported evaluating the utility of protein antigens in pleural tuberculosis. In a study by Kaisermann *et al* (2005), IgA antibody against two recombinant mycobacterial proteins-MTP-64 and MT 10.3 have been used. They obtained a sensitivity of 72 % for both antigens and a specificity of 96 %. In 2003, Kunter *et al* used the detection of IgG and IgM antibody detection against protein antigen A60 and obtained a sensitivity and specificity of 77% and 94% respectively.

Test used	Efficiency of the test					
	Sensitivity	Specificity	PPV	NPV	Positive DLR	Negative DLR
TBGL/IgG	**65.21**	87.30	83.33	72.1	5.142	0.3983
Cord factor/IgG	59.42	88.73	83.67	69.23	5.273	0.457
SL-I/IgG	52.17	92.96	88.09	67.34	7.4105	0.514
LAM/IgG	66.67	81.69	77.97	71.6	3.6428	0.4039
TBGL antigen detection assay	85.5	100	100	87.65	**α**	0.1449

Table 4.11 Data showing the sensitivities, specificities, PPVs, NPVs, positive diagnostic likelihood ratio and negative likely hood ratio of the diagnostic tests for the diagnosis of pleural tuberculosis.

Zou *et al* in 1994 showed 31 % and 88.5% sensitivity in IgG and IgM detection of A60. But detection of PPD antigen in ELISA showed less sensitivity (Zhang, 1990). Dhand *et al* reported false positive results in post cardiac injury syndrome patients. They suggested that the false positive reaction in ELISA of *M. tuberculosis* antigens in pleural fluids is due to cross-reaction and sharing of antigen between tumor cells and BCG (Dhand et al, 1988).

In this study, in all aspects, the best diagnostic test was antigen detection assay; it provided the highest sensitivity of 81.16 % with 100 % specificity. The positive DLR and negative DLR of the assay were infinity and 0.1884 respectively. Besides, due to the reasons described in section 4.3.5, this assay is suitable for routine diagnosis of tuberculosis in developing countries. Cord factor was also used in similar assays, but the results were not promising as it showed a very low sensitivity. Hence, antigen quantification ELISA was not attempted with other lipid antigens, and was limited to TBGL antigen alone.

4.4 CHEMOTACTIC RESPONSE OF POLYMORPHO-NUCLEAR LEUKOCYTES TOWARDS LIPID ANTIGENS

4.4.1 Chemotaxis of PMNS towards cord factor and sulfolipid-I antigens

The ability of sulfolipid-I and cord factor, two cell wall lipids of *mycobacterium tuberculosis* $H_{37}Rv$, to attract polymorphonuclear leukocytes (PMNs) was focused in this study. The results of the migration studies as showed in the figure 4.8 and figure 4.10 reveal that both the lipids are able to attract

PMNs. While cord factor showed a dose dependent effect on chemotaxis of PMNs, the effect of sulfolipid-I was dose independent.

Migration of cells towards cord factor could be seen even from a very low concentration of 5 μg /ml (Fig: 4.6). At 10-15μg/ml concentration, numerous migrating cells could be observed on the membrane (Figure 4.7), but the maximum migration was observed at 20μg /ml concentration of cord factor. The average number of cells per microscopic field was 102 ± 19. It is to be noted that, at increased concentrations of cord factor, a dramatic decrease in the number of migrating cells was observed.

In contrast to the above results, migrating PMNs in the Millipore membrane was observed at all concentrations of sulfolipid-I. The migrating PMNs towards 20 μg/ml of sulfolipid are shown in figure: 4.9. A small decrease in the number of migrating PMNs at 20 μg/ml and a small increase in the number of migrating PMNs at 30 μg/ml concentrations were observed. The results are summarized in the fig: 4.10. A very few number of migrating PMNs were observed in the blank which contained no chemo-attractants in the lower chamber (fig: 4.5) and this could be due to random migration of PMNs. In the positive control containing 500 μg of casein/ml, numerous migrating PMNs were observed (95.6 ± 21).

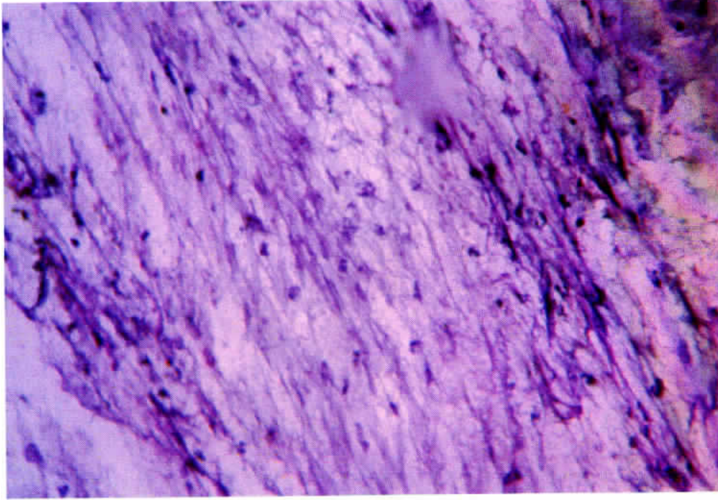


Fig: 4.5 Photomicrograph showing few number of randomly migrating PMNs on the nitrocellulose membrane in the absence of chemo-attractants

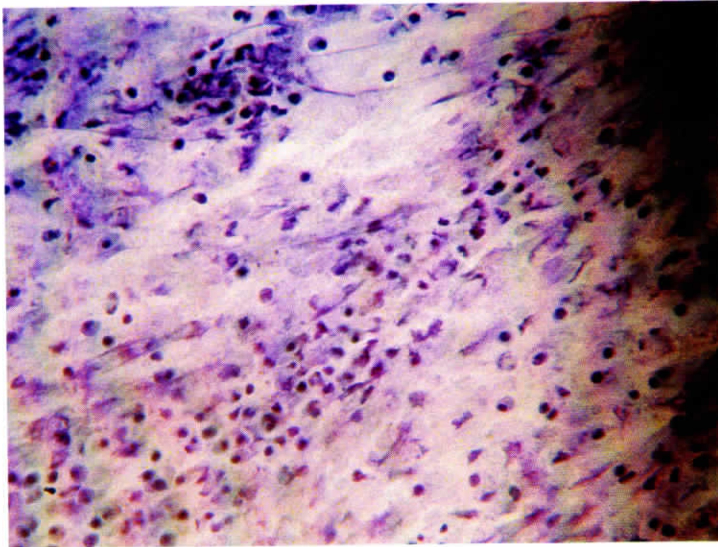


Fig: 4.6 Photomicrograph showing migrating PMNs on the nitrocellulose membrane in the presence of 5μg/ml of cord factor

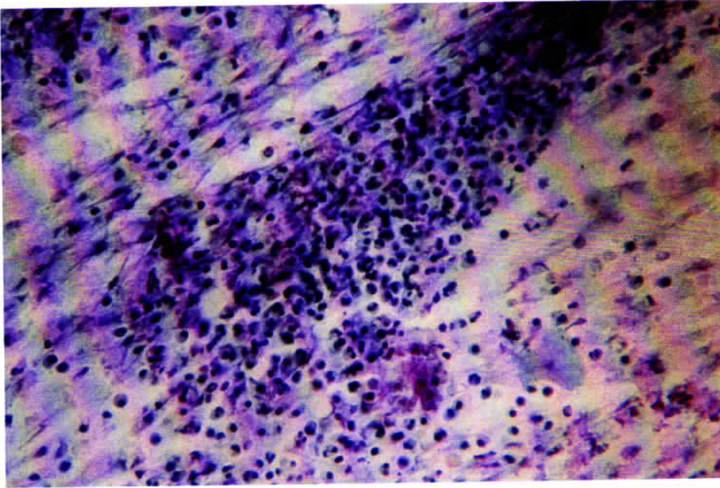


Fig: 4.7 Photomicrograph showing PMNs on the nitrocellulose membrane migrating towards 15 μg/ml of cord factor

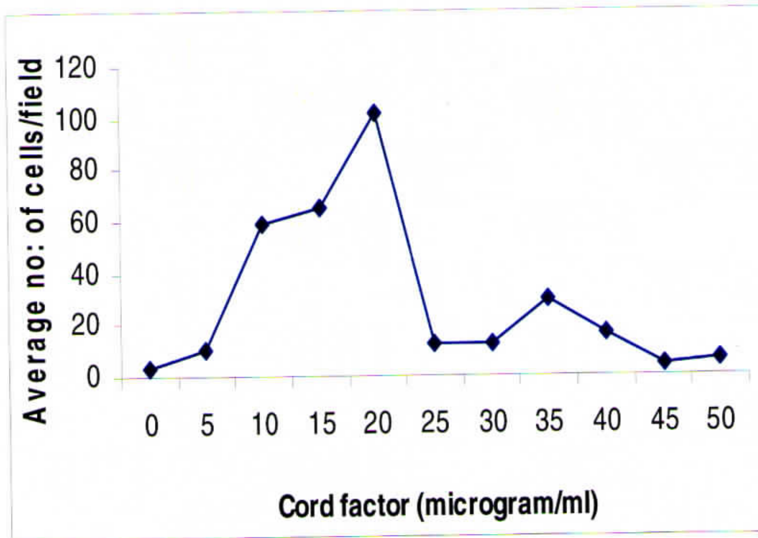


Fig: 4.8 Graph showing average number of PMNs migrating towards varying concentrations of cord factor

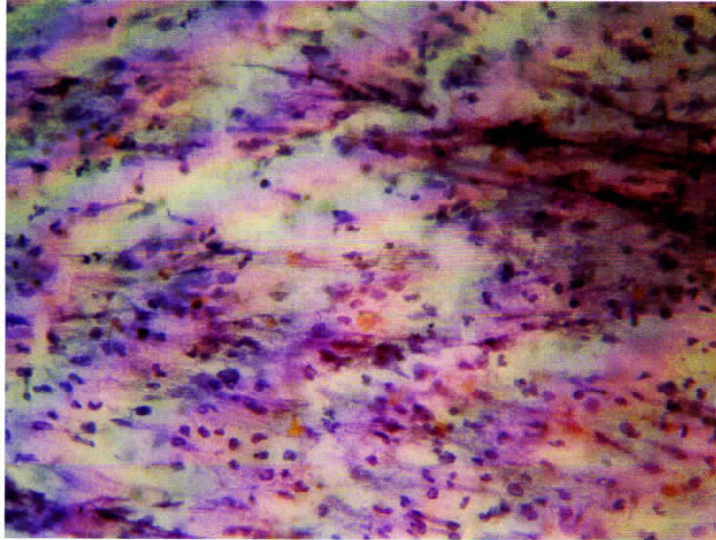


Fig: 4.9 Photomicrograph showing a few migrating PMNs on the nitrocellulose membrane in the presence of 20 μg/ml of sulfolipid-I antigen of *M. tuberculosis*

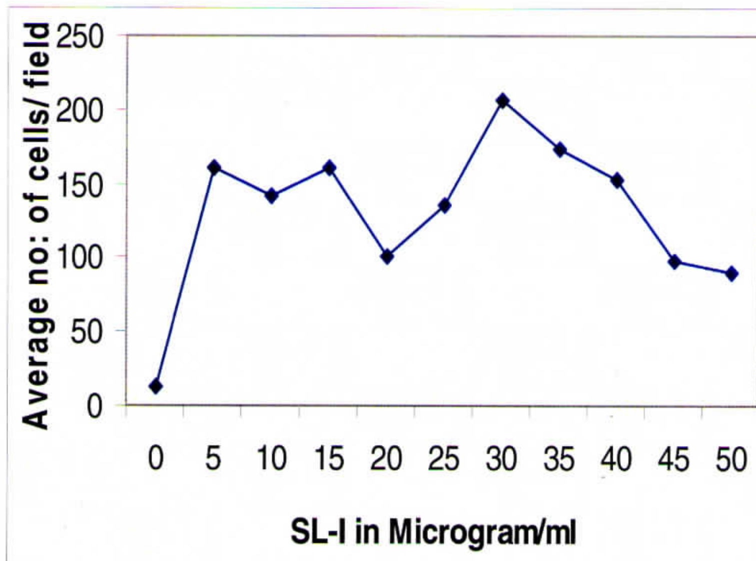


Fig: 4.10 Graph showing average number of migrating PMNs towards different concentrations of SL-I

During tuberculosis infection several immune cells play different roles, of which the role of macrophages and lymphocytes have been studied but the role of neutrophils were not clear. But studies show that the depletion of peripheral blood neutrophils and neutrophils in the lungs is associated with the increased risk of tuberculosis infection and/or enhanced mycobacterial growth (Martineau *et al*, 2007; Fulton *et al*, 2002). Furthermore, the macrophages which are the main effector cells in tuberculosis was shown to engulf apoptoptic neutrophils and use some of the neutrophilic peptides like cathelicidin and α defensins for their antimycobacterial activity (Martineau *et al*, 2007). Moreover, the cytokines produced by these neutrophils will help in activating macrophages for the effective removal of *M. tuberculosis*. Hence neutrophils are now accepted as having some key role in regulating the tuberculosis infection.

Mycobacterium tuberculosis is blessed with high amount of lipids comprising upto 60 % of bacterial cell-wall (Kolattukudy *et al*, 1997). These cell wall lipids play several important roles in the pathogenesis of tuberculosis. Cord factor is shown to induce granuloma formation (Baba *et al*, 1997), macrophage activation (Indrigo *et al*, 2002) and has anti-tumour and adjuvant activity. Similarly sulpholipid I, the principal sulfatide in *M. tuberculosis* is known to prevent phagosome lysosome fusion (Goren *et al*, 1976), induce macrophage activation and modulate macrophage and neutrophil functions (Zhang et al, 1991; Zhang et al 1988). But the role of the mycobacterial glycolipid antigens like SL-I and cord factor in attracting neutrophils has not gained enough attention.

To be potential immune effector cells, neutrophils must be recruited to infectious foci since other studies have shown that a peripheral blood neutrophilia does not confer protection from *M. tuberculosis* growth and dissemination in mice (Murray *et al*, 1998).

Neutrophils are attracted to the infection site by different components of the tubercle bacillus or by specific mediators produced in response to mycobacterial infection. From the results obtained, it is clear that cord factor and sulfolipid-I are two such factors that are responsible for the recruitment of neutrophils to infection area.

4.4.2 Inhibition of chemotaxis in tuberculosis patients:

The chemotaxis of the PMNs in the presence of sera and pleural fluids of tuberculosis patients showed that these body fluids contain certain inhibiting factors for the migration of PMNs. The average number of migrating cells towards the three chemoattractants- casein or cord factor or sulfolipid-I - in the presence of tuberculosis sera, diseased sera and in the absence of any sera is shown in the graph (fig: 4.12). The number of migrating PMNs in the presence of tuberculosis sera was significantly ($P<0.001$) less than the number of migrating PMNs in the presence of diseased control sera and normal sera. The chemotaxis of PMNs in the presence of sera from non-TB patients and healthy donors was more or less unaffected.

In the case of chemotaxis of PMNs in the presence of pleural fluids, a similar but more intense inhibition was observed in the presence of pleural fluids from tuberculosis patients. Chemotaxis of PMNs was not altered significantly in the presence of malignant pleural effusion but in the presence of 400 μl of pleural fluid from tuberculosis patients, number of migrating PMNs was literally absent in the millipore membrane. The result is summarized in graph (fig: 4.13).

The chemotaxis of healthy PMNs towards the well-known chemo-attractant casein was also found to be decreased significantly in the presence of body fluids (pleural fluid and sera) from tuberculosis patients. Hence, it is clear that this inhibition of the migration of PMNs is not solely due to the antibodies raised in these patients against the lipid antigens. The decrease in the number of migrating cells are evident in the order Cord factor > Sulfolipid-I > Casein. In the case of casein, the inhibition was not complete. Hence, the inhibition could not be due to cell death caused by the toxicity of the pleural fluids. From these data, it is concluded that, in tuberculosis patients, the migration of PMNs towards lipids like cord factor and SL-I is inhibited by certain soluble factors in the body fluids, which is derived either from the host or from the bacterium.

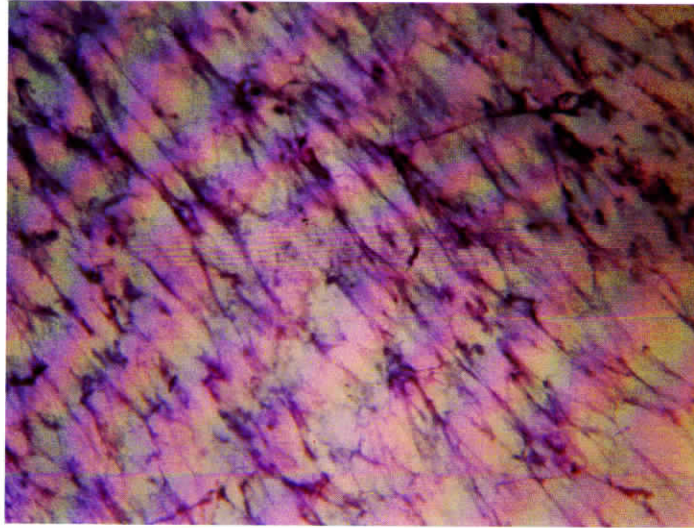


Fig: 4.11 Photomicrograph showing absence of migrating PMNs on the nitro-cellulose membrane in the presence of 400 μl of pleural fluid from a patient with pleural tuberculosis

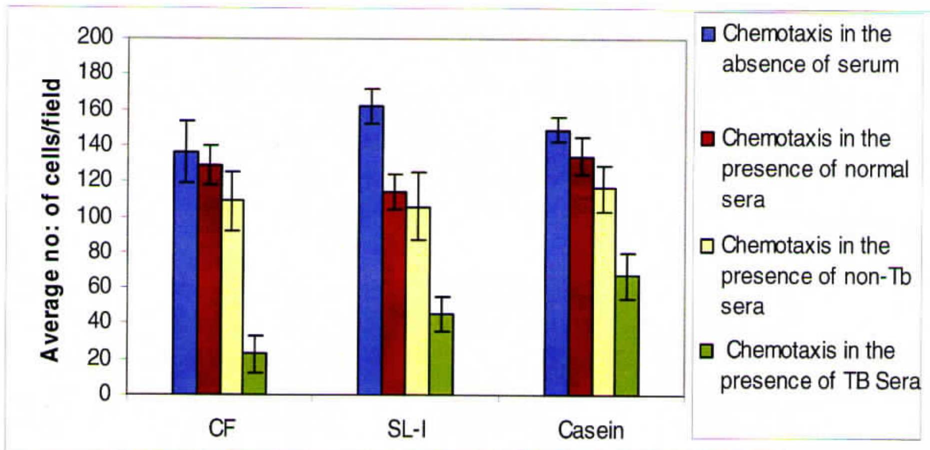


Fig: 4.12 Graph showing inhibition of migration of PMNs towards cord factor, SL-I and casein in the presence of sera from healthy donors, diseased controls and pulmonary tuberculosis patients

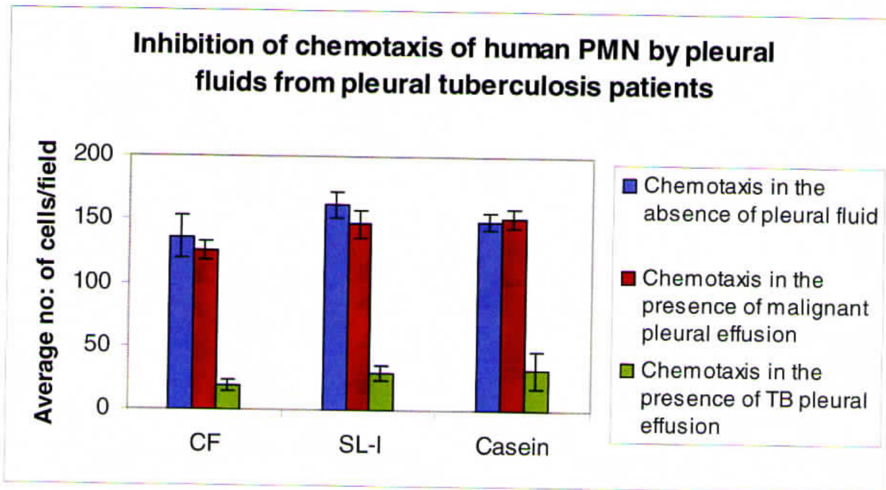


Fig: 4.13 Graph revealing inhibition of migration of PMNs in the presence of pleural fluids from patients with tuberculous pleural effusion and malignant pleural effusion

In conclusion, in the early stages of tuberculosis, when the amount is less, cord factor may induce PMN migration to the infected site but as the disease progresses, it may not induce further PMN migration, because more and more cord factor will be synthesized by multiplying bacteria. In immuno-suppressed stages also, the cord factor level will be high due to higher loads of bacteria. But SL-I will be active always in recruiting PMNs regardless of its amount in the tissue, which is also dependent on the bacillary load. This finding is very significant as SL-I but not CF is taken up by neutrophils and is thus primed for oxidative burst (Zhang et al, 1988). Moreover, neutrophils are recruited intensely to the infected site in the absence of cord factor (Lima et al, 2001). Thus, SL-I rather than cord factor (at high concentration) stimulate the accumulation of PMNs on *M. tuberculosis* infection and enhance neutrophil function.

If the PMNs are effectively recruited to the infection site, what is happening in tuberculosis patients, where the disease progresses to the next stages? For studying this, chemotaxis was performed in the presence of body fluids from pulmonary and pleural tuberculosis patients. The data showing the inhibition of chemotaxis of neutrophils in these patients suggests the secretion of some factors that inhibits the chemotaxis. As the inhibition was observed both in serum and pleural fluids samples, it should be a soluble factor. Thus, it is

concluded that in individuals who are susceptible to tuberculosis, certain soluble factors are produced to inhibit the chemotaxis of PMNs either by the host or the pathogen and in other competent individuals the neutrophils are effectively recruited to the infection site (which is succeeded by macrophages and lymphocytes). Thus the progression to the next stages of infection is stopped in those healthy individuals.

CHAPTER.5

SUMMARY, CONCLUSION & FUTURE PRSOPECTS

5.1 SUMMARY AND CONCLUSIONS:

India accounts for 1/5th of the global tuberculosis incidence. The control of tuberculosis mainly depends on early diagnosis and effective treatment and there is an urgent need for improved tools for laboratory diagnosis of active tuberculosis. In this study, four antigens were isolated from *the Mycobacterium tuberculosis*, the microorganism responsible for tuberculosis. The antigens isolated were tuberculosis associated glycolipid antigen (TBGL, that contains total mycolic acid containing glycolipids), trehalose dimycolate (Cord factor), sulfolipid-I and lipoarabinomannan. The purified antigens were then used for developing indirect ELISAs for the diagnosis of pulmonary tuberculosis and pleural tuberculosis.

Four indirect ELISA methods were described for the diagnosis of pulmonary tuberculosis and five ELISA methods were described for the diagnosis of pleural tuberculosis. The indirect ELISA methods for the diagnosis of pulmonary tuberculosis depends on the detection of IgG antibody towards four glycolipid antigens in tuberculosis patients – TBGL (tuberculosis associated glycolipid antigen), cord factor (trehalose dimycolate), sulfolipid-I and lipoarabinomannan (LAM) antigen. In pulmonary tuberculosis patients the best results were obtained by detection of IgG antibody towards TBGL antigen (83.33% sensitivity and 93.44 % specificity). The results obtained for anti-cord factor antibody detection was also promising as it gave 100 % specificity in the assay.

The four indirect ELISA methods for the diagnosis of pleural tuberculosis also depended on the detection of IgG antibody towards the four glycolipid antigens. The importance of measuring antibodies in the pleural fluid samples rather than in serum samples of pleural tuberculosis patients was also highlighted in the work. A novel modified indirect ELISA for the detection of TBGL antigen in pleural fluid specimens was also explained for the diagnosis of pleural tuberculosis. By this method, TBGL antigen concentration in the pleural samples of the pleural tuberculosis patients could be measured at nanogram levels. The assay yielded 100 % specificity and 85.5 % sensitivity in this study. Hence, best method for the diagnosis of pleural tuberculosis was concluded as TBGL antigen detection assay.

The role of two lipid antigens- cord factor and sulfolipid I -in recruiting polymorphoneuclear leukocytes (PMNs) to the site of active tuberculosis infection was also studied. Investigations were also carried out to find out whether PMN chemotaxis is inhibited in tuberculosis patients. Results suggested that both the lipid antigen were capable of recruiting neutrophil to infection area. But in tuberculosis patients, this chemotaxis was inhibited by certain soluble factors in the body fluids which is secreted either by the patient or the bacterium.

The major findings from this study are

i) The lipid antigens were found to be useful in the diagnosis of tuberculosis in developing countries as it is easy to prepare and thus is cost effective, having sufficient sensitivity, specificity and reproducibility.

ii) In pulmonary tuberculosis, TBGL/IgG can be used for the screening of tuberculosis patients but a definite diagnosis can be provided by TDM/IgG ELISA

iii) In pleural fluid, the best test for a definite diagnosis is TBGL antigen detection assay.

iv) Detecting antibodies in pleural fluid patients is beneficial in those patients in whom results of antibody detection in serum is not conclusive.

v) Cord factor and sulfolipid-I have the property of attracting polymorpho-nuclear leukocytes to the infection site during tuberculosis infection.

vi) But in tuberculosis patients, this chemotaxis of PMNs is inhibited. This could be due to the secretion of certain soluble factors either by the host or by the pathogen in these patients.

5.2. FUTURE PROSPECTS:

In this study, the most useful assay that was found for the diagnosis of tuberculosis in our population were TBGL/IgG detection and TDM/IgG detection in the sera samples for pulmonary tuberculosis and TBGL antigen detection of pleural fluids for pleural tuberculosis patients. As these assays are reproducible, reliable and cost-effective, it is well-suited to the laboratories of developing countries. Hence, evaluation of these assays in a large population in distinct parts of our country is invaluable prior their utilization in the clinical laboratories on a routine basis.

We have done the preliminary studies that documented the chemotactic potential of two cell wall lipid antigens – cord factor and sulfolipid-I – to attract the polymorphonuclear leukocytes, the first type of cells recruited to tuberculosis infection sites. To study the exact role of these lipid antigens in the granuloma formation in the tissues during tuberculous infection, the chemotactic potential of these lipid antigens for macrophages need to be assessed.

Another interesting area will be to search for the nature of the soluble component of the body fluids that inhibit the chemotaxis of PMNs and also their mechanism of action, which may give additional information about the disease progression. The study of cytokines released in response to these lipid antigens will also give information about their role in altering the functions of other important effector cells, the T cells.

CHAPTER.6

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CHAPTER.7

LIST OF PUBLICATIONS

PUBLICATIONS:

Anie Y, Sumi S, Varghese P, Madhavi L G K, Sathish M, Radhakrishnan V V. Diagnostic approaches in patients with tuberculous pleural effusion. Diagn Microbiol Infect Dis 2007: 59; 389-394

Papers published in conference Proceedings:

Anie Y, Sumi S, Radhakrishnan V V. Diagnostic utility of serum IgG antibody to lipid antigens of mycobacterium tuberculosis in patients with pulmonary tuberculosis. In the proceedings of the 19th Kerala Science congress held at Kannur from 29-31 January, 2007. pp 268-269

Anie.Y, Sumi. S, Radhakrishnan V V. Diagnosis of tuberculous lymphadenitis – Molecular and Immunological approaches, In the proceedings of the 18th Kerala Science congress held at Thiruvananthapuram from 29-31 January, 2006. pp 471-472

Sumi S, Anie Y, Radhakrishnan V V, Diagnostic approaches in pleural tuberculosis. In the proceedings of the 18th Kerala Science congress held at Thiruvananthapuram from 29-31 January, 2006. pp 444-445.

Award:

"Diagnostic significance of estimation of glycolipid antigen of *Mycobacterium tuberculosis* in patients with pleural tuberculosis", presented at the Annual meeting of National Academy of Medical Sciences held at Hyderabad on 28th to 30th October 2006, procured the Dr. S. S. Misra award for the best work for the year 2006-2007 by the National Academy of Medical Sciences (India), New Delhi

Annexure

List of media and Buffers

Lowenstein -Jensen medium

Mineral salt solution:

Mineral salt (Potassium dihydrogen phosphate)	2.4 g
$MgSO_4$. $7H_20$	0.24 g
Magnesium Citrate	0.6 g
L-Asparagine	3.6 g
Glycerol	12 ml
Distilled water	600 ml

Autoclave at 121°C for 25 minutes

Medium:

Mineral salt solution	600 ml
Beaten egg	1000 ml
Malachite green (2% in sterile water – Dissolve the dye in the incubator for 1-2 hours. Shake well before use)	20 ml

Sauton's Medium

Asparagine	4 g
Citric acid	1 g
Magnesium Sulphate	0.5 g
Dipotassium hydrogen phosphate	2.5 g
Ferric ammonium citrate	0.05 g
Glycerol	35 ml
Distilled water	1 litre

Mix well glycerol in 750 ml of distilled water. Add the chemicals and again mix properly. Then adjust the pH with liquor ammonia to pH 7.4. Adjust the volume to 1 litre and autoclave at 121°C for 15 minutes

DEA (Diethanolamine) buffer

Diethanol amine	97 ml
Sodium Azide	0.2 g
$MgCl_2 . 6 H_2O$	0.1 g

Dissolve in 800 ml distilled water, adjust pH to 9.8 with dil. HCl and make up to 1 litre.

PBS (Phosphate buffered Saline

NaCl	43 g
Na_2HPO_4	4.24 g
KH_2PO_4	2.7 g

Dissolve in 5000 ml water after adjusting the pH to 7.2

Hank's balanced salt solution

Phenol red indicator (0.4 per cent) – Dissolve 1 g of phenol red in minimum volume of 0.05 N NaOH and then bring the volume to 250 ml by the addition of distilled water.

Stock solution A

1. NaCl	160 g
KCl	8 g
$MgSO_4 . 7 H_2O$	2 g
$MgCl_2 . 6 H_2O$	2 g
H_2O	800 ml
2. $CaCl_2$	2.8 g
H_2O	100 ml

Mix these solutions slowly and adjust the volume to 1000 ml with water. Add 2 ml chloroform and store in a polythene bottle at 4°C.

Stock solution B

$Na_2HPO_4 . 12 H_2O$	3.04 g

KH_2PO_4	1.2 g
Glucose	20 g
Water	800 ml

Add 2 ml chloroform and store as with solution A.

Sodium Bicarbonate solution:

$NaHCO_3$	1.4 g
Water	100 ml

Sterilize by autoclaving in a container with a tightly closed screw cap for 10 minutes at 115°C.

Finally, Hank's Solution is made by adding 1 volume of stock solution A and 1 volume of stock solution B to 18 volumes of distilled water. Sterilize it. Immediately before use add 0.5 ml of sterile sodium bicarbonate solution to each 20 ml Hank's solution