

**PURIFICATION AND APPLICATION OF LIPOARABINOMANNAN
ANTIGEN AS A DIAGNOSTIC TOOL AND ITS COMPARISON WITH
PCR, RADIORESPIROMETRY AND OTHER CONVENTIONAL
METHODS FOR DIAGNOSIS OF TUBERCULOSIS**

**THESIS SUBMITTED
UNIVERSITY OF MUMBAI**

**FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN
MICROBIOLOGY**

**SUBMITTED BY
NEENA A. WALAWALKAR**

**UNDER THE GUIDANCE OF
DR. (MRS.) R. A. DESHMUKH**

**DEPARTMENT OF VIROLOGY
HAFFKINE INSTITUTE FOR TRAINING, RESEARCH & TESTING
MUMBAI – 400 012**

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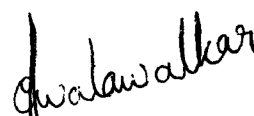
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DECEMBER 2005

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STATEMENT BY THE CANDIDATE

As required by the University Ordinances 770 and 771, I wish to state that the work embodied in this thesis titled, "Purification and application of Lipoarabinomannan antigen as a diagnostic tool and its comparison with PCR, Radiorespirometry and other conventional methods for diagnosis of tuberculosis" forms my own contribution to the research work carried out under the guidance of Dr. (Mrs.) R. A. Deshmukh at the Haffkine Institute for Training, Research and Testing, Mumbai. This work has not been submitted for any other degree of this or any other University. Whenever reference have been made to previous works of others, it has been clearly indicated as such and included in the Bibliography.


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Certified by,


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ABBREVIATIONS

Ab	Antibody
AFB	Acid Fast Bacilli
Ag	Antigen
AG	Arabinogalactan
AM	Alveolar macrophage
APC	Antigen presenting cell
APS	Ammonium persulfate
Ara-LAM	Arabinose capped lipoarabinomannan
CMI	Cell Mediated Immunity
CR	Complement receptor
CSF	Cerebrospinal fluid
CTL	Cytotoxic T Lymphocyte
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DOTS	Directly Observed Treatment Short-course
DTH	Delayed Type hypersensitivity
ELISA	Enzyme Linked Immunosorbent Assay
EMB	Ethambutol
HIV	Human Immunodeficiency Virus
IFN- γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
INH	Isoniazid
kb	Kilo bases
kDa	Kilo Dalton
LAM	Lipoarabinomannan
LJ	Lowenstein Jensen Medium
LM	Lipomannan
LSC	Liquid Scintillation Counter
Man-LAM	Mannose capped lipoarabinomannan
MBL	Mannose Binding Lectin
MDM	Monocyte derived macrophages
MMRc	Macrophage Mannose Receptor
MPI	Mannosy-phosphatidyl-inositol
MTB	Mycobacterium tuberculosis
M + 3SD	Mean + 3SD
NAG	N-acetylglucosamine
NAM	N-acetylmuramicacid
NK	Natural Killer cells
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate Buffered Saline
PBS-T	Phosphate Buffered Saline containing 2% Tween

PCR	Polymerase Chain Reaction
PI-LAM	Phosphatidylinositol capped lipoarabinomannan
PIM	Phosphatidylinositol mannoside
PZA	Pyrazinamide
RIA	Radioimmunoassay
RIF	Rifampicin
RNA	Ribonucleic acid
RNase	Ribonuclease
RNI	Reactive nitrogen intermediates
RNTCP	Revised National Tuberculosis Programme
ROI	Reactive oxygen intermediates
SDS	Sodium dodecyl sulfate
SM	Streptomycin
TBS	Tris Buffered Saline
TB	Tuberculosis
TBM	Tuberculous meningitis
TDB	Tris Deoxycholate Buffer
TEMED	N,N', N'-tetramethylethylenediamine
TH1	T helper cells type 1
TH2	T helper cells type 2
TNF	Tumor necrosis factor
ZNCF	Ziehl-Neelsen Carbolfuchsin

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INTRODUCTION AND REVIEW OF LITERATURE



Chapter 1.

1. INTRODUCTION & REVIEW OF LITERATURE

In 1882, Robert Koch said, *“If the number of victims which a disease claims is the measure of its significance, then all diseases, particularly the most dreaded infectious diseases such as bubonic plague, Asiatic cholera, etc. must rank far behind tuberculosis”* (1).

The above statement though, a century old, is of relevance even today. Tuberculosis, one of the oldest recorded human afflictions, is still one of the biggest killers among the infectious diseases, despite the worldwide use of a live attenuated vaccine and several antibiotics.

1.1. HISTORY

Tuberculosis can be present in various forms including one that attacks bone and causes skeletal deformities. Hard tissues like bone can be preserved for thousands of years allowing the almost certain identification of individuals with bone TB. The frequency of unearthed skeletons with apparent tubercular deformities in ancient Egypt, Italy, Denmark and countries in the Middle East indicate that TB was found throughout the world even 4000 years ago.

In recorded history, Assyrian clay tables describe patients coughing blood in the seventh century B.C., and Hippocrates has written of patients with consumption (Greek term: phthisis), i.e. wasting away associated with pain,

coughing frequently with blood in sputum. Tuberculosis was referred to by the writers of Hindu Upanishads (1500 BC) as both the king of diseases (rogaraj) and the disease of kings (rajayakshma). Throughout the centuries doctors and scientists have described TB in its many forms and sought to understand the origins of the disease, in order to use this information for better diagnosis, prevention and cure. Hippocrates thought the disease was largely inherited while Aristotle stressed its contagious nature. This opposing view of origin reemerged in the second half of 17th century when Mediterranean countries still maintained that TB was contagious. Conversely doctors in the Northern European countries favored constitutional or hereditary causes of this disease.

In 1865, Jean-Antonie Villemin reported that he had been able to demonstrate tuberculous lesions in rabbits by injecting them with tuberculous tissue from a cadaver. This report was immediately assailed by the French medical establishment, notably Herman Pidoux who maintained that there had to be more modern and social solutions to the problem of TB, which he felt arose in the poorer classes from external causes like malnutrition, poor sanitation and overwork. The report by Robert Koch 17 years later, which conclusively showed that TB was indeed caused by a bacterium, discredited many of Pidoux's arguments.

Starting with Edward Trudeau's work in the late 19th and the early 20th centuries, the apparent dichotomy in explaining the etiology of tuberculosis was resolved. In a classic experiment, he showed that TB could be induced in rabbits with a purified culture of virulent *M.tuberculosis* but that the environmental conditions in which the animals were maintained greatly influenced the course of the disease (2). This simple experiment gave scientific validity to the treatment of TB (fresh air and ample food) that was the basis of TB sanitarium movement started by Trudeau in his Saranac Lake TB treatment center that opened in 1884.

Thus "TB is caused by a bacterium but environmental factors play a major role" was rearticulated by Dubos 50 years ago (3). To Dubos, purely medical solutions alone would not work to cure and prevent TB. Unfortunately events of the last half of the 20th century have shown how prescient he was. Neither has the widespread use of BCG lowered the incidence of TB (4) nor has the use of antibiotics eliminated the disease. Clearly, new vaccines, better diagnostic methods, new drugs, social awareness and political commitment are needed for TB control.

1.2. EPIDEMIOLOGY

Europe with its population explosion and the growth of large urban centers became the epicenter for many TB epidemics starting in the 16th and 17th century. The disease peaked in the first half of the 19th century, but morbidity

and mortality rates due to TB steadily dropped during the 20th century in the developed world aided by better public health practices, widespread use of BCG vaccine as well as the development of antibiotics in the 1950s. This downward trend ended and the numbers of new cases started increasing in the mid 1980s. The major causes of this were homelessness, poverty and HIV. Only by massive expenditures of funds and human resources has this miniepidemic of TB cases been reversed in Europe and US (5).

However, the underdeveloped world is still suffering from TB as shown by following statistics. There were an estimated 8-9 million new cases of tuberculosis in 2000; fewer than half of which were reported. Most cases (5-6 million) were in people aged 15-49 years. The incidence of TB ranges from less than 10 per 100,000 in North America to 100-300 per 100,000 in Asia and Western Russia to over 300 per 100,000 in Southern and Central Africa but the most populous countries of Asia have the largest number of cases: India, China, Indonesia, Bangladesh and Pakistan together account for more than half the global burden. There is one death from TB every 15 seconds i.e. over 2 million die of TB every year. Without treatment, around 60% of people with the disease will die (6). Essentially all these cases are in the Third World countries (6), reflecting poverty, lack of healthy living conditions, inadequate medical care (7) and emergence of MDR-TB (8).

1.3.MYCOBACTERIOLOGY

1.3.1. Pathogens in the genus *Mycobacterium*

The genera *Mycobacterium* is grouped into the family *Mycobacteriaceae* within the order *Actinomycetales* based upon staining properties, lack of spore formation, lack of motility, presence of long chain mycolic acids and catalase production.

Mycobacterium tuberculosis complex

All the members of this group are known to be obligate pathogens and do not replicate outside the animal or human host. They are slow growing, showing little or no growth below 30°C and above 39°C. It includes *M.tuberculosis*, *M.bovis*, *M.microti* and *M.africanum*. Tuberculosis caused by mycobacterial species in the tuberculosis complex have been viewed as “typical mycobacteria” while other species of mycobacteria (except *M.leprae* and saprophytic species) have been viewed as atypical mycobacteria (Table 1.1).

Atypical Mycobacteria

In addition to the *Mycobacterium tuberculosis* group, other species of mycobacteria may cause human disease. Commonly referred to as opportunist, non-tuberculous, atypical, mycobacteria other than tuberculosis (MOTT), these organisms are ubiquitous in the environment. They are free living bacteria found in soil, animal dung, salt and fresh water and attached to algae and grass. They are potentially pathogenic to many animals, including cattle and pigs as well as fish and reptiles. In humans, infection

usually develops with pre-existing chronic lung disease or in immunocompromised state but rarely may also occur without any predisposing factors. The infection is most likely to be acquired from the environment, but the source and the portal of entry differs between individuals. As shown in Table 1.1, the major environmental mycobacteria that may cause human disease are classified into four Runyon groups based on pigmentation and rate of growth (9).

Group I- Photochromogens

Group II- Scotochromogens

Group III- Nonchromogens

Group IV- Rapid growers

Saprophytic species

Certain saprophytic species including *M.gordoniae*, *M.gastri*, *M.flavescens* have been occasionally isolated from sputum and other clinical specimens.

Table 1.1: Mycobacteria associated with human disease

Group and species	Disease caused
Strict Pathogens	
I. Typical Mycobacteria	
• <i>M.tuberculosis</i>	Pulmonary disease
• <i>M.africanum</i>	Pulmonary disease
• <i>M.bovis</i>	Pulmonary disease
II. <i>M.leprae</i>	Predilection for skin, nerves, etc.
Atypical Mycobacteria	
I. Photochromogens Nonpigmented when grown in dark; yellow pigment formed when grown in the light; slow-growing	
• <i>M.kansasii</i>	Causing a tuberculosis like disease
• <i>M.marinum</i>	Skin papules and ulcers; contracted from swimming, in fresh and salt water;
II. Scotochromogens Red-orange pigment formed when grown in the dark or light; slow growing.	
• <i>M.scrofulaceum</i>	Causing cervical adenitis in young children
• <i>M.szulgai</i>	Pulmonary disease, adenitis, bursitis.
III. Nonchromogens Nonpigmented in the dark or light; slow growing	
• <i>M.avium/M.intracellulare</i>	Tuberculosis like disease; resistant to anti-tuberculosis drugs
• <i>M.malmoense</i>	Causes pulmonary disease
IV. Rapid growers May be pigmented or non-pigmented	
• <i>M.fortuitum</i>	Causes tuberculosis like disease
• <i>M.chelonae</i>	Has been isolated from patients with chronic respiratory disease.
Saprophytic species	
• <i>M.flavescens</i>	Local tissue defects, causes general immunosuppression
• <i>M.smegmatis</i>	
• <i>M.gordonae</i>	

1.3.2. Properties and Morphology of the Tubercle Bacilli

M.tuberculosis is an obligate parasite, is nonmotile, noncapsulated, with a large cell wall containing high molecular weight mycolic acids. The mycobacterial cell envelope is not only different from all bacteria but is also thicker and uniquely lipophilic (described in details in Chapter 1.11.3). It is from this external barrier that arises the principal characteristics of the organism such as acid fastness, aggregation of cells and resistance to many bactericidal agents.

Tubercle bacilli are difficult to stain with usual staining methods. But when stained with the classic carbol fuchsin or Ziehl-Neelson method, it resists decolorization with strong mineral acids and alcohol, hence the term “acid-alcohol fast”. It appears as curved or straight, small, red or pink rods, 2-4µm long and 0.2-0.5µm wide when observed under the microscope.

In aging cultures or in clinical specimens from patients on chemotherapy, tubercle bacilli are frequently not solidly stained, an appearance probably related to loss of viability. Acid-alcohol fastness of *M.tuberculosis*, which is not constant at all stages in the growth cycle, may disappear when the organism is submitted to the action of isoniazid, a thioamide, or even high concentrations of penicillin.

1.3.3. Nutrition and Growth

M.tuberculosis has a slow rate of growth, the generation time of the cells in best conditions of culture being 17-18 hours while the first appearance of grossly visible colonies takes 3-5 weeks. The slow growth is largely due to thick lipoidal cell wall, which may retard the passage of nutrients into the cells. *M.tuberculosis* lacks pigment, produces niacin and reduces nitrates.

M.tuberculosis is a strict aerobe equipped with catalase, peroxidase and superoxide dismutase, whose growth rate is highly dependent on oxygen tension. Mycobacteria can also switch to micro aerophilic growth resulting in a slower rate of catabolism. When oxygen tension is high, as in the tuberculosis cavity of the lung, *M.tuberculosis* multiplies freely; when it is much lower, as in the caseous foci of the lung, *M.tuberculosis* multiplies slowly or not at all (10).

Enriched media like Lowenstein media is required for primary isolation after which growth occurs in simpler synthetic media like Long's liquid medium. *M.tuberculosis* can oxidize an extremely wide range of compounds. The preferred carbon sources for growth of *M.tuberculosis* are glycerol, pyruvate or glucose. Citrate used as a ferric ammonium citrate is usually part of the culture medium and provides a source of iron for the organism. Asparagine is usually the preferred source of nitrogen for the bacilli, however even glutamine; aspartic acid or even ammonium salts can substitute for

asparagine. *M.tuberculosis* requires four major inorganic elements for growth: potassium, magnesium, sulfur, phosphorous and trace elements such as iron, zinc and probably manganese.

1.3.4. Genetics of *M.tuberculosis*

Cole *et al* (1998) determined and analyzed the complete genome sequence of *Mycobacterium tuberculosis* H₃₇Rv (109). The genome comprises 4,411,529 base pairs with a G+C content of 65.6%. It consists of fifty genes coding for functional RNA and 3924 open reading frames encoding proteins. By using database, precise functions were attributed to 40% of the predicted proteins, they found information for 44% while no functions could be assigned to remaining 16% of the proteins. From the genome sequence, it was clear that the tubercle bacilli has the potential to synthesize all essential amino acids, vitamins and enzyme cofactors, to metabolize a variety of carbohydrates, hydrocarbons, alcohols, ketones and carboxylic acids. In addition to many functions involved in lipid metabolism, enzymes necessary for glycolysis, pentose phosphate pathway, TCA, glyoxylate cycle, and components of oxidative electron transport chain and of anaerobic phosphorylative electron transport chain are all present. Hydrolytic or drug modifying enzymes such as β -lactamases and aminoglycoside acetyl transferases are also present.

The genome is also rich in repetitive DNA sequences particularly insertion sequences (11). *IS6110* and *IS1081*, both transposons, are insertion sequences

around 1300bp that are inserted into chromosomal DNA. *IS6110* first identified in *M.tuberculosis* was then shown to be extensively present in *M.tuberculosis* complex. Because of its similarity in sequence, *IS6110* is grouped into IS3 family, which is found in enterobacteria. Between 0-20 copies of *IS6110* are found in almost all the strains of *M.tuberculosis* complex, and is not known to be present in other organisms. Around 5 copies of *IS1081* are also shown to be present in *M.tuberculosis* complex.

A repetitive sequence is a short DNA sequence of up to 40bp repeatedly located in the chromosomal DNA. Many such sequences are present in the MTB complex chromosome. This includes PGRS, DR and MIRU sequences.

The PGRS element appears to be the most abundant repetitive sequence in MTB. It occurs multiple times and has significant polymorphism. This sequence is characterized by high GC content and has been successfully used as a secondary marker to discriminate between MTB isolates with a low number of *IS6110* copies.

The DR is composed of multiple numbers of a 36bp sequence that are interspersed by unique spacers 35-41 in length. One DR and adjacent spacer sequence together is called direct variable repeat (DVR). Gronen *et al* (1993) have developed a reverse blot technique, termed spoligotyping, to determine

the presence or absence of DVR polymorphisms, a technique that can be used to differentiate different strains of mycobacteria (12).

MIRUs (40-100bps) consist of repetitive elements found in tandem repeats scattered throughout the genome of MTB complex. Variations in nucleotide sequence and copy number of the tandem repeats have been identified. The five exact tandem repeats, known as variable number of tandem repeats (VNTRs) used previously as a genotyping tool, are also part of MIRUs.

1.4. PATHOPHYSIOLOGY OF TUBERCULOSIS

The pathogenesis of tuberculosis can be considered as a series of battles between the host and the tubercle bacillus. When inhaled, only fine particles containing 1 to 3 tubercle bacilli are capable of starting the infection because they remain suspended in the air stream that enters the alveolar spaces (13). The heavier particles that get impinged on the mucosal surfaces of both the nasopharynx and bronchial tree are moved up the bronchial tree by cilia and expelled. Tubercle bacilli may vary in virulence both genotypically and phenotypically. Pulmonary infection begins only after a strongly endowed bacillus is ingested by a weakly endowed alveolar macrophage, initiating a cascade of events that results in either successful containment of the infection or progression to active disease (primary progressive tuberculosis).

Tuberculosis progresses in 5 stages (14, 15, 16, 17, 18):

Stage 1 of Pulmonary tuberculosis: Its Onset

The 1st stage of tuberculosis begins after the bacillus is inhaled into the alveolus. The alveolar macrophage (AM) ingests the inhaled bacilli and often destroys it. This destruction depends on the inherent microbicidal power of the AM and the genetic and phenotypic virulence of the ingested bacillus. Most AMs are activated cells. They have been activated non-specifically by many stimulants, including the ingestion of a variety of inhaled particles.

Stage 2 of Tuberculosis: Symbiosis

If the original AM fails to destroy or inhibit the inhaled particle, the bacilli multiply until that macrophage bursts. The early exudate contains chemotactic factors that attract circulating monocytes, lymphocytes and neutrophils, which further ingest the multiplying bacilli. Granulomatous focal lesions, composed of macrophage-derived epithelioid giant cells and lymphocytes, begin to form. Generally, the process of granuloma formation serves as an effective means for containing pathogens, preventing their continued growth and dissemination. Its success depends on both the number of macrophages at the site of infection and on the number of organisms present. A host's initial resistance to *M.tuberculosis* infection is directly proportional to granulomatous response. In time, macrophages from the circulation become completely responsible for the fate of the early lesion. In such a lesion, the alveolar macrophages rarely participate, because they

remain peripheral rather far from the bacilli, which are almost always located centrally (19). A symbiotic relation develops in which neither the macrophages nor the bacilli injure each other. The new macrophages have not yet been activated so, they cannot inhibit or destroy the bacilli and the bacilli cannot injure the macrophages because the host has not yet developed tissue damaging DTH. NK cells defense occurs early during the second stage and continues thereafter, but the broadly specific immunologic T-cell defense occurs soon after the second stage.

Stage 3 of Tuberculosis: Early stages of caseous necrosis and DTH

The third stage of the disease takes place 2 to 3 weeks after infection when the early specific immune response occurs. This response is primarily tissue damaging DTH. It kills macrophages that have allowed the tubercle bacillus to grow logarithmically in their cytoplasm, thereby producing the first stages of necrosis. The favorable intracellular environment for bacillary growth is eliminated and replaced by extracellular environment of solid caseum in which the bacilli cannot grow. Caseous necrosis as stated earlier is a DTH reaction produced by T cells, especially Cytotoxic CD8 cells (20, 21, 22) Clotting factors, anoxia, cytokines (23, 24), reactive oxygen and nitrogen intermediate from macrophages, (25, 26) Ag-Ab complexes, complement products and toxic products released from the bacilli all contribute in the formation of necrosis. Bacilli cannot multiply because of anoxic conditions, reduced pH and the presence of inhibitory fatty acids. Thus, the host locally

destroys its own tissues to control the uninhibited growth of bacilli which otherwise would be lethal to host (27). Only after such control is established can CMI prevent the progression of the disease. Later in the course of the disease, DTH may indeed be more harmful than beneficiary causing lung cavitation.

Stage 4 of Tuberculosis: Interplay of CMI and Tissue damaging DTH

During the fourth stage, the disease may become clinically apparent; atleast by radiograph or it may be arrested with little visible evidence remaining except for a positive tuberculin skin test. The fate of the caseous tuberculous lesion is mainly controlled by CMI. In resistant host, the caseous center of the lesion is surrounded by microbicidal macrophages developed by strong CMI (28). The bacilli that escape caseous center are therefore ingested and destroyed by the highly activated macrophages. Eventually the tubercle is walled off, the caseous center inspissates, and the disease is arrested, frequently for a lifetime. Unless, there is a subsequent defect in CMI, the infection generally remains contained and active disease may never occur. The few bacilli that enter the lymphatics or the bloodstream are rapidly destroyed at their site of lodgement by accelerated tubercle formation (a rapid CMI response producing many locally activated macrophages).

In susceptible hosts the caseous center is surrounded by poorly activated macrophages, and hence they permit the escaping bacilli to grow

intracellularly (14). These macrophages are again killed by DTH and the caseous center enlarges. Thus throughout the fourth stage, due to development of weak CMI, the host uses DTH to stop intracellular growth of tubercle bacilli in poorly activated macrophages and this process leads to further necrosis.

Stage 5 of Tuberculosis: Liquefaction and cavity formation

This stage follows if the bacilli evade the host defenses whereby liquefaction of the caseous center is followed by cavity formation (29). In the caseum, which is an excellent growth media, the bacilli grow uncontrollably and enter the bronchial tree followed by spread to other parts of the body and to the environment. Lung cavities are responsible for both; transmission of the bacilli as well as drug resistance. Macrophages do not survive in liquefied or solid caseous material, a reason why activated macrophages are completely ineffective at this stage. However, appropriate antimicrobial therapy can markedly reduce the number of bacilli and in time arrest the disease.

1.5. IMMUNOLOGY OF TUBERCULOSIS

1.5.1. Cell Types involved in the Immunological Reaction in Tuberculosis

1) Lymphocytes: their subgroups and their functions

Lymphocytes provide the immunologic specificity for the host's CMI and DTH. In tuberculosis, T cells appear to have two functions: 1) killing poorly activated macrophages in which the bacilli are multiplying and 2) producing cytokines that activate macrophages so that these macrophages can now kill

ingested bacilli. The bulk of experimental and clinical data suggest dominant role for CD4⁺ T cells in immune defenses against tuberculosis while CD8⁺ cells constitute the major cytolytic T cell defenses. B cells increase the production of IFN- γ by NK cells and via antibody-dependent, cell mediated cytotoxicity (ADCC), confer specificity to the killing of bacilli laden macrophages by NK cells (30).

CD4⁺ T cells can be divided into two subsets on the basis of cytokines they produce. i.e. Th1 and Th2 (31). The Th1 subset produces IL-12, which causes T-cell proliferation, as well as IFN- γ (which downregulates the Th2 response) and TNF- α both of which activate macrophages. The Th2 subset produces IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13, all of which promote antibody production by B cells. Th2 cytokines namely: IL-4, IL-10 and IL-13 downregulate Th1 response. IL-12 is to date, the major cytokine that specifically expands the Th1 population and upregulates its functions. IL-12 plays an important part in producing the CMI and DTH responses that control the pathogenesis of tuberculosis. Cytokines provide two-way signaling system between T cells and macrophages that apparently control the differentiation of both cell types into their various sub-types (32). CD4⁺ cells is also known to contribute to the immune defenses through direct cytolysis of macrophages and non-phagocytic cells infected with *M.tuberculosis* having low antimycobacterial potential, releasing bacilli to be engulfed by activated macrophages. Cytolytic

CD4⁺ T cells may also play a scavenger role by lysing dead macrophages, which are then metabolized by surrounding mononuclear cells (33).

Cytotoxic T-lymphocytes: CD8⁺ cells

CTLs are produced in thymus and most of CTLs are CD8 cells. CTLs recognize peptide antigens in context of MHC. CTLs require activation by exogenous antigens and cytokines to develop their full cytotoxicity. Most exhibit a Th1 cytokine profile (34). NK cells and CTLs kill the target cells by perforin dependent and perforin independent lytic pathways (35). In the later it involves apoptosis produced by cross-linking target cell Fas molecules with Fas ligands on NK or CTLs. CD4⁺ and CD8⁺ cells play complementary roles in the immune response to *M.tuberculosis*. CD4⁺ T cells may produce cytokines that activate macrophages to kill most mycobacteria and produce sufficient IL-2 to activate CD8⁺ T cells, which in turn lyse mycobacterium-infected cells.

γ/δ T-lymphocytes:

Most T cells have specific antigen α/β receptors. However, a subset of T cells exist with antigen γ/δ receptors. These lymphocytes are more prominent in organs directly exposed to the antigens from the outside environment, such as lung, intestine and skin. γ/δ T-lymphocytes produce IL-2 and IFN- γ and they can lyse antigen pulsed target cells (36). Their cytotoxic effects on non-activated macrophages containing tubercle bacilli probably play an important

role in limiting the symbiotic stage of tuberculosis. γ/δ T-cells do not require antigen processing or the presentation of antigenic peptides in a class I or class II MHC-restricted fashion (36). They also recognize non-protein antigens. By means of their cytokines, they influence and help regulate macrophages, NK cells, the Th1 and Th2 responses of α/β T-lymphocytes, and the IgE and IgG production of B-lymphocytes. Both NK cells and γ/δ cells are important in host defense during early stages of tuberculosis, whereas α/β T cells are important in the later, more immunologically specific stages (37). Finally γ/δ T-lymphocytes seem to play a role in the prevention of clinical tuberculosis in healthy tuberculin-positive personnel who are in constant touch with patients with tubercle bacilli in their sputum (37).

2) Natural killer cells:

NK cells make up to 10% to 15% of the blood mononuclear cell population. They proliferate in response to both monocyte and T cell derived cytokines (38). NK are defined as Cytotoxic cells that have the morphology of large granular lymphocytes but do not express surface CD3 or many other T-cell receptors. They are effective against cells that have no MHC class I expression (39). Macrophages containing intracellular microorganisms produce $\text{TNF-}\alpha$, IL-12, $\text{IFN-}\alpha/\beta$, IL-15 and IL-18 (40) that activate NK cells (41). NK cells then produce $\text{IFN-}\gamma$ that 1) activate macrophages non-specifically early during the infection and 2) favor a Th1 specific response over a Th2 immune response (42). NK cells also directly lyse cells in which tubercle bacilli are multiplying.

NK cells are probably a major force in antigen-independent host resistance that occurs early during infection before specific immunity develops.

3) Macrophages:

Non-activated macrophages /monocyte allow tubercle bacilli to multiply within them while highly activated macrophages destroy or inhibit tubercle bacilli. Activated macrophages produce reactive oxygen and nitrogen intermediates, lysosomal enzymes, and other factors that kill and digest tubercle bacilli. Macrophages produce both IL-12 a major Th1 activator, and IL-10, a major Th1 inhibitor. They produce IL-12 especially in the presence of *M.tuberculosis* and hence IL-12 plays an important role in producing the CMI and DTH that control the pathogenesis of tuberculosis.

4) Role of antibodies

The introduction of foreign antigen into an animal by natural infection or inoculation elicits a number of reactions in the immune system, one of which is production of antibodies. This requires the cooperation of B and T lymphocytes and the macrophages by which the B cells proliferate into antibody secreting plasma cells. Whole mycobacteria having complex antigenic structure and consisting of many different antigenic determinants, elicit a polyclonal response resulting in a large population of different antibodies. In progressive mycobacterial diseases the titre of antibodies tend to increase as the cellular immune response begins to diminish (43). Antibodies against mycobacterial antigens have been demonstrated in IgG,

IgM and IgA classes however, these specific immunoglobulins seem to play little or no role in the immunity of tuberculosis (44).

For several years, the detection of antibodies to mycobacterial antigens in circulation have been used as serodiagnostic test for the detection of active tubercular disease, as there were statistical differences found in antibody levels in patients with active disease and healthy controls. However, a large overlap is always evident as antibody levels are high in normal individuals residing in endemic areas, in BCG vaccinated individuals, in those exposed to environmental mycobacteria and in those who have been treated with antitubercular drugs (45,46,47). Nevertheless, with advances made in science and technology and with the availability of purified antigens, detection of antibodies in tuberculosis can prove to be a useful diagnostic tool.

1.5.2. The Immunological Reaction in Tuberculosis.

A) Binding and uptake of *M.tuberculosis*

M.tuberculosis is thought to enter the macrophage via specific binding to several distinct cell surface molecules. *M.tuberculosis* can bind directly via complement receptors i.e. CR1, CR3 and CR4 and the macrophage mannose receptor (MMRc) (Fig 1.1). IFN- γ has been shown to down-regulate MMRc expression while concomitantly increasing the capacity of the cells to kill microorganisms (48), in contrast IL-4 is a potent enhancer of MMRc

expression. Protein A, CD14 ligand are also involved in uptake of nonopsonized *M.tuberculosis* (49, 50).

B) Intracellular fate of *M.tuberculosis*

Following attachment and subsequent phagocytosis of the bacilli, sustained intracellular bacterial growth depends on the ability to avoid destruction by lysosomal enzymes, ROI and RNI. *M.tuberculosis* appears to have the ability to disrupt the normal functioning of phagosomes, preventing them from developing into acidic hydrolase-rich compartments. Several laboratories have reported a failure of mycobacterium containing vesicles to fuse with endosomal vesicles (51). Production of significant amount of ammonia and ammonium chloride by the bacilli diminishes the potency of the intralysosomal enzymes via alkalinization (52). Also presence of sulphatides and lipoarabinomannan in the cell wall inhibits phagosome-lysosome fusion (53). Moreover, containment of viable *M.tuberculosis* within these specialized vesicles may reduce the capacity of mycobacterial antigens to be processed, and/or get transported to the cell surface (54).

C) Microbicidal Action of Macrophages

Killing of ingested *M.tuberculosis* would most likely take place within macrophage phagolysosomes. Toxic constituents found within the acidic vesicle include lysosomal hydrolases, ROI such as H_2O_2 and O_2^- , and RNI such as NO and NO_2^- . In macrophages, NO and other RNI are derived from

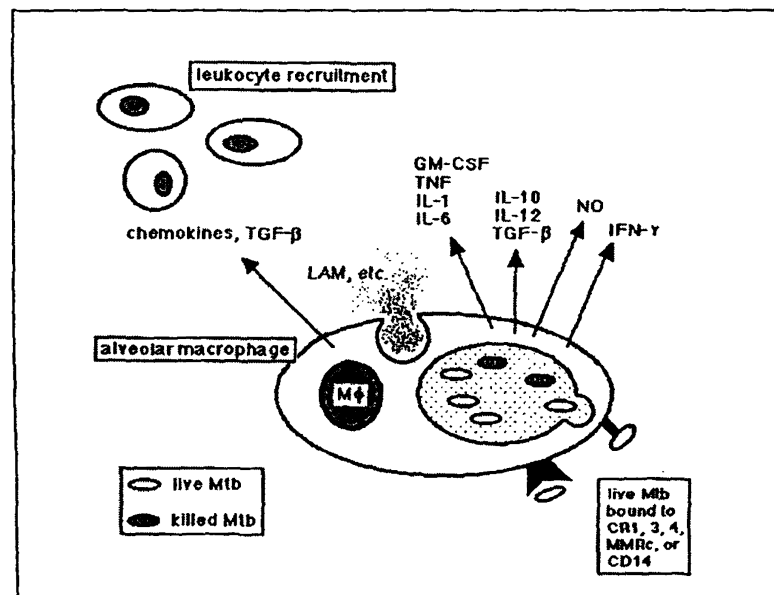
L-arginine via an enzymatic pathway controlled by an inducible nitric oxide synthase (55). TNF and IFN- γ are potent activators of *iNOS*, while IL-4 and IL-10 suppress it (56,57). Growth inhibition of mycobacteria by cytokine-stimulated murine macrophages strongly correlates with the generation of ROI (58). ROI alone may be insufficient to destroy *M.tuberculosis*, but ROI combined with RNI can significantly enhance mycobacterial killing (59).

Several mycobacterial products including LAM can scavenge ROI or inhibit the respiratory burst that generates them (60). Another mechanism by which the bacilli could evade the toxicity of ROI is to avoid binding the macrophage cell surface components such as Fc receptors that would provoke an oxidative burst; instead, it binds via complement receptors known not to trigger ROI production (61).

The capacities of resident alveolar macrophages and recruited monocytes to destroy mycobacteria differ significantly and progressively throughout the course of an infection. Initial infection by *M.tuberculosis* rapidly leads to the activation of alveolar macrophages, the induction of cytokines, which serve to limit the growth of the ingested organisms and the recruitment of additional leukocytes from the peripheral circulation. Enhancement of macrophage microbicidal function can be exerted by T cells, especially CD4⁺ $\alpha\beta$ ⁺ T cells that secrete IFN- γ and IL-2 (62). IFN alone cannot activate macrophages sufficiently to inhibit *M.tuberculosis* replication, although combined exposure

to IFN- γ and TNF was reported to be sufficient for effective killing. While activated alveolar macrophages may kill *M.tuberculosis* effectively, immature monocytes recruited from the periphery are thought to be less effective. The local production of IFN- γ and TNF by leukocytes is critical for the differentiation and activation of these recruited monocytes. Other macrophage-derived cytokines that may activate human cells to kill mycobacteria include GM-CSF and IL-12 (63, 64). In contrast, IL-1 and IL-6 have been reported to increase intracellular mycobacterial growth (Fig 1.1).

Fig 1.1: Macrophage responses to *M.tuberculosis*.



D) Presentation of *M.tuberculosis* antigens by macrophages

The main APCs for T cells are the dendritic cells located in the marginal zones of lymph nodes while dendritic cells in the lymphoid follicles are the major APCs for B cells (65). Macrophages serve as important antigen-presenting

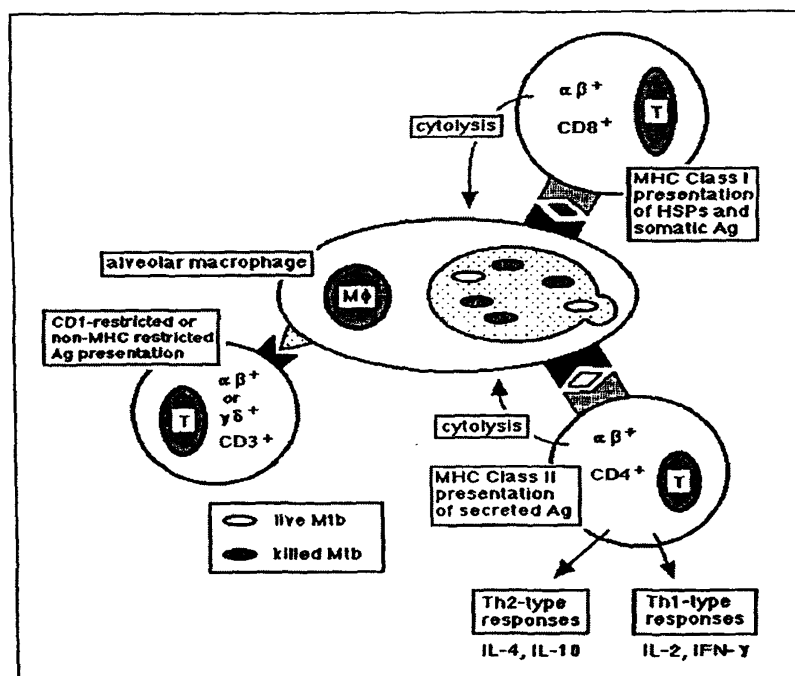
cells in the host response to *M.tuberculosis*. Mycobacterial antigens expressed in association with MHC class II or class I molecules can be recognized by CD4⁺ and CD8⁺ $\alpha\beta$ TCR⁺ T cells respectively. MHC independent recognition of *M.tuberculosis* antigens may be characteristic of the response of $\gamma\delta$ TCR⁺ T cells. Viable *M.tuberculosis* bacilli induce the replication of human peripheral blood T cells in a manner that favors the expansion of $\gamma\delta$ T cells (66). In contrast, PPD from *M.tuberculosis* predominantly induces a CD4⁺ $\alpha\beta$ T cell response with no or little increase in $\gamma\delta$ T cells. $\gamma\delta$ T cells have been shown to recognize nonpeptide ligands. CD1b restricted presentation of both mycolic acids and LAM to CD4⁺ CD8⁻ $\alpha\beta$ T cell clones has recently been demonstrated.

E) Interactions between T cells and macrophages

As shown in Fig 1.2, collaboration between macrophages and T cells is essential for eradication of *M.tuberculosis* via antigen-specific DTH responses. Initially, activated macrophages present mycobacterial antigens to T cells in association with MHC class I or class II proteins or with nonpolymorphic MHC-like proteins (CD1). Macrophage derived cytokines play critical roles as costimulatory molecules for antigen-specific T cells. Subsequently, cytokines produced by activated T cells further modulate macrophage function, although the net result may be either to inhibit or to augment intracellular *M.tuberculosis* growth. The presence of both antigen specific T cells and activated macrophages within the granuloma provides long-term surveillance and containment of infected macrophages. IL-12 may be important in

favoring the development of the DTH response by enhancing production of IFN- γ , facilitating the development of Th1 cells and augmenting the cytotoxicity of antigen-specific T cells and natural killer cells. In contrast Th2 responses may contribute to the immunosuppression often observed in advanced disease. This is particularly apparent in the context of TB patients coinfecting with HIV who have diminished Th1-type responses compared with HIV-seronegative patients. Macrophages are likely to contribute further to these responses through the production of the immunosuppressive cytokines IL-10 and TGF- β 1. Thus the cytokine network controls a complex set of responses that may be growth modulatory to *M.tuberculosis*, proinflammatory, or immunosuppressive.

Fig 1.2: Interactions between lymphocytes and *M.tuberculosis* infected macrophages.



1.6. IMMUNOGENETICS OF TB

In 1949, Haldane suggested that infectious diseases had been the main agent of natural selection for at least 5000 years and were responsible for the maintenance of the majority of human protein polymorphisms (67). Epidemiological data derived from twin studies clearly indicate that the susceptibility to TB has genetic determinants (68). Genes for HLA system have been extensively studied for association with susceptibility or resistance to mycobacteria. Studies of association between HLA and TB generally have resulted in variable conclusions. However, HLA-DR2 was shown to be associated with increased risk of progression to advanced pulmonary disease while HLA-DRB1* 1501 allele was associated with advanced disease and failure to respond to drug therapy whereas HLA-DRB1*1502 allele was shown to be associated with decreased risk (69). Recent interest also has focused on the role of polymorphisms in cytokine promoters (e.g. TNF- α) and cytokine receptors (e.g. IFN- γ) in susceptibility to mycobacterial disease (70).

The active metabolite of Vitamin D: (1,25D₃) activates monocytes, stimulates CMI and suppresses immunoglobulin synthesis. These effects are exerted by interaction with the vitamin D receptor (VDR), which is present on monocytes and on activated B and T lymphocytes. Experimental findings have led to the hypothesis that polymorphism in the gene for VDR may be involved in genetic susceptibility to tuberculosis (71, 72). These findings raise the

possibility that vitamin D supplementation may modify the immunity to tuberculosis.

Mannose binding lectin: MBL is a calcium dependent serum lectin which acts as an opsonin to promote phagocytosis and which can activate complement via the classical pathway. Three co-dominant single-base substitutions results in reduced serum MBL concentrations and individuals with two mutant alleles have extremely low circulating MBL levels. In a study conducted in Gambia, it was found that the MBL allele with a variant codon was relatively under represented among tuberculosis cases as compared to healthy donors (73). This result will require confirmation in other populations, but it would be fascinating if MBL deficiency, which is frequently described as world's commonest immune deficiency, has been selected for by heterozygote resistance to tuberculosis.

Among inbred strains of mice resistance to infection with *M.bovis* BCG is controlled by gene designated as *Nramp1* (74). The human homologue of *Nramp1* designated *NRAMP1* has been cloned and mapped to chromosome 2q35 (75). In a population study, four polymorphisms in the *NRAMP1* gene were found to be associated with tuberculosis (76). This demonstrates that *NRAMP1* is an important gene in individual variability in susceptibility to tuberculosis.

Association between genetic polymorphisms and tuberculosis susceptibility differ according to ethnic origin but the extent to which genetic polymorphisms contribute to the global tuberculosis burden is unclear because of the great difficulty of separating lifelong environmental influences from genetic predisposition.

1.7. CLINICAL MANIFESTATIONS

1.7.1. Primary Progressive Pulmonary TB

The initial infection with the tubercle bacilli is known as primary infection and affects the middle or lower lung zones with the involvement of the draining lymph nodes. This forms the primary or Ghon's complex, while all other lesions are post primary. The clinical manifestations vary depending on the age of the patient occurring more often in children than in adults. In approximately 10% of the patients, the initial infection with *M.tuberculosis* is manifested by pleural effusion. Local progression of the initial pneumonia with or without caseation and cavity formation may occur called progressive primary tuberculosis.

1.7.2. Post-Primary Pulmonary Tuberculosis

There are two routes to a repeat episode of TB (post primary pulmonary TB): either by inhalation of additional *M.tuberculosis* organisms or by reactivation of a dormant primary lesion. The existence of post-primary TB means that an infection can proceed inspite of existing immunity.

In reinfection TB, a hypersensitivity reaction is the characteristic response accompanied by tissue necrosis and caseation. In an attempt to seal off the necrotic site, lymphocytes and other cells converge upon the site and direct formation of a wall of fibrous tissue. Some lymphatic spread may occur, but in this case the hilar lymph nodes are not implicated. Most often caseated granulomas heal over a period of time, shrinking as they become fibrotic and then calcified. However if healing is impaired, the growing lesions may erode adjacent bronchi, resulting in the formation of cavities. *M.tuberculosis* organisms multiply freely in these cavities leading to huge numbers of bacilli. An open cavitated lesion can leak infectious material directly into the bronchus, resulting in the continuous discharge of bacilli into the sputum. Leaked bacilli can also be inhaled into other portions of the host's lungs, resulting in tuberculous bronchopneumonia. If the growing granulomatous lesion erodes the wall of a vein, organisms can spread in the circulating blood, resulting in miliary disease (77).

M.tuberculosis can persist for decades in a dormant state inside a granuloma, particularly in the apical regions of the lung. Although the effective host immune response can initially contain *M.tuberculosis* infection, several factors can trigger subsequent development of active disease from reactivation of remote infection. HIV is the greatest single risk factor for progression to active disease in adults. Other medical conditions that can also compromise the immune system and predispose to development of active disease include

poorly controlled diabetes mellitus, renal failure, underlying malignant disease, chemotherapy, malnutrition, deficiency of vitamin A or D (78), defects in the production of IFN- γ or TNF- α (79) etc. Reactivation of these latent organisms can also lead to post-primary disease, even in persons who successfully fought their initial battle against TB. The mechanisms, which govern dormancy and reactivation, within either the organism or the host, are not yet understood. The host typically mounts an inflammatory response to reactivation TB, with the lesion appearing circumscribed and necrotic.

1.7.3. Pulmonary and Extrapulmonary tuberculosis

The most common clinical manifestation of tuberculosis is pulmonary disease. Extrapulmonary tuberculosis accounts for about 20% of disease in HIV-seronegative people but is more common in HIV-seropositive individuals (80). Among people not infected with HIV, extrapulmonary disease, particularly lymphatic tuberculosis, is particularly common in women and young children (81).

Pleural tuberculosis occurs as a result of either pulmonary progressive *M.tuberculosis* infection or reactivation of latent infection. A chest radiograph generally reveals a unilateral pleural effusion. Unlike other clinical manifestations of tuberculosis, pleural disease probably represents an increased, rather than diminished, immune response. Infact, primary serofibrinous pleural effusion resolves without treatment in upto 90% of

cases; however, if untreated, nearly two-thirds of patients will subsequently have relapses with tuberculosis at other organ sites (82).

The most serious clinical manifestation of tuberculosis is involvement of the CNS. Such involvement can include inflammation of the meninges, as well as space occupying lesions (tuberculomas) of the brain. The clinical manifestations are due to the presence of *M.tuberculosis* as well as the inflammatory host immune response. Children under 5 years of age and HIV-infected individuals are at increased risk of tuberculous meningitis (83), which can present clinically as chronic meningitis, with headache, fever, and changed mental status. Neurological manifestations can include cranial nerve palsies and motor, sensory and cerebellar defects. According to the location of the tuberculomas; seizures can also occur. Meningitis is fatal in almost all cases without chemotherapy, and prompt identification and treatment are essential to prevent serious neurological sequelae.

Tuberculosis can affect any bone or joint, but the spine (i.e. Pott's disease) is the most common bony structure involved. In the spine, the most common location is the thoracic section. Vertebral body involvement can be followed by disease of an adjacent intervertebral disc.

Genitourinary tuberculosis is uncommon and is difficult to distinguish from other infections of the genitourinary tract. In men, manifestations include

those of prostatitis or prostate enlargement, epididymitis and orchitis, but disease can also be present as a painless scrotal mass. Urine analysis may show red or white blood cells, or both, with a negative urine culture for bacteria (sterile pyruvia).

Disseminated tuberculosis is defined as involvement of many organs simultaneously and can occur as a result of primary progressive disease or reactivation of latent infection. Mortality in disseminated tuberculosis is high despite chemotherapy and may be related to delays in diagnosis and other commonly present medical conditions (81).

1.7.4. Tuberculosis and AIDS

The Global Programme on AIDS estimated that 16 million adults and 1 million children were infected with HIV by mid-1994, with nearly 90% of infections occurring in the developing world. The interaction between TB and HIV infection was investigated initially because of the high incidence of TB and HIV co-infection in sub-Saharan Africa and other parts of the developing world, which together accounted for 95% of new TB cases and 98% of TB deaths (84).

1.7.4.1. Clinical TB disease in AIDS patients.

Patients infected with MTB and HIV are more likely to progress rapidly to clinical TB (85), with an annual risk of 8% compared to a lifetime risk of 10%

for HIV seronegative subjects (86). TB results primarily from reactivation of endogenous infection, usually many years after primary infection. In developing world TB precedes HIV infection, and reinfection occurs against a high background of endemic disease. Pulmonary disease is the most common presentation in HIV seronegative patients but in HIV infected individuals, focal and disseminated extrapulmonary disease is more common. In immunosuppressed patients, clinical disease may be present with atypical clinical and radiological features including fewer lung cavities.

1.7.4.2. Immunology of TB and HIV coinfection.

CD4⁺ cells have an important role in inducing cytotoxicity, leading to lysis of infected monocytes and to panoply of cytokines released. Two subsets of T-helper CD4⁺ lymphocytes, TH1 and TH2, co-ordinate the overall response and inhibit the presence of the other subset. TH1 cells secrete IFN- γ and other cytokines responsible for macrophage activation in response to mycobacterial antigens, whilst TH2 cells produce IL4, 5 and 10 that stimulate B cells and antibody production. As HIV disease progresses, the number of TH1 cells declines, correlating with a reduction in macrophage activity against mycobacterial antigens, the loss of Langhans giant cells, epithelioid macrophages and loss of granuloma formation, resulting in a shift from a paucibacillary to a multibacillary state (87, 88).

Conversely, HIV replication may be enhanced by the multiplication of MTB in monocytes or macrophages through the up-regulation of transcription, or increased release of TNF- α and β 2-microglobulin (89, 90, 91). The levels of HIV-1 p24 antigen in culture supernates of monocytes taken from patients with active pulmonary TB who were infected subsequently with HIV-1 were significantly higher than for culture supernates of monocytes from non-tuberculous individuals (92). Enhanced susceptibility to HIV infection or reproduction may be a direct result of monocyte activation by exposure to MTB.

1.7.5. MDR TB

The epidemic of AIDS and TB are complicated by a third epidemic of MDR-TB. This has been defined as a case of tuberculosis caused by a strain of *M.tuberculosis* that is resistant to two or more antituberculosis drugs. Factors that contribute to increased incidence of MDR-TB include the AIDS pandemic, deterioration of public health infrastructure and non-compliance by tuberculosis patients, which is associated with homelessness, poverty, alcoholism and drug users. In persons with HIV and MDR-TB the outcome is dismal as the combination of decreased immunity and the loss of most effective antituberculosis drugs make effective treatment difficult and lengthy leading to prolonged periods of infectivity and high mortality rate.

1.8. PROPHYLAXIS

Two prophylactic measures are available. Immunization, or Bacille Calmette-Guerin (BCG) vaccination must be considered for the uninfected when there is a high risk of becoming infected. Preventive therapy or chemoprophylaxis is directed at those already infected, especially when the risk of infection is slight or where the risk of disease, given infection, is high.

1.8.1. BCG vaccination

In 1908, Calmille Guerin and Albert Calmette initiated their attempts to produce an antituberculosis vaccine from a virulent bovine strain. Thirteen years and 230 subcultures later, an attenuated bacillus unable to form progressive TB in various animal models was developed.

Vaccination against tuberculosis currently consists of the intracutaneous inoculation of BCG. Adequately controlled trials on its usefulness and efficacy were reported only after millions had been vaccinated. Unfortunately the results of these trials do not agree. In the 20 years after vaccination, the British Medical Research Council found 77% less tuberculosis among the vaccinated children than the unvaccinated controls (93), and the trials by US Public Health Service showed a 29% reduction attributable to BCG in Puerto Rico (94). During a 15-year period after vaccination, a large and well-conducted trial in South India showed a 12% reduction among vaccinated persons below 15 years of age (95) but a 21% increase among older vaccinated persons.

Although large variation has been found, the major conclusion drawn from these trials is that BCG protects against childhood manifestations of TB (military or meningeal) whereas the efficacy in preventing pulmonary TB is very limited (96, 97).

Several explanations have been suggested for the variation in the results of above-mentioned trials. From a review of multiple studies, it seems likely that discrepant results of the various trials are largely due to differences in the potency of the strains (98, 99). This might be because after the first vaccination, lots of BCG was distributed worldwide and propagated in various laboratories resulting in both phenotypic and genotypic differences not only compared to the original parent strain but also between various daughter strains (100, 101). Recent data indicate that a large number of mutations have taken place during the long invitro propagation of this strain (102, 103). It is known that these mutations have resulted in the deletion of open reading frames encoding several important T-cell antigens such as the immunodominant molecules ESAT-6 and CFP-10 (104).

The hypothesis that has gained most widespread acceptance attributes this variation to higher levels of exposure to environmental mycobacteria in the areas where the vaccine affords no protection (105). The precise nature of this interference is not known, however, based on animal experiments, it has been suggested that the protection provided by environmental mycobacteria partly

masks the effect of a subsequent BCG vaccination or that environmental mycobacteria have a direct influence and skew the immune response towards a Th2 direction (105, 106).

It has been known that susceptibility to tuberculosis is determined in part by genetic factors (68, 107, 108) and thus it has been reasoned that there might be genetic differences in response to BCG vaccination.

Studies by Lurie *et al* 1964 (14) and Sutherland *et al* 1979 (110) provide evidence that BCG imparts greater protection against disease than against primary infection with the tubercle bacilli. It produces localized ulcerations that are limited in extent and duration. However, drainage from these ulcerations contains viable organisms, an important consideration for immunosuppressed patients.

One of the most important disadvantages of vaccination is the production of sensitivity to tuberculin. In areas with a high risk of infection, most persons become tuberculin reactors in the course of time and the premature production of tuberculin sensitivity by vaccination is rarely of any consequence, however, in areas with a low risk of infection, interference with the diagnostic value of the tuberculin test constitutes an added contraindication to BCG vaccination.

1.8.2. New Vaccine Developments

Exploiting the benefits of new vaccine technology, a number of novel methods for improving or substituting the existing BCG vaccine have appeared over the last 5 years. Development in vaccines for tuberculosis has been summarized below:

Table 1.2: Summary of new Tuberculosis vaccine development (111)

Vaccine types	Examples	Results
Avirulent/ Saprophytes	<i>M. vaccae</i>	No activity (m)
	<i>M. microti</i>	Marginal activity (m, gp)
	<i>M. smegmatis</i>	No activity (m)
Auxotrophs	<i>M. tuberculosis</i> BCG	As protective as wild type but may not persist long enough to generate adequate memory (m).
Recombinant	BCG	Good as BCG (m)
Subunit	Culture filtrates	Good activity (m, gp)
	Individual proteins	Marginal activity (m, gp)
	Protein mixtures	Good activity (m, gp)
DNA vaccines	Ag 85	Promising results in short and
	Hsp 60	long term assays (m, gp).
gp: guinea pig, m: mouse		

It is obvious that vaccination must be accompanied by other means of controlling tuberculosis viz., diagnosis, effective treatment and raising living and educational standards of the society.

1.9. TREATMENT

In a patient with tuberculosis disease, there are three populations of MTB organisms that need to be considered (112). The first population is the actively growing extracellular organisms. Huge numbers of organisms can grow extracellularly in pulmonary cavities, and it is in this population that drug resistance develops readily. The second population consists of slow growing or intermittently growing organisms that are inside macrophages. There are fewer of these organisms, but the intracellular environment is acidic and many drugs are not active in these conditions. The third population is made up of slow growing organisms that grow in caseous material. The environment is neutral in pH, but the penetration of drugs into this area may be compromised by a poor blood supply.

1.9.1. Standard First line Drugs, its mechanism and regimen

Five standard drugs used in the treatment of tuberculosis are Isoniazid (INH), Rifampicin, Ethambutol, Pyrazinamide and Streptomycin. The mechanism of action of INH is to inhibit a cell wall biosynthetic pathway, probably mycolic acid biosynthesis (113). Therefore, it is only bactericidal for actively growing extracellular organisms. Rifampicin (RIF) is the only drug that is bactericidal

against all three populations of bacteria. RIF inhibits the DNA-dependent ribonucleic acid (RNA) polymerase of MTB (114) and is able to interfere with RNA synthesis even when the organism has minimal metabolic activity. Pyrazinamide (PZA) is bactericidal only to intracellular organisms in an acidic environment (115). It is uniquely effective in eliminating potential persisters and is used during the first two months of chemotherapy to reduce the total length of therapy. Ethambutol (EMB) is only bacteriostatic (116) and although its mechanism of action is not completely understood, metabolic labeling experiments suggest it inhibits glucose incorporation into cell wall components of MTB (117). Streptomycin disrupts bacterial protein synthesis but is bactericidal against only actively growing extracellular organisms.

Almost all recommended treatment regimens have two phases on the basis of extensive evidence from controlled clinical trials. There is an initial intensive phase designed to kill actively growing and semi dormant bacilli followed by continuation phase. The continuation phase eliminates most residual bacilli and reduces the number of failures and relapses (118, 119). For each patient, the recommended regimen depends on the treatment category, which is based on severity of disease and history of previous treatment. The short course or 6 month regimen is preferred for the treatment of tuberculosis. This regimen consists initially of a 2-month period of treatment with INH, RIF and PZA followed by a 4-month period with INH, RIF and EMB or SM. Intermittent drug regimens can also be given.

In Extrapulmonary tuberculosis treatment is same as pulmonary tuberculosis except that it may constitute of longer courses of therapy. Special regimens should be charted out for pregnant and breast-feeding women, for patients with prior liver disease or renal failure and for HIV-infected patients (118, 120, 121, 122). In order to prevent relapse and drug resistance, clinicians must prescribe an adequate regimen and ensure that patients adhere to therapy. Widespread drug resistance is the result of over 40 years of effective antibiotics that have been ineffectively administered.

1.9.2. Development of drug resistance

Primary drug resistance involves transmission of an already resistant strain from a source case patient to a secondary case patient who has never previously received treatment for tuberculosis. *Secondary or acquired resistance* refers to the on-treatment emergence of resistant disease in a patient with an initial susceptible isolate. This typically occurs as a result of inadequate drug therapy, which may arise from patient noncompliance, from incorrectly selected medications, or from suboptimal drug dosing.

On developing drug resistance, a patient's drug susceptibility pattern is studied and second-line drugs are then included in the treatment schedule. Common second line drugs are Rifabutin, Ethionamide, Cycloserine, PAS, Clofazamine, Aminoglycosides (other than Streptomycin) and Quinolones. Rifabutin inhibits DNA-dependent RNA polymerase, Ethionamide and PAS

inhibit cell wall synthesis, Cycloserine inhibits folic acid pathway whereas Clofazamine binds to mycobacterial DNA. Aminoglycosides like Kanamycin, Amikacin, Capreomycin and Viomycin are bactericidal and they disrupt bacterial protein synthesis while Quinolones like Ciprofloxacin, Ofloxacin, and Levofloxacin disrupt the bacterial chromosome by inhibiting the supercoiling action of DNA gyrase.

1.9.3. DOTS

Tuberculosis is one of India's most serious health problems. Despite a long and distinguished tradition of TB research in India and the existence of a national programme since 1962, tuberculosis is a common cause of morbidity and mortality. DOTS known also as Revised National Tuberculosis Control Programme in India, was introduced in 1993. DOTS is a systematic strategy which has five components: 1) Political and administrative commitment, 2) Good quality diagnosis, 3) An uninterrupted supply of short-course chemotherapy drugs, 4) Standardized intermittent drug regimens administered under direct supervision and 5) Systematic monitoring and evaluation thus preventing the drug default problem, which is a major cause of MDR-TB. The RNTCP will be implemented in a phased manner in a population of nearly 300 million in the next few years. DOTS should be considered for all patients because there is no way to predict which patients will adhere to treatment (123). Many factors may influence patient compliance, including the patient's social characteristics, personality,

understanding of illness and understanding of the treatment, the patient-practitioner relationship and the practitioner's attitudes (124). When DOTS is not possible, pills containing a combination of tuberculosis medicines can be used to prevent a patient from taking a single drug and thus help prevent the development of drug resistance. e.g. Rifamate, Rifater.

1.9.4. Preventive therapy

Preventive therapy usually consists of the oral administration of isoniazid. The generally accepted regimen for preventive treatment is 5-10mg of isoniazid per kilogram of body weight, not exceeding a total dose of 300mg/day, given orally in a single daily dose for 6-12 months. In the U.S. Public Health service trials among household contacts and Alaskan villagers, the optimal duration of treatment appeared to be 9 to 10 months (125). A committee convened by the American Thoracic Society recommended that certain screening procedures be carried out before starting prevention therapy (126). The presence of active tuberculosis should be ruled out so also people who have already undergone an adequate chemotherapy course and those with previous side effects to isoniazid or acute liver disease should be excluded. Preventive therapy is recommended to people with known or suspected infection with HIV, to close contacts of persons with infectious tuberculosis or to those with increased tuberculosis risk. If patients have been infected with multi-drug resistant tubercle bacilli then multidrug preventive therapy should be considered. This should consist of at least two anti-

tuberculosis drugs depending on the resistance pattern of the organisms. Possible regimen would include a regimen of two drugs selected from Ethambutol, Pyrazinamide or a Quinolones prescribed for 12 months at standard dosages.

1.9.5. Development of new drugs and drug targets:

- Enzymes involved in synthesis of the complex cell wall components represent attractive potential drug targets.
- Presence of porin like molecule in cell wall of *M.chelonae* (127) and detailed analysis of the uptake mechanisms required for transport of nutrients across the cell wall has given new insights to design drugs. E.g. transporters specific for essential nutrients have themselves become targets for novel inhibitors.
- Concept of using a pro-drug, which is subsequently converted to active form within the bacteria, may represent a useful mechanism for achieving efficient drug uptake.
- Designing reagents that would interfere with development of generalized stress resistance and disrupt synthesis of stress proteins that help survival of some organisms during therapy.

1.10. DIAGNOSIS

Diagnosis, with respect to *Mycobacterium tuberculosis*, may be viewed as having two general components; the diagnosis of tuberculous infection with

or without concomitant disease and the diagnosis of the disease due to the organism (i.e.) tuberculosis. The diagnosis of tuberculous infection has traditionally been accomplished by means of tuberculin skin testing, while on the other hand, the diagnosis of tuberculosis has used a variety of clinical and microbiological methods.

1.10.1. Traditional Diagnostic Techniques

A) Confirmatory Direct Tests

i) Microscopy

Direct microscopy of sputum is the norm for diagnosis of tuberculosis in developing countries today. The thick impervious lipid cell wall of mycobacteria prevents easy staining. However, prolonged exposure to heat and surface active agents allow penetration of stains but once stained these bacilli resist decoloration even when exposed to concentrated acids or alcohols and hence are termed as acid fast bacilli. The acid fastness property is due to the ability to form acid alcohol stable complexes with certain aryl methane dyes like carbol fuchsin and fluorescent dyes like Auramine and/or Rhodamine (128).

AFB staining technique has the advantage of being relatively rapid and inexpensive to perform. Since the risk of transmission of tuberculosis is related to the number of bacilli in sputum specimen, microscopic examination of smears for the presence of AFB is often the first test to be performed by mycobacteriological laboratories (129).

Despite their advantages, AFB staining is limited. Microscopic examination with either Ziehl-Neelson or fluorochrome stained smears is insensitive, detecting acid-fast bacilli only when there are more than 10^4 organisms/ml of sputum. Further it does not provide information concerning the identification or viability of the organism.

ii) Culture and Biochemical identification

Culture remains the “gold standard” for diagnosis of tuberculosis. Growing cultures permits speciation and drug susceptibility testing. Most commonly used mycobacterial culture media include egg-based (Lowenstein Jensen), agar-based (Middlebrook 7H10 and Middlebrook 7H11) and liquid based media (Sautons, Longs, Kirchners, Youmans, Dubos etc).

A number of inspissated egg-based solid media including Lowenstein Jensen and Petragani have been used for primary isolation of tubercle bacilli from clinical specimens. They tend to yield a higher proportion of positives from direct clinical specimens than do semi synthetic agar media (130). The egg media are very rich and contain phospholipids and proteins that tend to bind to and/or neutralize toxic products in clinical specimens. However, this definitive diagnosis of *M.tuberculosis* from clinical samples is hampered by the slow growth of the tubercle bacilli. Traditionally, the identification of *M.tuberculosis* has relied on acid-fastness, niacin production, nitrate reduction and inactivation of catalase at 68°C.

B) Indirect Tests**i) Tuberculin Skin testing: Mantoux test**

Tuberculin testing is used to diagnose tuberculous infection in contrast with tuberculous disease. Its reactivity separates the infected person from the exposed without infection. The implication of a positive tuberculin test result is infection with tubercle bacilli.

Infection with *Mycobacterium tuberculosis* results in sensitivity to antigens derived from that organism. Tuberculin is composed primarily of tuberculo-protein obtained from cultures of the tubercle bacillus. When the material is injected intracutaneously, a classic delayed type hypersensitivity reaction occurs in the infected patient. The initial process of sensitization following infection takes about 6-8 weeks with sensitized T-lymphocytes developing in regional lymph nodes and entering the circulation. Restimulation of these lymphocytes by intracutaneous injection of tuberculin results in the indurated skin reaction. The induration is due to cellular infiltration mediated by the sensitized lymphocytes. The reaction is maximal at 48-72 hours and then slowly fades. Almost all infected patients yield a positive reaction.

This test is performed by injecting 0.1ml of PPD tuberculin (5TU) intracutaneously into the skin of the volar or dorsal area of the forearm. The test is read in 48-72 hours. The presence of induration is determined by touch

and the diameter of the induration is measured transversely. Currently, a reaction of greater than 5mm to 5TU PPD with the Mantoux test after 48 hours is considered positive for those with HIV infection, for those with chest films consistent with old nonactive tuberculosis and for those with recent, close contact with infectious tuberculosis cases. A reaction of greater than 10mm is positive in drug users, residents of long term facilities like prisons, nursing homes, mental institutions, children under 4 yrs of age, and those with conditions in which risk of tuberculosis is high like diabetes mellitus, silicosis and immunosuppressive therapy. In all others a reaction greater than 15mm is considered positive (131).

The tuberculin reaction produced by BCG vaccine cannot be distinguished from that of due to *M.tuberculosis* infection. While interpreting induration in vaccinated individuals, the age at which vaccination is been given is important (132). However, a negative reaction does not rule out tuberculous infection. Problems with unresponsiveness and anergy have been appreciated for many years. It can be due to chronic renal failure, malnutrition, bacterial or viral infection, HIV infection etc (133, 134).

ii) Imaging Procedures: Chest Radiographs

Because the majority of tuberculosis patients will have a pulmonary component to their disease, chest X-ray films and to a lesser degree, newer imaging procedures such as computed tomography of the chest have played

an important role in differential diagnosis and determining extent of tuberculosis. Despite the fact that no chest radiographic pattern is unique to tuberculosis and that an abnormality does not prove the presence of active disease, certain configurations like hilar and mediastinal adenopathy, fibronodular changes involving the apical or posterior segments of the upper lobes and/or superior segments of lower lobes, cavitation etc have traditionally been viewed as highly suggestive of tuberculosis. Nevertheless, in pre HIV era, as many as one third of newly diagnosed pulmonary tuberculosis patients were found to have atypical findings on chest radiograph (135, 136). The occurrence of tuberculosis among HIV co-infected persons has produced an even larger proportion of patients with unusual chest radiographic findings (137).

iii) Fluid and Secretion Analysis

Tuberculosis, particularly extrapulmonary disease, frequently produce aberrations in body fluids and hence, analysis of these fluids may be helpful in the diagnosis. Hematological changes of a wide variety have been reported in association with TB. Thus cytopenias, monocytosis, lymphocytosis, have all been attributed to tuberculosis (138, 139). Abnormalities of serum calcium have also been observed and its occurrence has been attributed to enhanced vitamin D metabolism by activated macrophages and monocytes. Pleural, peritoneal, cerebrospinal and joint fluid abnormalities associated with tuberculosis share several characteristics e.g. protein elevation, reduction in

glucose, domination of polymorphonuclear cells and lymphocytes over the first few days of involvement (140).

1.10.2. New Technologies

A variety of new methods and technologies have been applied in recent years to the diagnosis of tuberculosis. The impetus for their application has generally been a desire to make more rapid diagnosis.

A) Direct tests

i) Improved AFB detection

This technique involves an immunomagnetic separation strategy for capturing and concentrating mycobacteria from processed specimens (141). In this procedure, magnetic polystyrene microspheres coated with antibody to the mycobacteria are added to a sample; the beads and bound organisms are recovered using a magnet and captured cells detected by microscopic examination.

Murtagh and colleagues showed that magnetic beads coated with a rabbit IgG polyclonal antibody to LAM; could capture organisms from dilute suspensions of mycobacteria (approx 100/ml) and concentrate the organisms sufficiently to be detected by fluorescence microscopy. This sensitivity represents a 2-3-log improvement over the detection limit of the standard AFB smear (141).

ii) Radiometric assays and other rapid culture methods

Because the growth rate of *M.tuberculosis* on egg/agar media is slow, there has been a great interest in developing techniques that could identify tubercle bacilli more rapidly. The BACTEC 460 TB uses liquid Middlebrook 7H12 medium containing ^{14}C labeled palmitic acid for the radiometric detection of growth (142). The sediment from the processed samples is inoculated into BACTEC 12B bottle and when the organism grows, $^{14}\text{CO}_2$ is released into the headspace of the bottle and detected by the sensor in the system. Such systems can indicate the presence of growing mycobacteria weeks before conventional systems. One advantage for this system is that deoxyribonucleic acid identification and antibiotic susceptibility testing can be performed from this system.

A similar system of detecting mycobacterial growth using ^{14}C labeled acetate source in a biphasic vial assembly forms the basic principle of Radiorespirometry. In this method, radioactivity detected in a Liquid Scintillation Counter from accumulated $^{14}\text{CO}_2$ respired from metabolically active bacteria and absorbed on an alkaline absorbent paper is taken as index of growth. (143,144). However, one major drawback for such rapid radiometric systems is disposal of radioactive waste, which makes it impracticable for use as a routine procedure in all diagnostic laboratories.

The ESP Myco System consists of a bottle containing a modification of Middlebrook 7H9 broth with growth factors and cellulose sponge, which is thought to increase the surface area. It measures the change in oxygen consumption. The MB/BacT Alert uses bottles containing modified Middlebrook 7H9 medium with growth factors and a sensor at the base of each bottle, which detects CO₂ production via a colorimetric detection system. The MGIT tube system contains a 7H9-based medium with enrichment, antibiotics, and an oxygen-labile fluorescent indicator at the bottom of each tube. As mycobacteria grow and use oxygen, the indicator compound is excited and the resulting fluorescence can be visually examined with an ultraviolet source. An automated version Bactec 960 which also uses a fluorescent indicator is under evaluation.

iii) Molecular methods for detection and identification of *M.tuberculosis*

Because of the slow-growing nature of *M.tuberculosis*, prompt detection and identification has been a challenge for clinical microbiological laboratories. Extensive studies have shown that nucleic acid amplification methods are promising and may be a good solution to the problem. These methods include signal and target amplification systems. Ribosomal RNA or different parts of genomic DNA are selected as targets. Although many in house developed assays reported by academic scientists show promise, commercial tests have also demonstrated good performance. These commercial systems have less

carryover contamination, are more amenable to quantification, and provide high specificity (145).

iiia) Signal amplification methods

Is a procedure that amplifies the detection signal to an easily measured level. These methods include branched DNA signal amplification (146), Q β signal amplification (147) and reporter phage systems (148).

iiib) Target amplification methods.

Another strategy that increases the sensitivity of an assay is the amplification of a characteristic component of an organism to a detectable level, which includes PCR amplification of DNA and isothermal gene amplification of RNA (149, 150), Strand displacement amplification (151) and Ligase chain reaction (152).

For the PCR, oligonucleotide primers are used to direct the amplification of target nucleic acid sequences via repeated rounds of denaturation, primer annealing and primer extension. The specificity of this amplification lies in the choice of primers. Various PCR assays targeting different template sequences have been developed. Some of the most commonly used target sequences are *IS6110* (153), 16S rDNA gene (154), 65kDa protein gene (155) and MPB64 gene (155).

In commercially available Amplicor PCR assay, mycobacterial DNA is amplified with genus specific primers formulated on the basis of the 16srRNA gene. After amplification, the amplicons are denatured and added to a microtiter plate containing a bound *M.tuberculosis* complex specific oligonucleotide probe. Further an avidin-horseradish peroxidase conjugate is added which binds to the bound labeled amplicons and forms a color complex after reacting with substrate (156, 157)

In isothermal amplification (also known as transcription mediated amplification) of rRNA, the initial RNA strand is degraded, and cDNA duplex is formed which is then transcribed by DNA directed RNA polymerase to produce more rRNA molecules. The new transcripts are templates for reverse transcription and further amplification. The specificity of the assay primarily depends on the specificity of the oligonucleotide probes used in the hybridization assay. A TMA based kit for the detection of *M.tuberculosis* in sputum specimens has been developed. : Mycobacterium Tuberculosis Direct Test by Gen-Probe. This test combines the amplification of ribosomal RNA with a detection method that involves an acridium-ester-labeled DNA probe specific for *M.tuberculosis* in a homogenous solution hybridization assay (150).

It should be mentioned that although these nucleic acid amplification methods provide rapid results, mycobacterial cultures are still required. They

not only support the growth of the organisms from all the specimen sources but also are essential for organism identification as well as antimicrobial susceptibility testing. The greatest problem facing the diagnostic application of PCR is the false positives due to contaminating nucleic acids or amplicon build up (155). Another drawback is false negatives due to interference. Further, a survey that evaluated the reliability and performance of nucleic acid amplification for the detection of *M.tuberculosis* internationally showed that among the 30 laboratories in 18 countries using different tests (including both in house and commercial kits) only five laboratories were able to identify correctly the presence or absence of mycobacterial nucleic acid in all 20 samples distributed (158). These results demonstrate the variation of tests from laboratory to laboratory and the need for a good laboratory practice.

In general, amplified tests currently are not stand-alone tests. They must be used in conjunction with conventional cultures. When specimen volume is an issue, conventional cultures are performed over amplified tests. Currently the cost of these tests is excessive and must be considered when their use is contemplated.

iiic) Molecular Based techniques in species identification

PCR-based sequencing consists of PCR amplification of mycobacterial DNA with genus specific primers and sequencing of the amplicons. The organism is

identified by comparison of the nucleotide sequence with the reference sequences (159).

A still developing technology that appears promising for clinical laboratory involves use of oligonucleotide arrays or DNA chips. High-density oligonucleotide arrays offer the possibility of rapid examination of large amounts of DNA sequences with a single hybridization step. This technique is based on hybridization of fluorescently labeled PCR amplicons generated from bacterial colonies to a DNA array containing nucleotide probes. The bound amplicons emit a fluorescent signal that is detected with the scanner (160).

It is now possible to identify chromosomal DNA or ribosomal RNA from mycobacteria growing in culture. These tests involve the use of complementary DNA or RNA probes that are highly specific for nucleic acid within certain species of mycobacteria. At present, these commercially available probes (Accuprobe, Gen-Probe Inc., San Diego, CA) can identify *M.tuberculosis* complex, *M. kansasii*, *M. avium intracellulare* and *M. gordonae*. After lysis of cells, the cDNA or cRNA will hybridize with nucleic acid sequences producing a radioisotopic or other signal depending on system in use. Nonetheless, to obtain adequate mycobacterial nucleic acid, perhaps 10^5 to 10^6 bacilli must be present and application has generally been limited to the probing of growing cultures. This test has been successfully used with Bactec

12B medium (161) and therefore, more and more clinical laboratories are using the nucleic acid probes alone or in combination with other identification methods (162).

iv) Chromatographic Identification

The gas-liquid chromatography (GLC) procedure provides a rapid preliminary and sometimes definitive identification of cultures (163). Mycobacterial cell wall lipids are extracted using an ether-hexane extraction technique requiring only one loopful (0.001 ml calibrated) of organism. The extracts are analyzed using gas chromatography. The procedure is simple and results are available in as little as 2 hours. GLC coupled with mass spectrometer has been used to detect minute amounts of indolic-type compounds in CSF from patients with tuberculous meningitis, thereby distinguishing them from patients with other forms of lymphocytic meningeal disease (164).

Many laboratories currently advocate HPLC for rapid identification of cultures (165). The long chained fatty acids, mycolic acids obtained from extraction are species specific and yield different peaks when separated by HPLC. The number, heights and position of the peaks are used for identification of mycobacteria. HPLC is particularly useful because it can definitely identify any of more than 50 species in less than 4 hours (this does not include the time required to produce a pure culture). Thus, not only is this

HPLC testing a rapid procedure but it can also replace an entire battery of biochemical tests. However, HPLC require presence of around 10^7 to 10^8 tubercle bacilli, hence growing cultures several weeks in age must be available for its use.

B) Indirect tests

i) Adenosine deaminase

ADA, a pure degrading enzyme, is necessary for the maturation and differentiation of a variety of cells, including monocytes, macrophages and T lymphocytes. Measurement of ADA activity in CSF has been reported as a useful test in the diagnosis of TBM even in patients with AIDS (166, 167). Another study however showed considerable overlap with levels obtained in the nontuberculous and tuberculous patients (168).

ii) Serological testing

The earliest demonstration of circulating antibody to mycobacterial antigens in tuberculosis patients was probably the study of Arloing published in 1898 (169). With the help of many classical techniques like agglutination of sensitized erythrocytes and other carrier particles, complement fixation, precipitation, immunofluorescence and radioimmunoassay (45), antibodies were repeatedly demonstrated in tuberculosis patients however no satisfactory technique for serodiagnosis emerged. Following the first use of ELISA for the serodiagnosis of tuberculosis by Nassau *et al* (1976) and the general availability of conjugated second antibodies specific for individual

immunoglobulin isotypes, rapid advances in understanding of humoral immune responses in tuberculosis and in the development of serodiagnostic techniques occurred (170).

Daniel and Debanne (1987) have summarized many studies reporting sensitivity and specificity of serological assays (171). The reported sensitivity of these assays ranges from 60-80%. In general higher sensitivity have been reported by studies of populations from less well-developed countries endemic for tuberculosis. Reported specificities have also varied widely, probably reflecting many different mycobacterial antigens used as well as the type of control populations studied. A study by Ziess and colleagues (1984) combined ELISA and flurochrome stain and attained a sensitivity of 86%. When both tests were negative, there was a 97% predictive value that tuberculosis was not present (172). It should be noted that while studies have applied serodiagnosis to the diagnosis of pulmonary tuberculosis, its application to extrapulmonary tuberculosis has also been promising. Preliminary studies in AIDS patients have suggested that the serological response in HIV/TB coinfectd patients is complex with an initial high antibody titer following infection with *M.tuberculosis* followed by declining titer as tuberculosis progresses. This raises the possibility that serological assay in that setting might be most useful as an adjunct in identifying tuberculous infection.

Various types of serological assays have been studied by researchers all over the world and which have been summarized below:

a) Immunoassays for Mycobacterial Antigens

Several groups have studied detection of mycobacterial antigen. Sada *et al* (1983) described the detection of *M.tuberculosis* antigen in CSF using direct ELISA (173). Subsequently a variety of techniques including a competitive inhibition ELISA, a latex agglutination test, a double antibody sandwich ELISA and an inhibition ELISA have been used (174, 175, 176). Krambovitis *et al* (1986) assayed sera of pulmonary and extrapulmonary tuberculosis for plasma membrane antigens and obtained a sensitivity of 45% (177). Double antibody sandwich ELISA for the detection of 43kDa antigen demonstrated 100% sensitivity for CSF, pleural fluid and ascitic fluid and the specificities were 96% to 97% (178). On application of inhibition ELISA for the detection of antigen 5, it was observed that 80% of the patients with tuberculous meningitis had a significant concentration of antigen 5 in their CSF and the specificity was 100% (179). Using co-agglutination test to detect the presence of lipoarabinomannan, 85% positivity was seen in the sera of cases of tuberculosis with AIDS whereas 90% positivity was seen in cases of TB without AIDS; the specificity of the test was 93% (180).

b) Spectrum of antibodies

Levels of immunoglobulin G antibody detectable by ELISA or other immunoassay are usually an indicator of active tuberculous disease. Studies

with many antigens using many techniques have found that few control subjects have measurable IgG antibody levels (171). Studies have also shown that asymptomatic primary infection (181) and minimal pulmonary disease (182) are usually not sufficient to induce a significant antibody response. During the course of treatment, antibody levels rise for the first 1-2months, falling thereafter but remaining detectable for 1 to several years (183). Patients with remote healed tuberculosis do not have readily detected IgG antibody to mycobacterial antigens.

IgM responses are directed chiefly at nonspecific polysaccharide antigens. They develop early but never reach high titer, and their level does not correlate well with the presence or absence of active disease (171). Many healthy persons probably develop low levels of IgM antibody in response to contact with environmental mycobacteria. IgA antibody has been recognized at low levels in the serum of patients with active tuberculosis but not in control subjects (171).

c) Immunoassays to detect antibodies in serum/fluids

It is possible to devise simple serodiagnostic tests using any of the several antigens, but that the choice of antigen is a major determinant of diagnostic test specificity and predictive accuracy. The early attempts to diagnose TB by ELISA by way of estimation of antibodies in the serum were based on the use of crude antigens e.g. PPD, mycobacterial culture filtrates or sonicates (171,

172, 184) and invariably, there was an unacceptably high overlap between the results of tests on proven cases of tuberculosis and healthy control subjects. This overlap was even more evident in comparisons of antibody levels between patients with smear negative tuberculosis and patients suffering from other diseases likely to be confused clinically with TB. As the tubercle bacillus contains both shared and species-specific antigens, attempts were made successfully to purify and extract the latter by various physiochemical techniques (185).

The following purified antigens have been tested in various serological trials for estimating specific antibodies of IgM, IgA and IgG classes in the serum of patients of pulmonary and extrapulmonary tuberculosis viz, glycolipids from *Mycobacterium bovis* BCG (186), antigen 6 from *Mycobacterium tuberculosis* (187), antigen 5 from *Mycobacterium tuberculosis* (188), purified lipoarabinomannan from *Mycobacterium tuberculosis* (189), a plasma membrane antigen from *Mycobacterium tuberculosis* (190), and a 38kDa protein antigen from *Mycobacterium tuberculosis* (191). The antibodies of IgA and IgG subclasses were found to be much more specific than the IgM type (191).

In a number of studies, both in India and abroad, specific antibodies (IgG type) against Mycobacterial antigens (crude, semipurified and purified) have been demonstrated in CSF of patients suffering from tubercular meningitis using Radioimmunoassay, Western blot and ELISA techniques with

reasonably good results as mentioned below. Studies have shown antibodies against soluble mycobacterial extract (174), against lipoarabinomannan, 38kDa, 19kDa, 14kDa (192), and against antigen 5 (193). As is evident, the tests were found to be 100% specific for diagnosing tubercular meningitis except where the crude mycobacterial extract was used as the antigen, thereby demonstrating use of purified antigens in the ELISA test. A reasonably high sensitivity was also obtained.

Studies regarding estimation of antimycobacterial antibodies in other body fluids are limited. In an Indian study, using RIA and *Mycobacterium tuberculosis* sonicate as the antigen for the estimation of antimycobacterial antibody levels in the pleural and ascitic fluids, a specificity of 100% and a reasonable sensitivity of 63.4% was obtained (194).

Though ELISA is very simple, cost effective and rapid test, the genetic makeup of the population that undoubtedly plays an important role in the antibody response and the geographic location, which determines the extent of exposure of the population to cross-reacting organisms, can interfere with the sensitivity and specificity of the test.

1.11. MYCOBACTERIAL ANTIGENS

1.11.1. Identification of Mycobacterial Antigens

The search for improved diagnostic reagents, such as more species-specific skin test antigens and serological markers, has been a driving force in many of the efforts devoted to the fractionation and identification of mycobacterial antigens.

Florence Seibert's first major attempt to purify isolated antigens from mycobacteria by chemical fractionation using precipitation with alcohol and acetic acid resulted in 4 proteins (A, B, C and D) and 2 polysaccharides (I and II) (195). In attempts to isolate mycobacterial antigens various physico-chemical methods like ion-exchange, molecular exclusion, density gradient ultracentrifugation, immunodiffusion, isoelectric focusing, crossed immunoelectrophoresis, zonal electrophoresis, affinity chromatography and HPLC were used (195, 196, 197, 198, 199).

Monoclonal antibodies have made it possible to identify single antigenic determinants, which have further helped in the characterization and purification of the complex mycobacterial antigens. Several monoclonal antibodies have been developed for *M.tuberculosis* and *M.leprae*. Cloning relevant genes from mycobacteria in recombinant DNA expression vectors and its overexpression for antigen purification allows detailed investigation of the proteins. The use of monoclonal antibodies and recombinant DNA

technology has resulted in the identification of several protein antigens of *M.tuberculosis*. While it seems likely that monoclonal antibodies defined proteins are immunodominant for murine B cells, it is not clear that same pattern of immunodominance will be reflected in human T cell response to mycobacteria. A relevant parameter to screen is the ability of a protein to induce invitro proliferation of T lymphocytes from diseased or vaccinated humans or animals and to study the release of specific cytokines.

1.11.2. Mycobacterial Protein Antigens

Indeed when compared to other pathogens, mycobacteria have an extraordinarily large panoply of antigens. This may be related to the fact that these bacteria are rich in adjuvants, so that many minor protein constituents are presented to host cells under circumstances that favor antigenicity. The protein profiles of the mycobacterial sonicate or culture filtrate will expectedly vary in composition according to the conditions in the culture vessel. The details of the metabolic conditions within an activated macrophage and what consequence these may have on the phenotypic appearance of *M.tuberculosis* is not known.

Proteins discussed below are divided into groups in accordance to their subcellular location. They may be cytoplasmic, cell membrane or cell wall associated, or secreted to the surrounding medium. Given below is an introduction to some of the major groups of mycobacterial protein antigens.

A) Cytoplasmic proteins

i) Enzymes involved in Amino Acid and Protein Biosynthesis.

A renewed interest in the metabolic pathways of mycobacteria has arisen in recent years. The idea of improving the existing BCG vaccine by genetic manipulation has prompted a demand for selectable cloning markers, which do not imply the biohazard problems of antibiotic resistance genes. Inactivation of genes involved in the synthesis of amino acids may prove to be a suitable way to obtain attenuated vaccine strain. The search for new drug targets and the fact that the enzymes of the cytosol may be as antigenic as any other protein are the reasons to be interested in vital metabolic pathways.

The *aroA* gene from *M.tuberculosis* involved in the synthesis of aromatic amino acids, the *lysA* gene from *M.tuberculosis* that encodes the diaminopimelic acid decarboxylase and catalyzes the conversion of diaminopimelic acid to lysine and L-alanine dehydrogenase catalyzing the reversible conversion of pyruvate to L-alanine in a NAD/NADH-dependent were cloned, expressed and studied (200, 201). Studies prove that the enzymes are not of immunological importance but might be of interest to researchers as a way to attenuate virulent mycobacteria and might also help in synthesizing new antimycobacterial drugs.

ii) Stress proteins

Among many stress proteins, 65kDa antigen of *M.tuberculosis* and other mycobacteria has been extensively studied and found to be a heat shock

protein with substantial homology with other well-known heat shock proteins, including the *E.coli* GroEL protein. Heat shock proteins are widely distributed in nature and highly conserved with substantial structural similarity crossing taxonomic lines. Named because they are produced in increased quantity by cells growing under stressful culture conditions such as high temperature, they are thought to have important functions in maintaining cell integrity and are produced by bacteria under conditions that are less than optimal.

The gene for the 65kDa antigen has been cloned and its structure is well known (202). This protein may exist in polymeric form in mycobacteria. The 65kDa contains many helical structures and hydrophobic regions, characteristics typical of highly antigenic proteins. Studies with monoclonal antibodies have demonstrated that this protein has multiple epitopes, many of which are widely shared among mycobacteria, some of which appear to be species specific. Some epitopes appear to be shared with epitopes of mammalian proteins; these have been implicated in adjuvant arthritis and might be related to human autoimmune diseases (203). This association has reduced interest in these antigens from the vaccine point of view. However, the study of this group of proteins in relation to pathogenesis and in improving our understanding of the physiology of mycobacteria has been and still is of great importance.

B) Proteins secreted across the Cell boundary

Proteins synthesized in the cytoplasm may be destined to function outside the cytoplasmic membrane either in association with the cytoplasmic membrane or cell wall or exported all the way to the surrounding medium. Mycobacteria secrete a number of secreted proteins to the surrounding medium (204) and these proteins are extremely potent in generating a cellular immune response in mice infected with live *M.tuberculosis* (204). T lymphocytes directed toward these proteins may be responsible for the initial recognition of the infected macrophage leading to efficient control of the infection at a very early stage (205). The emergence of ³⁵S-labeled proteins in the culture medium at very early times studied by SDS-PAGE (206) as well as studied by the formula of localization index (207) has helped in identifying many secreted proteins.

i) Proteins associated with cell membrane or cell wall.

Lipoproteins are generally associated with cell surfaces in a way that the lipid moieties of these proteins are integrated into the cell surface and the protein part is exposed on the exterior, thereby allowing the proteins to carry out functions like binding of certain nutrients, etc. The 38kDa and 19kDa are two of the major mycobacterial lipoproteins.

Daniel and Anderson (1978) used immunoabsorbent affinity chromatography to isolate an antigen referred to as antigen 5 (197). Anderson and Hansen

(1989) also purified and characterized 38kDa designating it antigen b (208). Haslov and colleagues (1990) demonstrated that protein b and the proteins purified by Daniel and Anderson were identical (209). Anderson and coworkers (1990) later proposed that this protein is necessary for phosphate metabolism of the organism (210). It has a signal sequence, which allows the protein to be transported across the cytoplasmic membrane. With Daniel and others, the immunobiology of this protein was studied extensively and it was found to be restricted to *M.tuberculosis* and *M.bovis* (211). This antigen elicited T and B lymphocyte responses in sensitized guinea pigs and infected humans (212, 213).

Another lipoprotein of importance is the 19kDa antigen, which also has been shown to be involved in phosphate metabolism. This protein seems to be structurally much more conserved within mycobacterial species as judged by the cross-reactivity of the monoclonal antibodies (214).

ii) Proteins exported to the exterior.

Superoxide dismutase released by the bacteria is an example of an enzyme that serves a very distinct function, i.e. paralyzing a host defense mechanism by inactivating the toxic superoxide radicals generated by the activated macrophage (215). SOD is an example of a protein exported to the surrounding medium by a mechanism that is not dependent on a consensus signal sequence.

One of the prominent bands observed among the secreted proteins is a double band of molecular weights 30/31kDa (216, 217). Wiker and colleagues have used elegant crossed immunoelectrophoretic techniques to show close immunologic relationships between three antigens designated by them as 85A, 85B and 85C (216, 217, 218). Although the proteins of 85 complex do possess true signal sequences that are cleaved off before release of the proteins to the exterior, a fraction of the proteins remains attached, at least temporarily, to the bacterial surface. This fraction is believed to be of importance for the adhesion of the bacteria to and subsequent infection of eukaryotic cells. This antigen is present in all mycobacteria that have been tested and there is substantial evidence that the molecule contains both species specific and shared epitopes (199).

The best known of these antigens is 85B, which has been previously described as alpha antigen (219), antigen a₂ (220), antigen 6 (221), and MPB-59/MPT-59 (216, 217). With a molecular weight of 30kDa, this antigen is the predominant antigen in culture medium and is secreted by growing mycobacteria (216, 217). Measuring IgG antibody to the 30kDa antigen provides the basis for a good serodiagnostic test for active tuberculosis (222). It is of considerable interest that T lymphocytes of healthy *M.tuberculosis*-infected individuals react strongly to this antigen whereas those of patients with active tuberculosis do not (223). This might suggest that decreased T-lymphocyte responsiveness to the 30kDa antigen is a feature of the immunoregulation in

active tuberculosis, or it might mean that individuals who do not mount T-lymphocyte responses are predisposed to the development of disease.

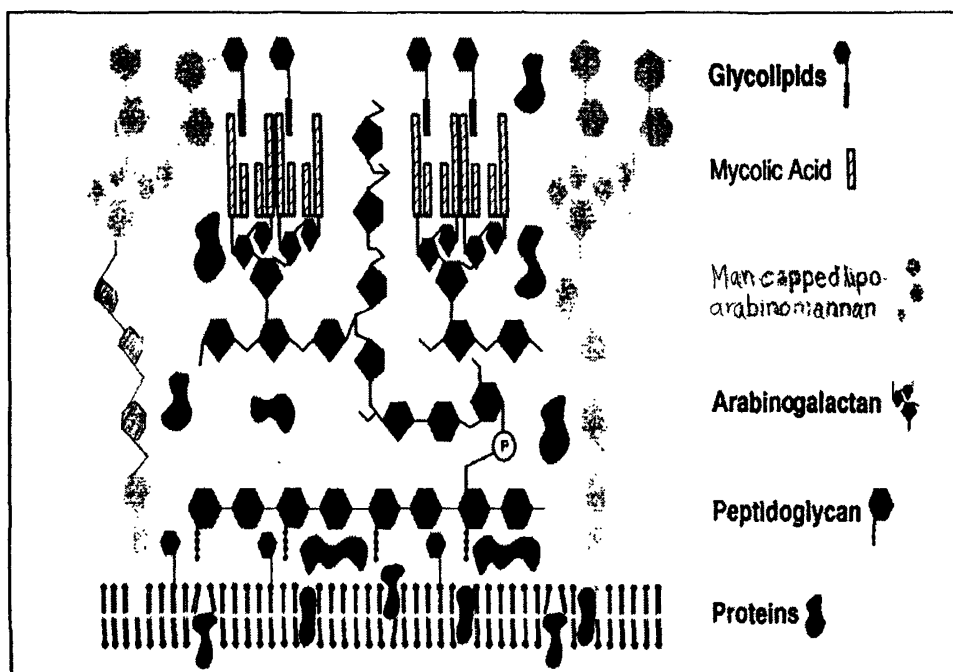
Other members of the 85 complex of antigens are less well studied. The 85 A component, also known as antigen P32 is also a fibronectin-binding, secreted protein. It has a molecular weight of 31-32kDa. This antigen evokes antibodies in patients with tuberculosis (224).

1.11.3. Mycobacterial Polysaccharide Antigens

1.11.3.1. Mycobacterial Cell Wall

Within actinomycete line, mycobacteria belong to one branch, often called *Corynebacterium-Mycobacterium-Nocardia* branch. These bacteria produce cell walls of a unique structure, sometimes called chemotype IV cell wall, containing *meso*-diaminopimelic acid as the diamino acid in the peptidoglycan. An important feature of the chemotype IV cell walls is the presence of unique polysaccharide, arabinogalactan (AG), which is substituted by characteristic long chain fatty acids, mycolic acids. In addition, large variety of lipids and glycolipids and possibly proteins are also known to be associated with the peptidoglycan-arabinogalactan skeleton (as shown in Figure 1.3).

Fig 1.3: Schematic representation of cell wall of *M.tuberculosis*



1.11.3.2. Ultrastructural features

The cell wall is composed of an inner layer of moderate electron density, a wider electron transparent layer and an outer electron opaque layer of variable appearance and thickness. The inner layer probably contains peptidoglycan; its moderate electron density is consistent with known staining properties of diaminopimelic acid, which contains carboxyl groups that bind metal ions (225) while the electron transparent layer appears to be the hydrophobic domain of the cell wall, which is dominated by the mycolate residues covalently bound to arabinogalactan. The transparency is caused by the failure of the water-soluble stains to penetrate into these hydrocarbon rich regions together with a lack of reactive chemical groups that might bind to commonly used electron microscopical stains. The outermost electron-opaque layer of the wall varies in thickness, electron density and appearance, which is attributable to differences among species. The dye ruthenium red allowed this layer to be consistently visualized suggesting presence of negatively charged head group of lipids (225). In some species, the additional carbohydrate material like LAM, other heteropolysaccharides or proteins may also contribute to the binding of stains.

1.11.3.3. Cell Envelope Components

A) Plasma Membrane

i) Phosphatidylinositolmannoside

The location of phosphatidylinositolmannoside (PIMs) is still an issue of debate. Some of the papers mention this being part of the plasma membrane

while some other authors believe it to be a part of the cell wall. The phospholipids in mycobacterial envelopes are almost invariably derivatives of phosphatidic acid. The most common are phosphatidylglycerol, diphosphatidylglycerol, and phosphatidylinositol and its mannosides. The PIMs are restricted to the bacteria of the actinomycete line. They are major plasma membrane components and also form the lipid base of lipoarabinomannan (LAM) and lipomannan (LM). The early use of H-NMR clearly established that the glycerol phosphate moiety was attached to the L-1 position of the *myo*-inositol ring and that the mannose residues were glycosidically linked to the 2 and 6 positions (Fig 1.5 and Fig 1.6). The structures of each of the higher homologs, PIM₃, PIM₄, PIM₅ and PIM₆, have been established. They contain a single α -D-mannopyranosyl group at the 2nd position, and a mannose oligosaccharide at the 6th position. The PIMs notably PIM₂ and PIM₅ contain acyl functions other than those of the diacylglycerols.

ii) Other components

Other components associated with the plasma membrane include carotenoids, menaquinones, etc that are involved in electron transport.

B) The Cell Wall Skeleton:

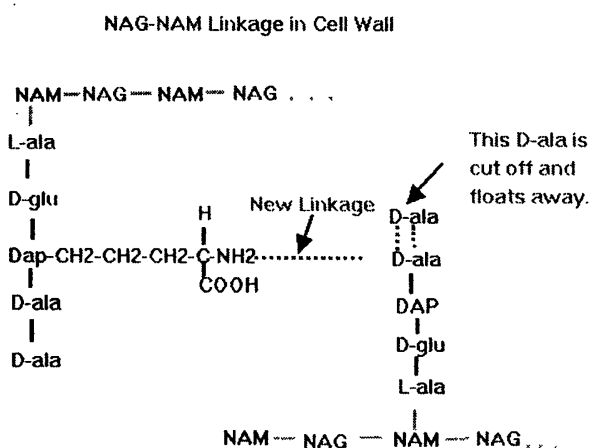
The covalently linked skeleton of cell wall may be described as a peptidoglycan to which are linked polysaccharide side chains esterified at their distal ends with mycolic acids, that is mycolyl-AG-peptidoglycan

complex, 40% of which corresponds to lipids in the form of mycolic acids (226). All the components of the cell wall including Peptidoglycan, Arabinogalactan, Mycolic acids, Lipoarabinomannan and Extractable lipids are described below:

i) Peptidoglycan

Peptidoglycan consists of a polymer of N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM). The type of peptidoglycan that is commonly found in bacteria contains peptide side chains consisting of L-alanyl-D-isoglutaminyl-*meso*-diaminopimelyl-D-alanine, in which the diaminopimelic acids are amidated. The mycobacterial peptidoglycan differs in two ways from the common type, in that the muramic acid is n-glycolylated and the cross-links include bonds between diaminopimelic acid and D-alanine and between two residues of diaminopimelic acid (Fig 1.4).

Fig 1.4: Structure of peptidoglycan in Mycobacteria



ii) Arabinogalactan

D-arabino-D-galactan serves as the backbone of the mycobacterial cell wall of mycobacteria and other actinomycetes and corynebacteria. It was also demonstrated early that arabinogalactan (AG) is attached to the peptidoglycan through a phosphodiester link and mycolic acids in turn are attached to the arabinogalactan (226). Arabinogalactan readily elicits antibodies in immunized experimental animals but this polysaccharide induces and elicits no or few cellular responses (226). Arabinogalactan together with the underlying peptides phosphodiesterified to it is a water-soluble adjuvant capable of potentiating a wide variety of immune responses (227). At the same time, it has major immunosuppressive properties (228).

iii) Mycolic acids

Mycolic acids are high molecular weight α -alkyl, β -hydroxy fatty acids, present mostly as bound esters of AG, where they appear primarily as tetramycolylpentaarabinosyl clusters but also in extractable lipids, mainly as trehalose 6,6'-dimycolate (cord factor).

iv) Lipoarabinomannan

Phosphorylated lipoarabinomannan was extensively studied by Hunter, Brennan and their colleagues and was shown to be antigenic (229). Hunter *et al* (1986) isolated a highly purified LAM by using anion exchange and gel filtration chromatography (230). On analyzing fractions by SDS-PAGE, LAM

yielded a broad, diffuse band with a mass of 30-40kDa. The broadness of the band on PAGE is perhaps due to the presence of microheterogeneity within the molecule. Studies have shown that LAM contains glycerol, inositol and phosphate in addition to arabinose, mannose, lactate, succinate, palmitate and tuberculostearate.

Circulating antibodies to lipoarabinomannan are easily demonstrated in experimentally immunized animals (231) and are found in the majority of patients with active tuberculosis (189). However delayed type hypersensitivity reactions are not induced or evoked by this compound. Many attributes of lipoarabinomannan may be important to the immunopathogenesis of tuberculosis and other mycobacterial diseases, perhaps functioning as a virulence factor (60, 232) {discussed in details in Chapter 1.12}.

v) Extractable Lipids

Lipooligosaccharides, Phenolic glycolipids, glycopeptidolipids, Waxes, Acylated trehaloses, sulfolipids, and some special proteins also form a part of the cell wall of the mycobacteria.

1.12. LIPOARABINOMANNAN AND RELATED LIPOGLYCANS

Lipoarabinomannan (LAM) as well as its related precursors, lipomannan (LM) and phosphatidyl-*myo*-inositol mannosides (PIMs), are found interspersed in the mycobacterial cell wall. PIMs, LM and LAM are major

lipoglycans that are non-covalently attached to the plasma membrane through their phosphatidyl-*myo*-inositol anchor and extend to the exterior of the cell wall (233, 234). These complex molecules are believed to play important roles in the physiology of the bacterium as well as in the modulation of the host response during infection.

1.12.1. Structure of mycobacterial LAM and its related precursors:

The PIMs and their multiglycosylated counterparts, LM and LAM are complex lipoglycans that are found ubiquitously in the envelopes of all mycobacterial species. They all share a conserved mannosyl-phosphatidyl-*myo*-inositol (MPI) that is presumably used to insert these structures into the plasma membrane, suggesting that they are metabolically related (233). In addition to the MPI, LAM possesses a mannan core with a branched arabinan polymer and in some cases; cap motifs decorate the termini of the branched arabinan (234). As shown in Fig 1.5 and Fig 1.6, the mannan core consists of a α 1,6-linked Man_p backbone, which is substituted at C-2 by single Man_p units in numerous species including *M.tuberculosis*, *M.leprae*, *M.kansasii* and *M.smegmatis* and at C-3 by single Man_p units in *M.chelonae* (235). The size and the degree of branching of the mannan core are species dependent. The arabinan polymer of LAM consists of a linear α (1→5)-linked arabinofuranosyl backbone punctuated with branched hexa-arabinofuranosides and linear tetra-arabinofuranosides (236, 237).

Fig 1.5: Schematic visualization of the structural relationship of PIM, LM and LAM.

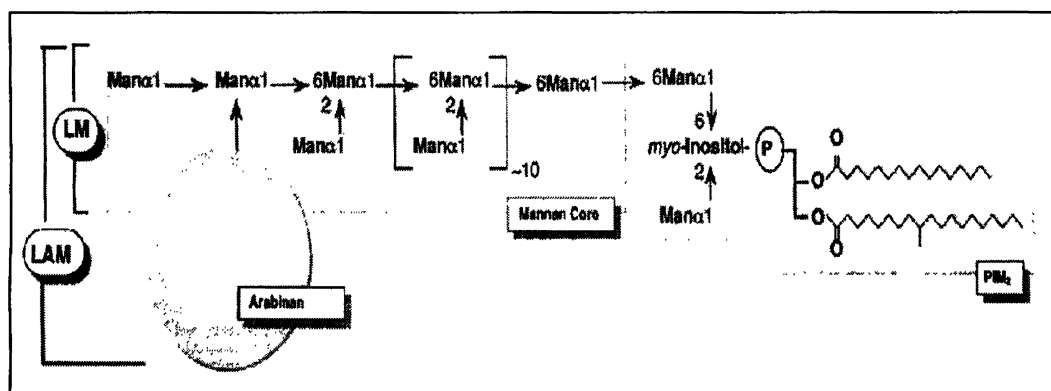
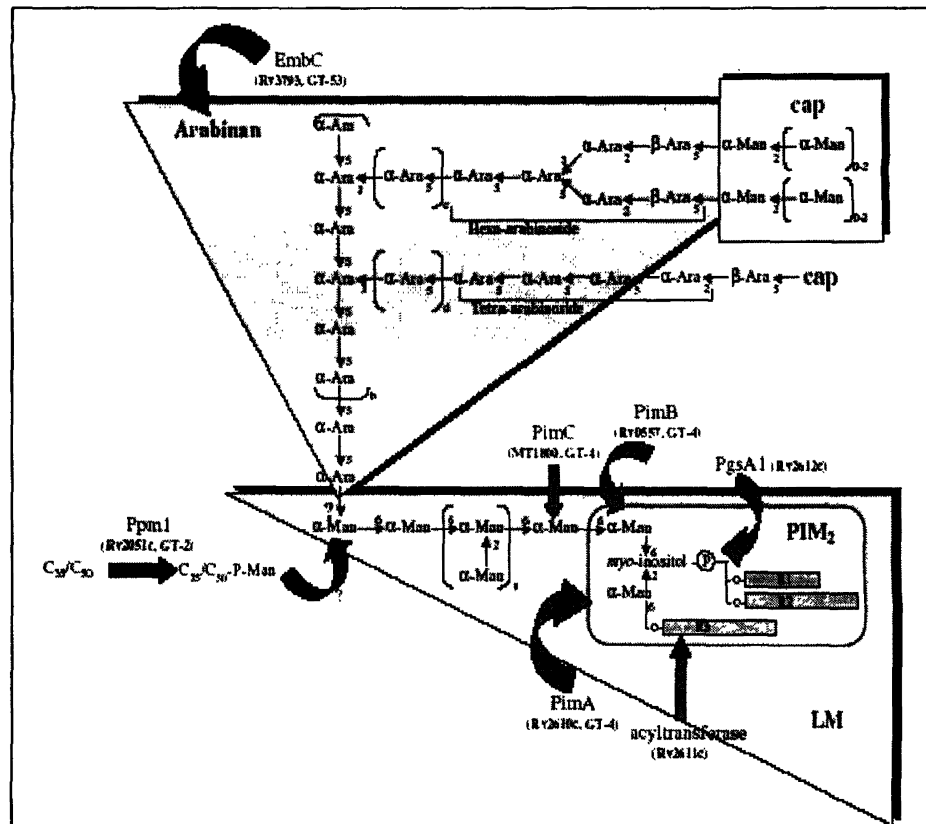


Fig 1.6: Structure of Man-LAM from *M.tuberculosis* and structural relationship between PIM, LM and LAM. Also biogenesis of PIM, LM and LAM has been diagrammatically represented below



LAM can be classified into three major structural families according to the capping motifs present on the non-reducing termini of the arabinosyl side chains. The arabinan termini in the pathogenic strains *M.tuberculosis*, *M.leprae*, *M.kansasii* and *M.avium* are modified with caps consisting of a single Manp, a dimannoside or a tri-mannoside, with dimannosides predominating (238, 239) resulting in molecules designated ManLAM (Fig 1.6). ManLAM contains about 50 Manp and 60 Araf units. In the fast-growing non-pathogenic species e.g. *M.smegmatis*, branches of the terminal arabinan are terminated by inositol phosphate caps, characterizing the PILAM family (240). A third family, designated AraLAM, recently identified in *M.chelonae*, comprises a LAM molecule devoid of both the manno-oligosaccharide and inositol phosphate caps (235).

Two possible situations have been hypothesized: 1) LAM is anchored in the plasma membrane by MPI and protrudes through the thickness of the wall so that its terminal arabinose or mannose capped arabinose units are accessible to the outside (241) 2) LAM is incorporated by its PI anchor into the outer leaflet of a proposed outer membrane analogue in mycobacteria (242). Both these hypothesis are consistent with external presentation of terminal arabinose or mannose caps. The relatively strong conditions needed to release LAM from mycobacteria seem to favor first hypothesis but there is no strong evidence to support either hypothesis.

1.12.2. Biogenesis of PIMs, LM and LAM (Ref Fig 1.6)

A) Biogenesis of Phosphatidylinositolmannoside (PIM)

PIM biosynthesis is initiated by two distinct mannosyltransferases that uses GDP-Man ν as the sugar donor. The first step involves the transfer of a mannose residue from GDP-Man ν to the 2-position of the myo-inositol ring of phosphatidyl-my ν -inositol (PI) to form phosphatidyl-my ν -inositol monomannoside (PIM $_1$). This reaction is catalysed by the α -mannosyltransferase PimA (243). Interestingly *pimA* is the fourth gene in an operon of five genes that are potentially involved in PIM biosynthesis (243). The first gene in this cluster encodes a protein of unknown function, while the second encodes PgsA1, the PI synthase that catalyses the condensation of inositol and the di-glyceride of CDP-diacylglycerol (244). The third gene Rv2611c of this operon encodes a protein with high similarity to bacterial acyltransferases. This protein has been shown to be responsible for the acylation of the 6-position of the Man ν residue linked to the position 2 of the my ν -inositol in PIM $_1$ and PIM $_2$, with the mono-mannosylated lipid acceptor being the primary substrate of the enzyme (245). The last gene Rv2609c, encodes a putative GDP-Man ν hydrolase that awaits further characterization. The second mannosylation step catalysed by PimB allows the transfer of another Man ν residue to the 6-position of the myo-inositol ring of PIM $_1$ leading to PIM $_2$. A third Man ν unit is finally introduced on the growing molecule to form PIM $_3$ in a reaction carried out by the product of the *pimC* gene identified in *M.tuberculosis* as CDC1551 (246, 247). The mannose unit at

the position 6 of PIM₃ is then further elongated with mannose residues to generate PIM₄₋₆.

B) Biogenesis of Lipomannan (LM)

Besra *et al* (1997) established that PIMs are extended with additional Man_p residues from the alkali-stable C₃₅/C₅₀ polyprenyl monophosphomannose donor to form linear LMs, containing only $\alpha(1\rightarrow6)$ Man_p residues (248). C₃₅/C₅₀-P-Man_p is synthesized from GDP-Man_p and polyprenols by the polyprenol monophosphomannose (*ppm*) synthase, encoded by *ppm1* gene (249). In *M.tuberculosis* and other mycobacteria analyzed till date, mature LM consists of linear LM bearing single $\alpha(1\rightarrow2)$ -linked Man_p residues. Nevertheless, one exception is the LM of *M.chelonae*, which has $\alpha(1\rightarrow3)$ -linked Man_p residues (235). None of the specific genes encoding these branching mannosyltransferases has been identified.

C) Biogenesis of arabinan domain of LAM

The mature LM is then subsequently glycosylated with arabinan to form LAM. Two forms of arabinans are found in mycobacterial cell wall: one is part of the heteropolysaccharide arabinogalactan (AG) and other is part of LAM. The two forms of D-arabinan differ; mycolic acids esterify arabinan in AG, thus constituting the basis of the lipid barrier of mycobacteria. In contrast, in *M.tuberculosis* LAM, the arabinan moiety is further capped with mannose residues responsible for some of its biological functions. Therefore arabinan

represents a valid target for the generation of anti-mycobacterial drugs because blocking of its biosynthesis would lead to dual disruption of both the mycolyl-AG-peptidoglycan cell wall complex and LAM.

As mentioned above the arabinan domains consist of a linear $\alpha(1\rightarrow5)$ -Araf backbone substituted by two kinds of arrangements, linear tetra-arabinofuranosides (Ara₄) and hexa-arabinofuranosides (Ara₆). In both cases, the non-reducing end is characterized by the disaccharide unit β -D-Araf-(1 $\rightarrow$ 2)- α -D-Araf-(1 $\rightarrow$) (233, 250). The only Araf sugar donor identified so far is the C₃₅/C₅₀ polyprenyl monophosphoarabinose (251).

D) Biogenesis of Mannose cap

LAM is modified further by either manno-oligosaccharides or phosphoinositol caps, according to the species, resulting in ManLAM or PILAM respectively. As mannose capping is a major structural entity engaged in receptor binding and subsequent immunopathogenesis, inhibition of this motif may directly affect the biological functions associated with ManLAM. Genes participating in the synthesis of these caps have not been reported and the identification of mannosyltransferases involved in this reaction remains a challenge.

1.11.3. Immunomodulation by PILAM/AraLAM/ManLAM

PILAM/AraLAM/ ManLAM as well as LM and PIM exhibit a wide spectrum of immunomodulatory functions.

A) Inflammation inducing activity

The virulence of a particular *Mycobacterium tuberculosis* strain is generally inversely related to its capacity to elicit production of proinflammatory cytokines in infected hosts. PIMs and PILAM but neither ManLAM nor AraLAM consistently induces the secretion of proinflammatory cytokines IL-8 and TNF- α (235, 252, 253). PILAM, but not ManLAM or AraLAM significantly induces IL-12 expression and apoptosis (254). These results support the hypothesis that the phosphoinositol caps of PILAM are potent proinflammatory constituents.

LMs of mycobacteria in general are also strong proinflammatory factors (254) and as LAM and LM are part of the cell wall, one could argue that it is important for virulent bacteria to minimize the amount of LM present in the cell wall in order to reduce host's proinflammatory response. Consequently one might expect a direct correlation between virulence and a high LAM/LM ratio. The arabinan domain of LAM inhibits the proinflammatory activity of LM on macrophages by masking mannan core. Consistently, gradual chemical reduction in the amount of arabinan domain of *M.kansasii* ManLAM correlated with increased proinflammatory cytokine expression of the truncated LAM molecules, thus revealing the proinflammatory activity of the

LM core (253). As a consequence enzymes modifying the LM core by addition of arabinose residues should be important targets for the creation of attenuated strains for vaccine and for the discovery of new drugs.

B) Inhibition of cellular responses

The first demonstration of the capacity of LAM to inhibit a host response involved in defense against bacterial infection was conducted by Sibley *et al* (1988), who reported the inhibition of the interferon- γ response of macrophages by ManLAM (255). Subsequently live *M.tuberculosis* infection was shown to inhibit IFN- γ signalling as demonstrated by the reduction in the IFN- γ mediated cell-surface expression of MHC class II after infection of macrophages with *M.tuberculosis* (256, 257).

Furthermore, ManLAM from *M.tuberculosis* inhibited the *M.tuberculosis* infection induced apoptosis of macrophages and the secretion of IL-12 induced by LPS in dendritic cells and macrophages (258, 259). The activity of ManLAM reflects the capacity of whole bacterium to inhibit infection induced apoptosis and IL-12 secretion of macrophages (260, 261). ManLAM has the capacity to avoid the triggering of several macrophage early response genes at mRNA (c-fos, JE, KC) and protein levels (TNF- α) that may be involved in cell anti-microbial activities (262). TNF- α , one of the most important early response genes is one of the key cytokines in the induction of macrophage RNI production. By avoiding macrophage activation, ManLAM may act as a

virulence factor that allows the organism to evade cytokine-mediated mechanisms of elimination.

One of the hallmarks of the host-pathogen interaction between macrophages and *M.tuberculosis* is the ability of the bacilli to inhibit the fusion of phagosomes and lysosomes (263). Latex beads coated with ManLAM inhibit phagosome-lysosome fusion (53) suggesting that ManLAM is an important mediator of the inhibition of phagosome maturation in the context of infection with live bacteria. Thus mannose caps on ManLAM may mediate efficient binding and entry and regulate the initial events of phagocytosis as well as survival within the host macrophages.

C) Receptors involved in inhibition and activation processes.

Toll-like receptors are important initiators of the innate immune response that are specific for pathogen associated molecular patterns such as peptidoglycan, flagellin etc. Interaction of agonists with TLR-2 induces IL-12 secretion and cell apoptosis (264). PILAMs and LM but not ManLAM have been shown to interact with TLR-2 (254, 265). Binding of ManLAM to DC-SIGN on dendritic cells induces secretion of IL-10, a known inhibitor of IL-12 secretion (266). Thus DC-SIGN appears as a major mediator of IL-12 inhibition by ManLAM on dendritic cells. Macrophage mannose receptor and CD14 are important LAM receptors on macrophages involved in activating macrophages and inducing T cell chemotaxis (267).

D) Intracellular mediators of inhibition

Very little is known about the intracellular effectors that have been triggered by ManLAM binding to the receptors. However it has been proposed that *M.tuberculosis* mediated inhibition of the increase in cytosolic Ca^{2+} , (which is usually associated with phagocytosis of bacteria), is a central mediator of the inhibition of three major macrophage responses to infection: phagosome maturation, macrophage apoptosis and IFN- γ signalling (53, 258, 268, 269, 270, 271).

Man-LAM coated beads have shown a similar inhibition of macrophage responses. Man-LAM but not PIMs coated beads have been shown to inhibit the recruitment of intracellular markers syntaxin 6 and cathepsin D, which are part of the vesicular fusion complex that mediates the fusion of phagosomes with late endosomes and subsequently with lysosomes (53). Regulation of programmed cell death via calcium fluxes has been reviewed recently and one report provides evidence of a possible link between the activity of ManLAM in inhibiting infection-induced apoptosis and its capacity to inhibit Ca^{2+} accumulation in macrophages (258). In addition to inhibition of the cellular (Ca^{2+}) response, ManLAM directly activates Bad/AKT signalling pathway and thus promotes cell survival (272). It also increases the activity of Src homology 2-containing tyrosine phosphatase 1, which inhibits IFN-signalling.

E) T-cell recognition by LAM

Uptake of LAM by monocyte-derived macrophages (MDMs) via the macrophage mannose receptor (MMRc) transports LAM to the late endosomes, lysosomes and the compartment for MHC class II antigen loading (273). MDMs were shown to preferentially phagocytose Man-LAM coated microspheres when compared with Ara-LAM coated microspheres (274). Another study (275) also implicates role of the MMRc in the LAM dependent uptake of virulent *M.tuberculosis* strains. Once in the acidic environment of the late endosome, LAM binds to CD1b and gets transported to the surface. CD1b/LAM complexes can be recognized by antigen specific T lymphocytes bearing either the $\alpha\beta$ or $\gamma\delta$ T cell receptor (276). This function may serve to attract lymphocytes to the site of infection and/or suppress lymphocyte activation.

B cells can also present LAM to T cells via CD1c. This B cell specific antigen presentation pathway may recruit lymphocyte help, which leads to production of anti-LAM antibodies (277).

F) Other important functions

It has been shown that ManLAM significantly induces TGF β production in macrophages (278). TGF β is a potent antagonist of macrophage and lymphocyte activation and it also contributes to Th2 type of immune response (279).

LAM has been designated a possible virulence factor of mycobacteria by virtue of its ability to effectively inhibit effector functions of macrophages by scavenging potentially cytotoxic oxygen free radicals (60) and inhibition of Protein kinase C activity (60).

The biological properties of LAM are dependent on the complete native structure of LAM. Upon treatment of LAM with mild alkali to remove acyl functions, the capacity of the molecule to induce cytokine production and the suppression of antigen-induced proliferation are removed. This confirms that the phosphatidylinositol anchor along with its acyl groups is also crucial to some of the biological functions of LAM (252). Due to the presence of repeating D-arabinofuranose residues it has a direct B cell stimulatory capacity. Mycobacterial LAM stimulates a humoral immune response of IgG2 subclass (280).

Fine structural differences among LAMs from different sources can be discriminating and important when considering their biological functions and potency in eliciting host immune responses. The seemingly heterogeneous nature may perhaps conceal a well-evolved adaptation enabling the intracellular pathogen to fine-tune a perfect balance against the host defense system for its survival and propagation.

As discussed in above sections, various diagnostic tests have their own merits and demerits. Considering the known facts that smear results are relatively insensitive, culture results involve a lengthy time duration to get reported as positive while PCR, Bactec and Radiorespirometry techniques are costly and require excellent skills, it was decided to evaluate the efficacy of immunodominant Man-LAM based ELISA kit in serodiagnosis of active TB, in order to find a cheap and a reliable diagnostic solution to TB.

AIMS AND OBJECTIVES



Chapter 2.

2. AIMS and OBJECTIVES

A fast, reliable and inexpensive method to diagnose TB is a prerequisite to hasten the identification of patients and contribute to the success of DOTS programme in India. A serologic test could comply with these requisites. In recent years with the development of ELISA and availability of reagents and techniques to purify antigens, it's been possible to increase the sensitivity and specificity of a serological test.

Lipoarabinomannan is a lipopolysaccharide, which constitutes one of the dominant antigens of the mycobacterial cell wall. Presence of repetitions of D-arabinofuranose residuals in the structure of LAM molecule induces strong and extremely pure immune reactions.

Therefore, the chief goal of this study was to prospectively evaluate under field conditions the programmatic use and accuracy of Lipoarabinomannan based ELISA kit in serodiagnosis of tuberculosis and to compare the efficacy of this kit with other available methods like Smear, Culture, Radiorespirometry and PCR. In short the objectives of this study were:

- To purify Lipoarabinomannan (Man-LAM) from mycobacterial (Std virulent *M.tuberculosis* strain: H₃₇Rv) cell wall.

- To develop an ELISA for the detection of anti-LAM antibodies using purified Lipoarabinomannan antigen.
- To demonstrate the suitability of Lipoarabinomannan ELISA kit for the detection of antibody in clinical specimens.
- To compare the results obtained from Lipoarabinomannan ELISA kit with other techniques like
 - I. Smear examination of clinical samples by Acid Fast Staining
 - II. Isolation and identification of *M.tuberculosis* by Culture & Biochemical testing
 - III. Radiorespirometry and
 - IV. PCR (in-house PCR for *IS6110* gene target).

(In this project Man-LAM [i.e. LAM molecule capped with mannose residues] was purified from *M.tuberculosis* standard strain H₃₇Rv, however for convenience and easy understanding Man-LAM would be referred to as lipoarabinomannan or as LAM in all the following sections).

MATERIALS AND METHODS

Chapter 3.

3. MATERIALS AND METHODS

3.1. SPECIMENS

A total of 120 patients were included in this study. Of these, 100 were radiologically and clinically diagnosed pulmonary tuberculosis patients from Group of T.B Hospital, Sewree. The rest 20 (comprising of 12 males and 8 females) were patients clinically suspected of tuberculous meningitis from K.E.M Hospital, Parel.

3.1.1. The Criteria of Patient Selection

For pulmonary tuberculosis, patients fulfilling the following criteria were included in the study:

- A. Clinical: Cough, night sweats, fever, headache, vomiting, etc.
- B. Radiological: X-ray positive

For tubercular meningitis, patients fulfilling the following criteria were included in the study:

- A. Clinical: Fever, headache, neck stiffness, vomiting, etc
- B. CSF: Pleocytosis with more than 20cells/mm³, predominantly lymphocytes. Proteins raised above 80mg% and hypoglycorrhachia.
- C. Abnormal CT scan findings.

3.1.2. Controls

Sera from a group of 50 healthy individuals, comprising of students, technicians constantly involved in tuberculosis research work, patients suffering from upper respiratory tract problems or allergy were included in this study. Of these 50 controls, 5 were cured of tuberculosis 3-5 years back and at the time of serum collection had no clinical/radiological symptoms of tuberculosis, 15 were suffering from upper respiratory problems, 2 had one of their family members treated for tuberculosis before, 3 had undergone surgeries in last two years and 5 were addicted to alcohol and smoking. Control CSF samples were collected from 20 patients i.e. confirmed cases of viral meningitis, epilepsy and pyogenic meningitis.

3.1.3. Data collection and recording

Detailed history such as age, sex, clinical symptoms, addictions, family history, education/ socioeconomic status, etc from all the patients and controls were recorded. Informed consent was obtained from all the study subjects.

3.1.4. Ethical Committee Approval

The study design was approved by Haffkine Institute's Ethical Committee.

3.1.5. Sample Collection

A) Collection of sputum samples

All the patients suffering from pulmonary tuberculosis and all of the sera controls were instructed to expel a deep productive cough. Early morning

sputum samples were collected and then transported to the laboratory and processed further to detect and isolate the causative mycobacteria. These samples were used for Smear, Culture, Biochemical Identification, Drug Susceptibility, Polymerase Chain Reaction and Radiorespirometry.

B) Collection of blood samples

Blood (4ml) was collected by aseptic vein puncture from all the pulmonary tuberculosis patients as well as controls and transferred to a sterile test tube. The blood was allowed to clot at room temperature and serum was separated. It was stored in sterile plastic vials at -20°C for subsequent testing by an in-house ELISA for the detection of antibodies against lipoarabinomannan. These samples were also used for specific detection of anti-HIV antibodies.

C) Collection of CSF samples

The cerebrospinal fluid was collected from the patients by lumbar puncture and was obtained in screw-capped, sterile, glass containers and transported to the laboratory. These samples were also subjected to Smear, Culture, Biochemical Identification, Drug Susceptibility, Polymerase Chain Reaction and Radiorespirometry. CSF fluids were also tested for the presence of anti-lipoarabinomannan antibodies.

3.2. CONCENTRATION OF SPUTUM/CSF SAMPLES

All the sputum samples were homogenized and decontaminated using NaOH-NALC method (281). The Sputum samples were mixed with double the quantity of homogenizing and decontaminating agent (Appendix A.1) with constant shaking and later incubated at 37°C for 20min. The samples were then centrifuged at 3000 rpm for 20min and the supernatant was discarded. Subsequently, they were given three washes with Phosphate Buffered Saline (PBS) (Appendix A.2), and the sediment obtained after third centrifugation was used for further analysis.

CSF samples were concentrated by centrifugation at 3000xg for 15-20min. The supernatant was discarded and the deposit was used for the various examinations as mentioned above. In case, a pellicle was present in the fluid, it was teased into pieces with the help of a sterile nichrome loop after centrifugation.

3.3. DIRECT SMEAR EXAMINATION

All clinical samples (controls as well as test samples) were examined for the presence of acid-fast bacilli after staining the smears by Ziehl-Neelson technique (282). A smear was prepared from the caseous or purulent part of the specimen using a 5mm internal diameter 24 SWG nichrome wire loop. The smear was then air-dried, heat fixed, submerged with Ziehl-Neelson Carbol fuchsin (ZNCF: Appendix A.3) stain and steamed gently for 7-10min. Care

was taken to avoid boiling or evaporation of stain by replenishing it whenever needed. Smear was then washed under tap water and was later decolorized using acid alcohol until no more stain appeared in washing. Following this step, the smear was thoroughly rinsed with water and counter stained with 1% methylene blue for 1-2min. The slide was again rinsed, air-dried, examined under oil immersion lens by scanning nearly 300 fields and average number of AFB was calculated. Smear was then graded depending on the average number of AFB per field (283)

No of organisms	Report
None in 100 or more fields	No AFB found
1-9 per 100 fields	1+
1-9 per 10 fields	2+
1-9 per field	3+
10 or more per field	4+

3.4. CONVENTIONAL CULTURE METHOD

A loopful of each deposit was inoculated in duplicate onto a Lowenstein Jensen medium slant (Appendix A.4) and was evenly spread with the help of a nichrome loop. The slants were then incubated at 37°C for a period of 8 weeks with the caps screwed tightly to prevent drying of the slant due to evaporation of water of condensation, as moisture is requisite for the growth of tubercle bacilli. The slants were examined once every week for growth of

Mycobacteria over the 8-week period. Smears were prepared from the growth and stained by ZN technique to confirm the presence of AFB (283).

3.5. IDENTIFICATION TESTS

The cultures of mycobacteria obtained on LJ medium were identified to the species level with tests based on their biochemical properties. They were also characterized on the basis of pigment production in dark as well as after exposure to light and colony characteristics. The identification tests were carried out as per the CDC directives. The method for performance and interpretation of key tests for identification of the mycobacterial strains (284, 285, 286) are detailed below

3.5.1. Niacin test

Principle: Niacin is a precursor in the biosynthesis of co-enzyme, Nicotinamide Adenine Dinucleotide Phosphate. A blocked enzymatic pathway prevents *M.tuberculosis*, *M.simiae*, and occasional strains of *M.marinum* and *M.bovis* from utilizing niacin to niacin ribonucleotide, and thereby leads to accumulation of niacin as a water soluble byproduct in the culture medium. Niacin, which is a pyridine compound, is detected by its reaction with cyanogen bromide in the presence of primary aromatic amine like aniline to produce yellow colored Schiff's base.

Procedure: One ml of sterile distilled water was added to a culture slant and allowed to remain in contact with the medium for 15min. In case the growth on the medium was completely confluent, the surface was punctured. Thereafter, 0.5ml of the liquid extract from the culture slant was removed and placed in a screw-capped, clean test tube. Subsequently, an equal volume of 4% aniline was added to it followed by an equal volume of 10% cyanogen bromide (Appendix A.5) and observed for a color change. The test was reported as positive in the case of development of yellow coloration and as negative if it remained colorless.

3.5.2. Reduction of Nitrate

Principle: Mycobacteria produce an enzyme nitroreductase, which reduces nitrate to nitrite, which is subsequently broken down to ammonia. The presence of nitrite in the medium is detected by its diazotization following the addition of sulphanilamide and subsequent coupling with n-naphthyl ethylene diamine dihydrochloride to form a red coloured complex. If nitrite has been reduced further the reaction remains colourless. This reduction is detected by the addition of zinc powder, which develops no colour, whereas true negative test is indicated by the development of red colour, due to immediate reduction of nitrate to nitrite catalysed by zinc. Clinically significant mycobacteria like *M.tuberculosis* and *M.kansasii* are positive for nitrate test.

Procedure: A loopful of actively growing culture was emulsified in a tube containing 2ml of 0.01 M sodium nitrate in M/45 phosphate buffered solution (Appendix A.6), and incubated at 37°C for 2hrs. Later one drop of dilute Hydrochloric acid was added followed by 2 drops each of 0.2% sulfanilamide solution and 0.1% n-naphthyl ethylene diamine dihydrochloride and the tube was observed for colour change. A positive test showed red color while a negative test remained colorless. Development of red colour on addition of zinc dust confirmed negative result.

3.5.3. Growth on p-nitro benzoic acid

Principle: p-nitro benzoic acid is an aromatic compound that has the property to specifically inhibit the growth of *M.tuberculosis* and *M.bovis*, thus aid in differentiating typical from the atypical mycobacteria.

Procedure: A loopful of the cell suspension was inoculated onto the LJ medium slopes containing 500µg/ml p-nitro benzoic acid (Appendix A.7) and incubated at 37°C. Slopes were observed for growth daily for 1 week and weekly thereafter upto 4 weeks. Presence or absence of growth on the slopes was recorded.

3.5.4. Catalase

Principle: *M.tuberculosis*, *M.bovis*, *M.gastri* and some strains of *M.avium* complex lose catalase activity when suspended in pH 7.0 buffer and heated to 68°C. This test is performed for all non-chromogenic slow growers.

Procedure: Colonies from LJ slant were added to a test tube containing 0.5ml of phosphate buffer (pH 7.0) and the tube was then placed at 68°C for 20min. After cooling the tubes to room temperature, 0.5 ml of Tween-Peroxide mixture was added. A positive reaction was determined by formation of bubbles on the surface of the liquid.

3.6. DRUG SUSCEPTIBILITY STUDY (287)

The mycobacterial isolates were tested for their susceptibility to the first line anti-TB drugs namely Isoniazid, Rifampicin, Streptomycin, Pyrazinamide and Ethambutol by Proportion method. The Proportion method consists in calculating the proportion of resistant bacilli present in a strain. The ratio of the number of colonies obtained on the drug-containing medium to the number of colonies obtained on the drug free medium indicates the proportion of resistant bacilli present in the strain. Below critical proportion of resistance (given below), the strain is classified as sensitive; above as resistant.

3.6.1. Culture medium

Lowenstein Jensen medium slopes used were 17mm x 170mm and contained 7ml of medium. The drugs dissolved in distilled water were incorporated in the medium before coagulating. The control medium without drugs was prepared at the same time as the drug-containing medium. After the drug was added to the medium and after the medium was distributed in the slopes, the medium was coagulated at 80°C for 1hr. The slopes were left at room temperature for 24hrs and then stored at 4°C.

3.6.2. Concentrations of the drug employed

Isoniazid	0.2µg/ml
Streptomycin	4µg/ml
Rifampicin	40µg/ml
Pyrazinamide	100µg/ml
Ethambutol	2µg/ml

3.6.3. Inoculum

With a spatula, a representative sample of 5-10mg was taken from the primary culture and placed in a spherical, flat bottomed flask containing 30 glass beads 3mm in diameter. The flask was shaken for 20-30sec; 5 ml of distilled water was added slowly until continuous shaking. The opacity of the bacterial suspension was then adjusted by the addition of distilled water to

that of a standard suspension containing 1mg/ml of tubercle bacilli. For further processing and or inoculation, a calibrated loop was used.

3.6.4. Loop

The loop had an internal diameter of 3mm; so that it delivered 0.01ml. The two bacterial dilutions prepared were 10^{-2} mg/ml and 10^{-4} mg/ml; so that two inocula had 10^{-4} mg and 10^{-6} mg of bacilli for each slope. The dilution 10^{-2} mg/ml was produced by discharging two loopful of the bacteria standardized at 1mg/ml into a small tube containing 2ml of distilled water. Two slopes of the medium without drug and 2 slopes of medium with drug were inoculated with a loopful of each dilution. The inoculated slopes were plugged with cottonwool; no rubber caps were placed on them. The slopes were placed in a stand at a very slight angle from the horizontal and placed in the incubator at 37°C. When the liquid part of the inoculum had evaporated the slopes were covered with rubber caps and left in the incubator at 37°C.

3.6.5. Reading of tests

The results were read for the first time on the 28th day. The average number of colonies obtained for the 2 control slopes indicated the number of culturable particles contained in the inoculum. The average number of colonies obtained for the drug containing slopes indicated the number of resistant bacilli contained in the inoculum. The ratio between the second figure and the first indicated the proportion of resistant bacilli existing in the strain. Below a

certain proportion (the critical proportion) the strain was classified as sensitive; above as resistant. The proportions were reported in terms of percentages. If, according to the criteria indicated below, the result of the reading made on the 28th day was resistant, no further reading of the test for that drug was required; the strain was classified as resistant. If the result at the 28th day was sensitive, a second reading was made on the 42nd day: this provided the definitive result.

3.6.6. Criteria of resistance

For the calculation of the proportion of resistant bacilli, the highest count obtained on the drug free and on the drug containing medium was, regardless of whether this count is obtained on the 28th day, on the 42nd day or with 28th day with one medium and on the 42nd day with the other.

Any strain showing a proportion of resistant bacilli equal or superior to that indicated below is classified as resistant to the corresponding drug:

	Drug concentration ($\mu\text{g}/\text{ml}$)	Critical proportion for resistance
Isoniazid	0.2 $\mu\text{g}/\text{ml}$	1
Streptomycin	4 $\mu\text{g}/\text{ml}$	10
Rifampicin	40 $\mu\text{g}/\text{ml}$	1
Pyrazinamide	100 $\mu\text{g}/\text{ml}$	10
Ethambutol	2 $\mu\text{g}/\text{ml}$	10

3.7. THE RADIORESPIROMETRY TECHNIQUE

A biphasic vial system as described by Shah *et al* (144) was used for early radiometric detection of *M.tuberculosis*. This method is based on quantitation of $^{14}\text{CO}_2$ released by bacteria during metabolism of ^{14}C -U-acetate.

3.7.1. Description of the Biphasic Scintillation Vial System

The Outer Vial: A screw capped, plastic scintillation vial of approximately 20ml capacity served as the outer scintillation chamber. A Whatman No.40 filter paper strip of size 4cmx4cm impregnated with a mixture containing 12 parts of scintillation cocktail and 1 part of 2N methanolic NaOH was placed within the outer vial so as to form a closely fitting, hollow cylinder lining the inner circumference of the vial.

The Inner Vial: A small glass tube of 12mm diameter x 30mm length with two side holes near its open end for gas phase communication between the inner and outer vials served as the inner growth chamber.

Assembly: The inner vial was placed inside the outer vial such that the outer circumference of the inner vial was covered by the scintillation fluor impregnated filter paper.

3.7.2. ¹⁴C- Labeled Substrate

Sodium-acetate-1-2-¹⁴C (49.3 mCi/mM from Bhabha Atomic Research Center, Isotope Group-Board of Radiation Isotope Technology, Mumbai) was incorporated into the nutrient media as the radiolabeled substrate for the microorganisms to utilize.

3.7.3. The Scintillator

A scintillation cocktail containing 10gm 2,5-diphenyloxazole (PPO) and 0.125 gm 1-4-bis-2-(5-phenyloxazole)-benzene (POPOP) was used.

3.7.4. Nutrient Media

1.8ml of glycerol free Lowenstein Jensen medium was added to inner vials and the vials were inspissated at 80°C for 60min in an upright position for three days.

3.7.5. Methodology

The inner vials were layered with 100µl of 1µCi of sodium-acetate-1-2-¹⁴C and 100µl of redigested clinical sample. For positive control, a suspension of the standard of *M.tuberculosis* H₃₇Rv was inoculated into the vials while for negative control; vials were inoculated with heat-killed *M.tuberculosis* H₃₇Rv, sputum collected from sera controls as well as CSF collected from non-TBM patients. Following inoculation, all the inner vials were incubated at 37°C for 72hrs to allow primary growth of organisms to take place. At the end of 72hrs

vials were transferred into the outer vial-filter paper assembly and an initial base-line reading was taken on a beta scintillation counter. Thereonwards, the entire assembly was incubated at 37°C and readings were taken every alternate day in a Packard Liquid Scintillation counter for a period of four weeks.

3.7.6. Reporting of results

A specimen was scored as positive if the counts per minute (cpm) recorded in a scintillation counter for a sample was above mean cpm counts of the blanks +3SD ($M+3SD$).

3.8. THE POLYMERASE CHAIN REACTION:

In addition to the samples from clinically suspected patients of tuberculosis, the test was also carried out with multiple positive and negative controls. The negative controls included 5 sterile distilled water samples, sputum collected from 50 sera controls and 20 CSF specimens from non-TBM patients. A positive control comprising of Standard strain of *M.tuberculosis* H₃₇Rv was processed with every batch of samples.

3.8.1. Extraction of DNA

DNA extraction was carried out as per the method described by Soolingen *et al* (288). A 100µl aliquot of sputum/CSF/control specimens was heated at 80°C for 60min. Lysozyme (50µl of 10mg/ml) was added to the tubes and

kept at 37°C for 1hr, followed by treatment with 75µl of SDS-Proteinase K mix (70µl of 10% SDS and 5µl of 10mg/ml Proteinase K) at 65°C for 10min. Subsequently, 100µl of 5M NaCl and 100µl of 10% CTAB in 4.1% NaCl solution were added to the tubes, tubes were vortexed and further incubated for another 10min at 65°C. Following this, 750µl of 24:1 Chloroform/isoamylalcohol mixture was added, tubes were vortexed again and centrifuged at 12000g for 5min. The supernatant was carefully transferred to a fresh tube and DNA precipitated with 0.6vol of isopropanol for 30min at -20°C. DNA was pelleted (12000g at 25°C for 15min), washed in 70%v/v ethanol in water, air-dried and redissolved in Tris-EDTA buffer (10mm Tris pH8.0, 1mm EDTA). The DNA solution was then quantified and stored at -20°C till further use.

3.8.2. Primers

Primers flanking a 123bp fragment of the *IS6110* locus: a highly repetitive insertion sequence of the *M. tuberculosis* genome was obtained from Bangalore Genei, India.

(Forward Primer: 5'CCT GCG AGC GTA GGC GTC GG3' and Reverse Primer: 5'CTC GTC CAG CGC CGC TTC GG 3').

3.8.3. PCR mix

A PCR reaction mixture was prepared as a master mix and then dispensed into different vials in order to ensure uniformity in composition. PCR was

carried out in a 25 μ l reaction mixture containing 1.5mM MgCl₂, 50mM KCl, 10mM Tris HCl (pH 9.0), 0.01% gelatin, 200 μ M of each of the dNTPs, 0.5 μ M of each primer, 1unit of Taq polymerase (Bangalore Genei, India) and 5 μ l of the extracted DNA. The mixture was overlaid with 50 μ l of mineral oil to prevent evaporation.

3.8.4. Amplification of DNA

Amplification protocol constituted of an initial denaturation step of 95°C for 5min followed by 40 cycles of 1min at 95°C, 1min at 67°C, 1min at 72°C. At the end an extension of 5min at 72°C was also included. During each amplification cycle, a negative control containing all the ingredients except the template DNA was included and a positive control was also included.

3.8.5. Detection and analysis of the amplified products

Ten microliter of the PCR product was resolved electrophoretically on 2% Agarose gel and the molecular size of the amplicon i.e. 123bp was estimated in comparison with the molecular size reference marker (Sigma, USA).

3.8.6. Precautions

The DNA extraction procedure was carried out in a separate room with minimum air circulation. The reaction mixtures were prepared in a vertical laminar flow hood equipped with ultraviolet lights, away from the place where the actual amplification was carried out. Also all the above operations

were physically separated from the place where analysis of the PCR product was carried out.

3.9. ELISA TO DETECT ANTIBODIES AGAINST HIV

SD HIV1/2 ELISA microplate 3.0 precoated with recombinant HIV 1/2 antigens (namely gp41, p24 and gp36 antigens) and marketed by PMC Medical, India was used for the qualitative detection of antibodies against HIV 1/2 as per the instructions provided by the manufacturer.

3.10. PURIFICATION OF LIPOARABINOMANNAN

Purification of LAM antigen from *M.tuberculosis* H₃₇Rv was carried out as per the method described by Severn *et al* (289). In order to improve the availability and purity of lipoarabinomannan for use in biological studies, extraction procedure based on Triton X-114 followed by gel filtration chromatography was used as it was shown to be a better technique in terms of speed than solvent partitioning techniques and in terms of yield and purity than aqueous phenol extraction method (289).

3.10.1. Cell Preparation

M.tuberculosis H₃₇Rv was grown for eight weeks on Youman's medium (Appendix A.8) and killed by heating to 100°C for 3hrs. Cells were washed twice with Tris buffered saline (TBS) 0.05 M, pH 7.5 (Appendix A.9), recovered by centrifugation (10,000g, 30min) and lyophilized.

Lyophilized cells (2g) suspended in TBS (20ml) were cooled to 4°C and sonicated in a Sonicator using a microtip at 80% power for bursts of 5min. The sonications were repeated with alternating cooling in an ice bath for a total sonication time of 15min and the solution of disrupted cells was made up to a volume of 40ml with TBS. After the addition of 10mM MgCl₂, the disrupted cells were digested with RNase and DNase (1 µg/ml) at 37°C for 60min and then at 60°C for additional 60min.

3.10.2. Isolation procedure

Triton X-114 was added to the lysed cells to a concentration of 8% (v/v) and after cooling on ice, the solution was stirred at 4°C for 16hrs. The cellular debris was removed by centrifugation (10,000g, 4°C, 30min) and the supernatant was incubated at 37°C to induce phase separation. The phases were recovered after centrifugation (4000g, 30°C, 20min) and the upper aqueous layer was mixed with the cellular debris and re-extracted as above. The detergent phases were combined and the lipoglycan was precipitated by the addition of cold ethanol (- 20°C, 95%, five times the volume of detergent phase) and collected by centrifugation (10,000g, 30min).

3.10.3. Partial purification by Ultracentrifugation

The crude lipoglycan obtained as above (3.10.2) was dissolved in water (10-20mg/ml) by stirring overnight and ultracentrifuged at 35,000g for 16hrs. The pellet was collected, dissolved in a minimal amount of water and treated with

Proteinase K (1mg/ml) for 2hrs at 60°C. The solution was ultracentrifuged twice more, reconstituted in water and lyophilized.

3.10.4. Purification of LAM by Gel filtration chromatography.

3.10.4.1. Calibration of Sephacryl S-200 column

Sephacryl S-200 was washed in water followed by two more washes in Tris deoxycholate buffer {10mM Tris-Cl pH 8.0, 10mM EDTA, 0.2M NaCl, 0.25% deoxycholate, 0.02% NaN₃, pH 8.0} (TDB). After removing the excess buffer, 70% slurry of Sephacryl S-200 in TDB was prepared. With continuous stirring, the gel was packed in a glass column (100 x1.5cm) at room temperature. All the protein standards and sample runs were also performed at room temperature.

Six protein standards namely, Blue dextran, Albumin, Alcohol dehydrogenase, Carbonic Anhydrase, Amylase and Cytochrome C were passed through the column and eluted with TDB in order to calibrate the column. After each protein run, the column was washed with the same buffer for a day. Elution volume (V_e) of all the protein standards and void volume (V_o) of the column was calculated. A graph of log molecular weight against V_e/V_o of the standards was plotted to determine molecular weight of the unknown sample.

3.10.4.2. Purification of LAM from crude lipoglycan extract by gel filtration chromatography

Lyophilized sample obtained after ultracentrifugation was prepared for column chromatography by resuspension in TDB to a concentration of 10mg/ml. The sample was applied to Sephacryl S-200 column (100 x 1.5cm) and eluted using the same buffer. The fractions were collected and analyzed colorimetrically for presence of arabinose (290) and by SDS-PAGE (291).

3.10.4.3. Colorimetric method for determination of sugars

Phenol in the presence of Sulphuric acid was used for quantitative colorimetric microdetermination of sugars, as this method is known to be simple, rapid, sensitive and stable (290).

Briefly, two milliliter of sugar solution containing 10 μ g to 70 μ g of arabinose/mannose was pipetted into a colorimetric tube and 1050 μ l of 4% phenol was added. Then 5ml of concentrated Sulphuric acid was added rapidly, the tubes were allowed to stand for 10min, and then they were shaken and placed for 10 to 20min in a water bath at 25 to 30°C before readings were taken. The absorbance of the characteristic yellow-orange color was measured at 480nm for arabinose and at 490nm for mannose. Blanks were prepared by substituting distilled water for the sugar solution. The amount of arabinose/mannose present in the eluted fractions was then determined by reference to a standard curve previously constructed for the respective sugars.

3.10.4.4.SDS-PAGE

Crude Lipoglycan extract obtained after ultracentrifugation, column eluents and concentrated LAM, LM and PIM were analyzed in a discontinuous SDS-polyacrylamide gel system using Tris-glycine buffer (291).

The glass plates, spacers and comb required for SDS-PAGE assembly were rinsed with Milli-Q water and wiped with methanol. The glass plates with spacers in between were placed together in an upright position on a stand tightly held by screws. A 12% acrylamide resolving gel solution was filled into the plate assembly and the gel solution was then overlaid with a few drops of water-saturated *n*-butanol to keep the gel surface flat and the interface sharp during polymerisation.

	12% Resolving Gel
Water	9.9ml
Acryl-Bisacryl mix (29:1)	12ml
Tris Buffer (1.5M,pH 8.8)	7.5ml
10%APS	0.3ml
10%SDS	0.3ml
Temed	12 μ l

After polymerisation of the resolving gel, the butanol was poured off and the top of the gel was rinsed with Milli-Q water and drained thoroughly.

Subsequently, the 4% Acrylamide stacking gel solution was overlaid on the resolving gel, the comb was inserted and the gel was left to polymerise.

	4% Stacking Gel
Water	7.2ml
Acryl-Bisacryl mix (29:1)	1.35ml
Tris Buffer (1.5M,pH 8.8)	1.25ml
10% APS	0.1ml
10% SDS	0.1ml
Temed	10 μ l

For electrophoresis, the plate assembly was placed into the buffer tank and subsequently the tank was filled with the chilled running buffer {25mM Tris, 190mM glycine (8.3), 0.1% SDS}. Meanwhile, 20 μ l of the column eluents along with 20 μ l sample buffer (62mM Tris, 10% SDS, 1%mercaptoethanol, 20% glycerol, 0.2% Bromophenol Blue) were mixed and heated to 100°C for 5min. The comb was removed carefully from the plate assembly, the wells were rinsed with running buffer and the samples were loaded into the wells. Molecular weight marker for silver staining (Bangalore Genei, India) was used as a calibration standard. The gel was run at 200V until the dye front was approximately 1 cm from the bottom of the gel. The apparatus was dismantled and the glass plates were prised apart very gently.

Recycling

- Staining 0.1% silver nitrate 30min
- Wash 1X dd water 10sec
- Developer 3% sodium carbonate,
0.02% formaldehyde 10min
- Stop 1% acetic acid 5min
- Wash 3X dd water 10min
- Reduction 0.3% sodium thiosulphate,
0.15% potassium ferricyanide
0.05% sodium carbonate 10-30sec
- Wash 2X dd water 10 min
- Store 7% acetic acid

3.10.4.6. Concentration of purified LAM samples

The appropriate LAM, LM and PIM fractions were pooled, desalted by ultrafiltration in a centriplus concentrator using a 3000 mol weight cut-off membrane, resuspended in water and lyophilized.

3.11. DEVELOPMENT AND STANDARDIZATION OF ELISA FOR LAM ANTIGEN

Different concentrations of LAM preparation (0.1µg/100µl - 8µg/100µl) was coated onto polyvinyl ELISA plates (Dynatech, UK) along with different concentrations of blocking agents [i.e. BSA {1%-3%} and lysine {.01%-.03%}],

various dilutions of sera/CSF (1:20 – 1:200) and anti-human IgG, IgM and IgA HRP conjugate (1:1000 – 1:20,000) by checker-board method to determine the optimum concentrations of the reagents for ELISA. Optimum concentrations thus determined were used for further analysis.

3.12. IN-HOUSE ELISA FOR DETECTING ANTI-LIPOARABINOMANNAN ANTIBODIES IN SERA/CSF SAMPLES

All the sera samples collected from pulmonary tuberculosis patients, CSF samples collected from suspected cases of tuberculous meningitis and control sera/CSF samples were tested for anti-LAM IgG, anti-LAM IgM and anti-LAM IgA antibodies.

Polyvinyl Microtiter plate (Dynatech, UK) was coated with 100µl of LAM antigen at the concentration of 5µg/ml in Carbonate-bicarbonate buffer (pH 9.6) and incubated overnight at 37°C. All appropriate blanks like conjugate blank and Antigen Blank were also run at the same time. The following day, after five washes with phosphate buffered saline containing 2% Tween-20 (PBS-T), the plate was blocked with 0.01% lysine in carbonate buffer for 2hrs at 37°C. Subsequently, the plate was washed 5 times with PBS-T and 100µl of test/control sera diluted 1:100 in PBS containing 1% Tween (dilution buffer) were added to the plate and incubated at 37°C for 2hrs. For CSF, 100µl of test/control samples diluted 1:20 in dilution buffer were added to the plate and incubated at 37°C for 2hrs. At the end of 2hrs, the plate was washed five

times with PBS-T and incubated with 100µl of anti-human IgG, IgM or IgA HRP conjugate (Sigma, USA) diluted 1:5000 in dilution buffer for 1hr at 37°C. Following this, the plate was washed five times more, 100µl of substrate (Sigma Fast OPD and Sigma Fast H₂O₂ tablets dissolved in distilled water) was added and the plate was left at room temperature in the dark for approximately 15min. Eventually the reaction was stopped by adding 50µl of 2NH₂SO₄ and the absorbance was measured at 490nm using an ELISA plate reader (Dynatech MR5000, UK).

3.13. STATISCAL ANALYSIS

All the data obtained was analyzed on computer using SPSS/PC package.

RESULTS

Chapter 4.

4. RESULTS

The present study included a total of 120 patients, who were divided into two groups. Group A consisted of 100 clinically and radiologically diagnosed patients of pulmonary tuberculosis, while Group B consisted of 20 clinically suspected cases of tuberculous meningitis (TBM). In addition to these, two control groups were also included in the study.

4.1. AGE/SEX DISTRIBUTION & OTHER CHARACTERISTICS

4.1.1 Age/Sex distribution in pulmonary tuberculosis cases (Group A)

Of the total 100 patients in Group A, 66 (66%) were males and 34 (34%) were females. Of 66 male patients, 57.6% belonged to the age group of 21-40 years. Similarly, of 34 females, 61.7% were within the third and the fourth decade of life (Table 4.1).

Table 4.1: Distribution of Age/Sex in Group A

Age groups	1-20 yrs	21-40 yrs	41-60 yrs	> 60 yrs	Total
Males	6 (9.09)	38 (57.6)	18 (27.2)	4 (6.06)	66 (66)
Females	8 (23.5)	21 (61.7)	5 (14.7)	-	34 (34)
Total	14 (14)	59 (59)	23 (23)	4 (4)	100

Figures in parenthesis indicate percentages

4.1.2. Age/Sex distribution in Tuberculous Meningitis cases (Group B)

In the case of Group B, of the 20 patients studied, 12 (60%) were males and 8 (40%) were females. Majority of the total patients i.e. 65% were children below the age of 10 yrs (Table 4.2).

Table 4.2: Distribution of Age/Sex in Group B

Age Groups	1-10 yrs	11-20 yrs	Total
Males	8 (66.6)	4 (33.3)	12 (60)
Females	5 (62.5)	3 (37.5)	8 (40)
Total	13 (65)	7 (35)	20

Figures in parenthesis indicate percentages

4.1.3. Age/Sex distribution in Controls

As shown in Table 4.3, of the 50 serum controls, 35 (70%) were males and 15 (30%) were females, while CSF controls included 15 (75%) males and 5 females (25%). In the case of the serum controls, 64% of the total were from the 21-40 year age group, while majority of the total CSF controls were (80%) in the age group of 1-20yrs.

Table 4.3: Distribution of Age/Sex in Controls

		1-20 yrs	21-40 yrs	41-60 yrs	Total
50 sera controls	Males	3 (8.5)	20 (57.1)	12 (34.2)	35 (70)
	Females	1 (6.6)	12 (80)	2 (13.3)	15 (30)
	Total	4 (8)	32 (64)	14 (28)	50
20 CSF Controls	Males	12 (80)	3 (20)	-	15 (75)
	Females	4 (80)	1 (20)	-	5 (25)
	Total	16 (80)	4 (20)	-	20

Figures in parenthesis indicate percentages

4.1.4. Demographic characteristics of Group A, Group B and Controls

The demographic characteristics of all the groups are listed in Table 4.4. As seen, 35% of the patients in Group A were illiterate, with 45% of the total population being unemployed. Twenty-seven% patients known to indulge in smoking, of which 15% were also alcoholics and 2% chewed pan. Eight % in Group A had at least one of their family members (father/mother/husband) being treated for tuberculosis in the past. In Group B, since all of the patients were within the age of 1-20 yrs, 50% of them were still studying. Only 15% of the total TBM population in Group B was unemployed.

Among the 50 serum controls, literacy rate was seen to be higher than in Group A, while none of the cases in this group were unemployed. This group comprised of 2 (4%) subjects with a family history of anti-tuberculosis treatment, 3 (6%) patients who had undergone invasive surgeries, 1 (2%) case of malaria and 5 (10%) patients who were cured of tuberculosis 3-5 years before the time of sample collection. It also comprised of 15 (30%) patients who suffered from upper respiratory diseases like pneumonia, allergy, etc. at the time of sample collection. Of the 20 CSF controls, 3 (15%) were married and 12 (60%) were students. All the sputum samples collected from sera controls and CSF samples collected from CSF controls were tested for Smear, Conventional Culture, Radiorespirometry and PCR in order to rule out tuberculosis.

Table 4.4: Demographic characteristics of Group A, Group B and Controls

Characteristics	Group A	Group B	Sera Controls	CSF controls
Total	100	20	50	20
<u>Marital Status</u>				
Married	32 (32)	-	12 (24)	3 (15)
<u>Education Status</u>				
Illiterate	35 (35)	6 (30)	-	-
Primary Education	21 (21)	7 (35)	-	-
Secondary Education	19 (19)	3 (15)	7 (14)	12 (60)
Matriculation	15 (15)	4 (20)	8 (16)	4 (20)
Graduation and above	10 (10)	-	35 (70)	4 (20)
<u>Employment</u>				
Unemployed	45 (45)	3 (15)	-	-
Students	5 (5)	10 (50)	21 (42)	12 (60)
<u>Addictions</u>				
Alcohol	15 (15)	-	2 (4)	-
Smoking/Tobacco	27 (27)	-	2 (4)	-
Pan	2 (2)	-	-	-
<u>Family contact</u>				
Family members treated for tuberculosis before	8 (8)	-	2 (4)	2 (10)
<u>Past ailments</u>				
Surgeries	-	-	3 (6)	-
Malaria	-	-	1 (2)	-
Tuberculosis (3-5 yrs back)	-	-	5 (10)	-
<u>Current ailments</u>				
Non-tuberculosis upper respiratory diseases	-	-	15 (30)	-
Viral/pyogenic meningitis	-	-	-	20 (100)

Figures in parenthesis indicate percentages

4.2. MICROBIOLOGICAL RESULTS

Comparative results of conventional methods and newer techniques for the diagnosis of tuberculosis.

Smear, Culture, Radiorespirometry, PCR and ELISA results in Group A and in Group B are explained in Table 4.5.

Table 4.5: Cumulative results of Smear, Culture, Radiorespirometry, PCR and ELISA in pulmonary tuberculosis (Group A) and tuberculous meningitis (Group B).

	Number of samples (%) positive by				
Group	Smear	Culture	Radioresp- irometry	PCR	+ve by either ELISA (IgG/ IgM/IgA)
A (n=100)	36 (36)	48 (48)	39 (39)	48 (48)	90 (90)
B (n=20)	2 (10)	5 (25)	4 (20)	12 (60)	9 (45)

Figures in parenthesis indicate percentages

As illustrated in Table 4.5, of the 100 pulmonary tuberculosis cases in Group A, 36% were positive by Smear, 48% by Culture, 39% by Radiorespirometry, 48% by PCR and 90% of the samples were positive by ELISA (samples tested positive for either IgG, IgM and/or IgA antibodies), while of the 20 tuberculous meningitis cases in Group B, 10% tested positive by Smear, 25% by Culture, 20% by Radiorespirometry, 60% by PCR and 45% by ELISA.

Diagrammatic representation of the number of samples positive by Smear, Culture, Radiorespirometry, PCR and either one of the ELISAs in Group A (Fig 4.1) and in Group B (Fig 4.2) is shown below.

Fig 4.1: Bar diagram showing comparative results of Smear, Culture, Radiorespirometry, PCR and ELISA in the pulmonary tuberculosis group (Group A)

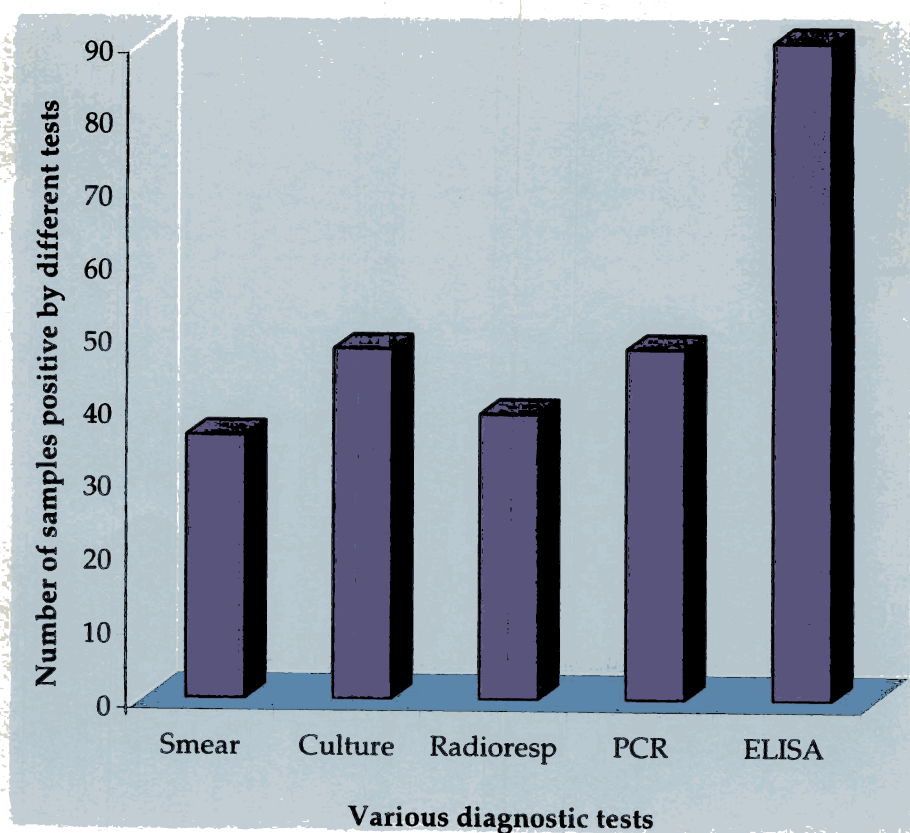
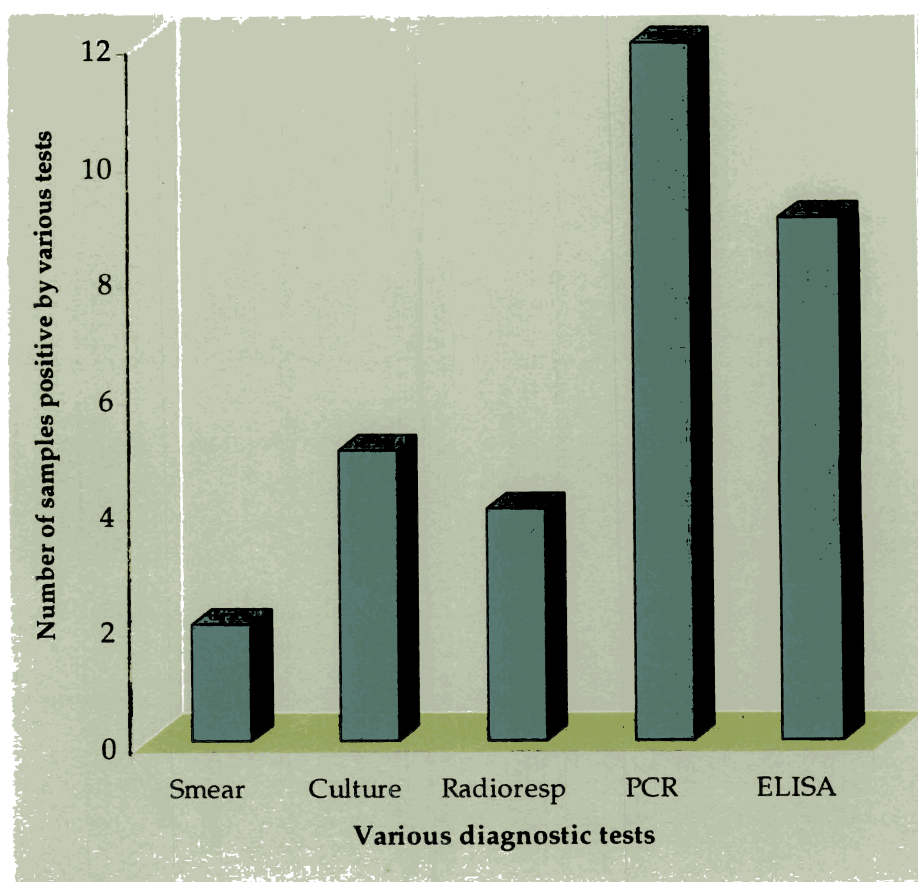


Fig 4.2: Bar diagram showing comparative results of Smear, Culture, Radiorespirometry, PCR and ELISA in the tuberculous meningitis group (Group B).



All the overlapping results of various diagnostic tests are shown in details in subsequent sections.

4.3. CONVENTIONAL DIAGNOSTIC METHODS

As illustrated in Table 4.6, in the case of Group A, 36% cases were positive by smear, while 48% were positive by culture. Of the 36 smear positive samples, 32 (88.8%) were positive by culture, while 16 (25%) of the 64 smear negative samples were positive by culture. Of the 20 patients in Group B, 2 (10%) samples were smear positive and 5 (25%) were culture positive, while 15 samples (75%) were smear and culture negative.

Table 4.6: Comparative analysis of smear and culture

Analysis of Smear and Culture in PTB (Group A)			
	Culture positive	Culture negative	Total
Smear positive	32 (88.8)	4 (11.1)	36 (36)
Smear negative	16 (25)	48 (75)	64 (64)
Total	48 (48)	52 (52)	100
Analysis of Smear and Culture in TBM (Group B)			
	Culture positive	Culture negative	Total
Smear positive	2 (10)	-	2 (10)
Smear negative	3 (16.6)	15 (83.3)	18 (90)
Total	5 (25)	15 (75)	20

Figures in parenthesis indicate percentages

All the mycobacterial isolates were identified as *Mycobacterium tuberculosis* by using biochemical identification tests like niacin test, nitrate test, catalase test, pigmentation and growth on PNB medium.

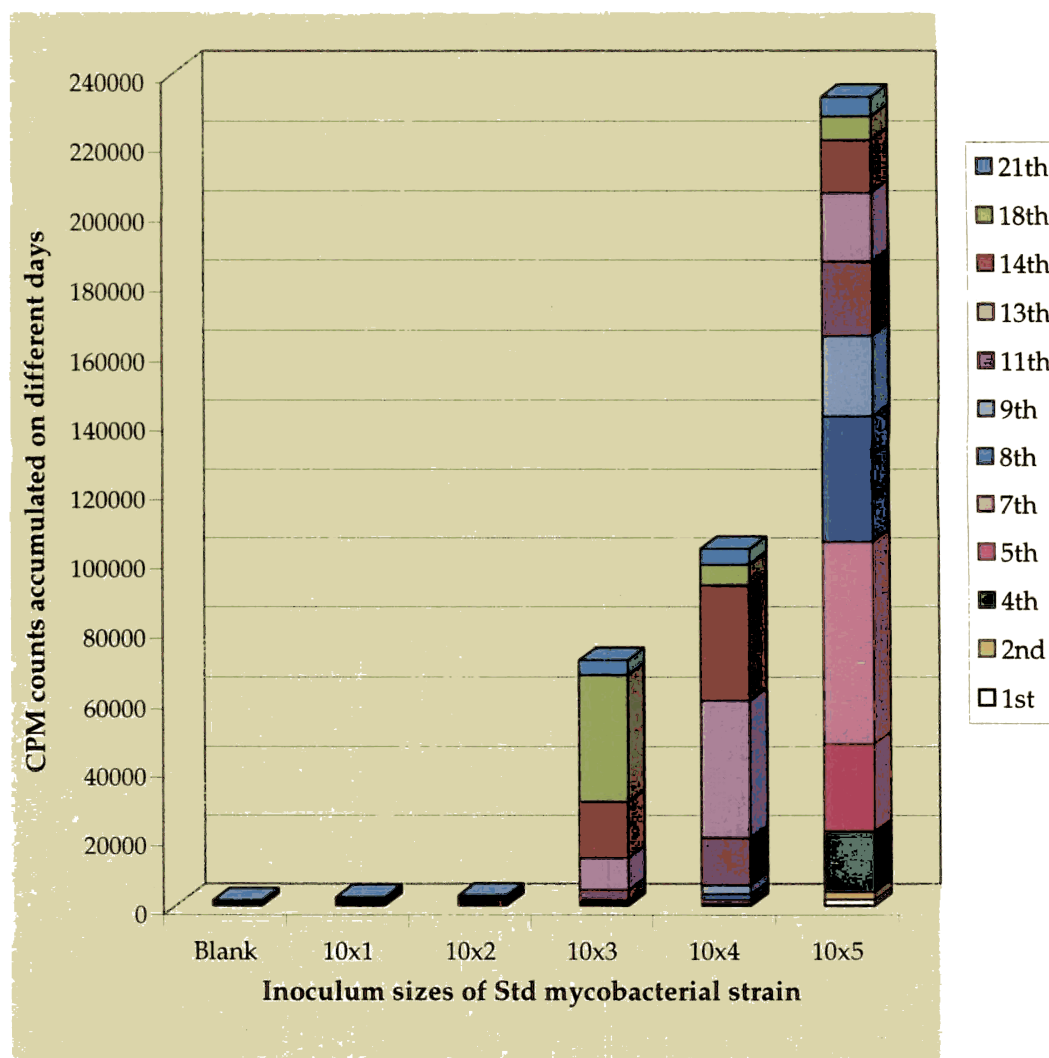
4.4. RADIORESPIROMETRY

4.4.1. Optimization of Radiorespirometry

Serial dilutions (10^1 , 10^2 , 10^3 , 10^4 and 10^5) of standard *Mycobacterium tuberculosis* strain H₃₇Rv mycobacterial cells (H₃₇Rv) as well as various blanks comprising of heat-killed std. strain and sputum samples & CSF samples (collected from controls and processed for Radiorespirometry as mentioned in materials and methods), were inoculated into the vials containing LJ media supplemented with ^{14}C Na-acetate as carbon source. The growth index was determined by monitoring $^{14}\text{CO}_2$ respired by the bacteria in a Liquid Scintillation Counter every alternate day for a period of 4 weeks.

As seen in Fig 4.3, a mycobacterial inoculum of 10^5 concentration accumulated 20,000 counts per minute (CPM) on the 5th day, while 10^4 and 10^3 inoculum dilutions accumulated the same number of counts by the 13th and the 14th day, respectively. It was also observed that once a maximum accumulation of counts was achieved, the counts would dip e.g. in the case of an inoculum concentration of 10^5 , beyond day 7, the counts per minute started decreasing and were seen to be minimal on day 21. Various blanks included in the study (as mentioned above) accumulated non-significant counts.

Fig 4.3: Counts per minute accumulated for various inoculum sizes of standard *Mycobacterium tuberculosis* strain i.e.H₃₇Rv



4.4.2. Results of Radiorespirometry

Samples were grouped on the basis of their smear and culture status and radiorespirometry results were compared with each group.

Table 4.7: Comparative results of Radiorespirometry with respect to smear and conventional culture for pulmonary tuberculosis (Group A) samples

		RADIORESPIROMETRY POSITIVE	TOTAL
SMEAR	Smear positive n= 36	19 (52.7)	39 (39)
	Smear negative n= 64	20 (31.2)	
CULTURE	Culture positive n= 48	25 (52.0)	39 (39)
	Culture negative n= 52	14 (26.9)	

Figures in parenthesis indicate percentages

As illustrated in Table 4.7, of the 100 sputum samples collected from the Group A subjects, 39 (39%) tested positive by radiorespirometry. These included 19 cases that were also positive by smear and 25 cases that were also positive by conventional culture. In addition to these, 20 samples that were negative by smear and 14 samples that were negative by conventional culture were found to be positive by radiorespirometry.

Table 4.8: Comparative results of Radiorespirometry with respect to smear and conventional culture for tuberculous meningitis (Group B) samples

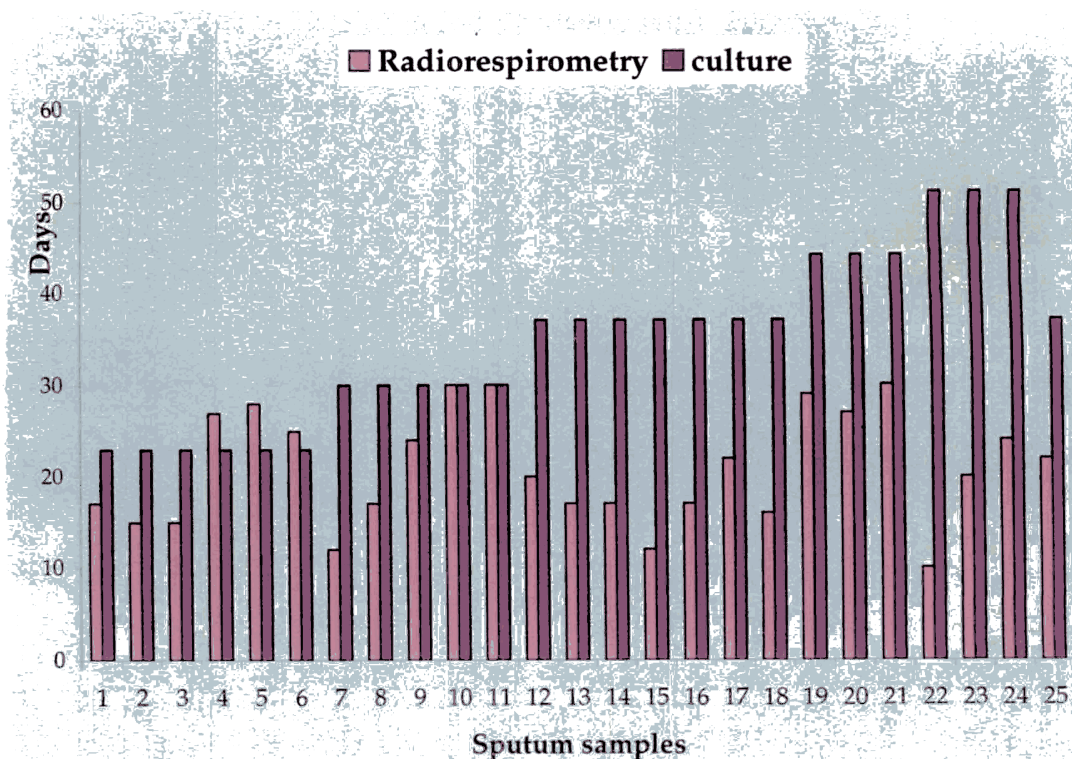
		RADIORESPIROMETRY POSITIVE	TOTAL
SMEAR	Smear positive n= 2	2 (100)	4 (20)
	Smear negative n= 18	2 (11.1)	
CULTURE	Culture positive n= 5	4 (80)	4 (20)
	Culture negative n= 15	-	

Figures in parenthesis indicate percentages

As explained in Table 4.8, in the case of the CSF samples collected from Group B, 4 (20%) were positive by radiorespirometry. These included 2 samples that also tested positive by smear and 4 samples that tested positive by conventional culture.

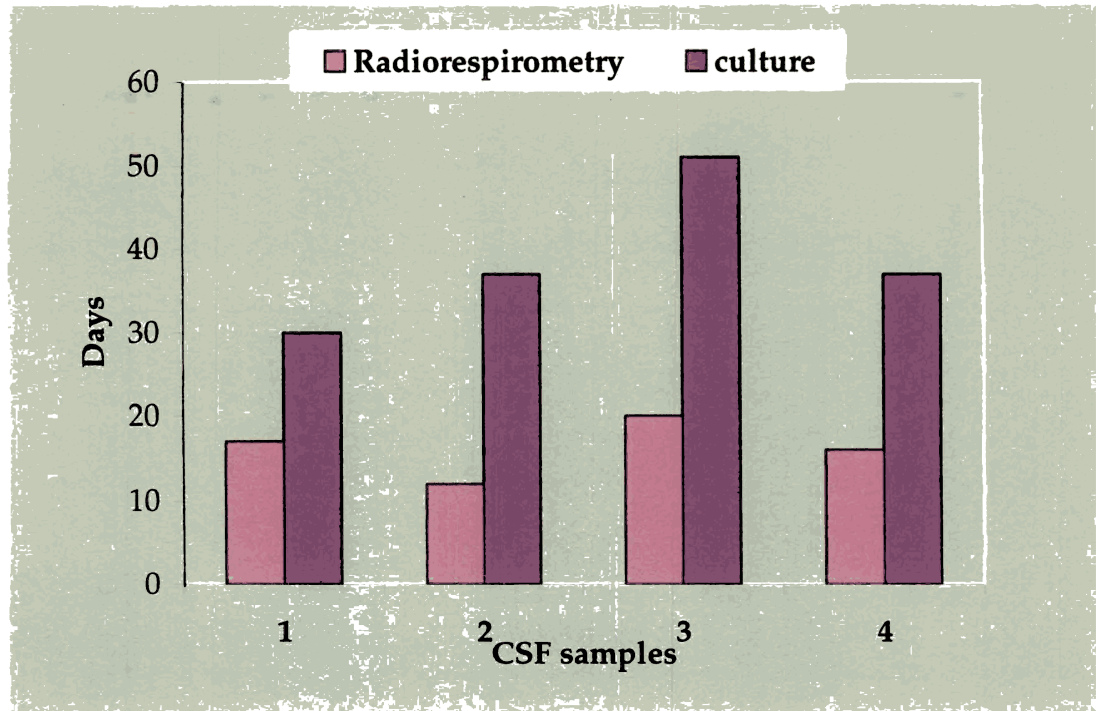
A comparative analysis of the detection time, in the case of the 25 Group A samples that were positive by both radiorespirometry and the conventional culture methods, showed that growth in 22 of the 25 sputum samples was detected earlier by radiorespirometry (Fig 4.4).

Fig 4.4: Comparison in detection time of 25 samples positive by both Radiorespirometry and conventional culture method (Group A).



A similar pattern was observed in the case of all the 4 samples in Group B that were positive by radiorespirometry as well as conventional culture, wherein growth was detected earlier by radiorespirometry than by the conventional method (Fig 4.5).

Fig 4.5: Comparison in detection time of 4 CSF samples in Group B positive by both Radiorespirometry and Culture.



The difference in the detection times of radiorespirometry and conventional culture in the case of both Group A and Group B samples was found to be significant using paired t-test ($p < 0.01$).

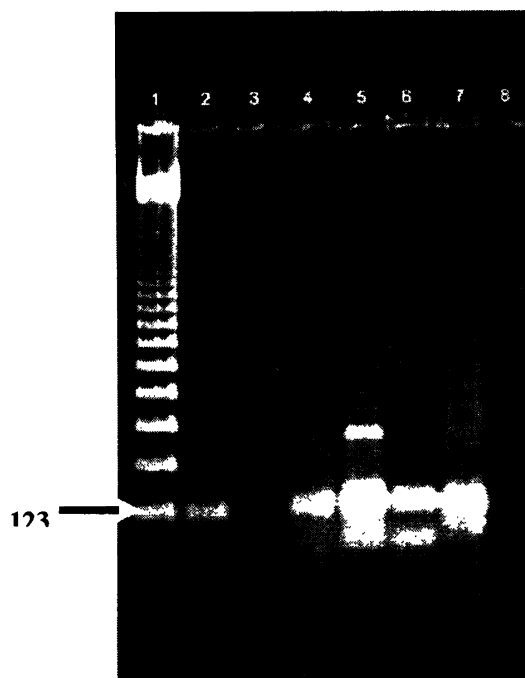
4.5. POLYMERASE CHAIN REACTION

4.5.1. Optimization of PCR

DNA was extracted from sputum samples and CSF fluids collected from clinically positive tuberculosis patients as well as from healthy controls and diseased controls (patients suffering from pneumonia, cough and non-TBM patients, etc.). To obtain optimal sensitivity in a diagnostic PCR, the reaction was carried out for 40 cycles. The PCR reaction for all the specimens was performed under stringent conditions, using a higher annealing temperature (67°C) in order to minimize the occurrence of primer-dimer and to eliminate non-specific reactions.

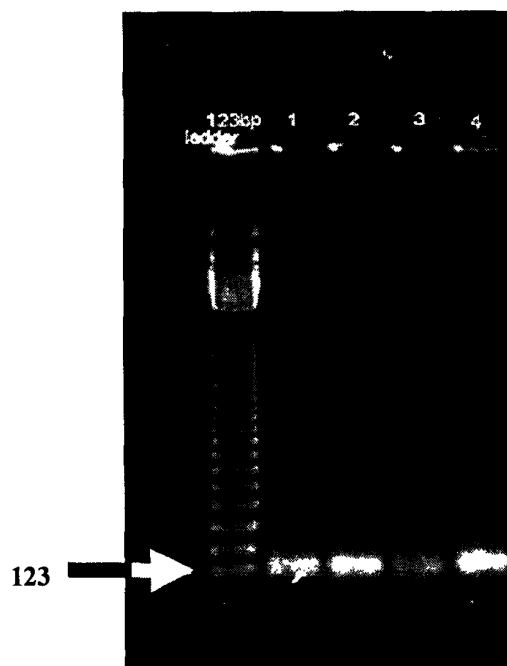
Fig 4.6 illustrates representative results of amplification analysis of *IS6110* gene fragment at 65°C annealing temperature. Fig 4.7 illustrates representative results of amplification analysis of *IS6110* gene fragment at 67°C annealing temperature. A specific band of 123bp (indicated by arrowhead) represents a positive reaction.

Fig 4.6: Representative results of amplification analysis of *IS6110* gene in Std *Mycobacterium tuberculosis* strain and clinical samples (PCR reaction carried out at 65°C annealing temperature.)



Lane 1: Std molecular weight marker (Sigma), Lane 2: Std *Mycobacterium tuberculosis* strain: H₃₇Rv = +ve control, Lane 3: Negative buffer control, Lane 4: Sputum sample showing 123bp PCR product, Lanes 5 & 6: Sputum samples showing a non-specific reaction and a primer -dimer along with 123bp PCR product, Lane 7: CSF sample showing 123bp product, Lane 8: Sputum sample of a healthy individual showing no 123bp product (negative control).

Fig 4.7: Representative results of amplification analysis of *IS6110* gene in Std *Mycobacterium tuberculosis* strain and clinical specimens at 67°C annealing temperature



Std molecular weight marker (Sigma), Lane 1: Std *Mycobacterial tuberculosis* strain: H₃₇Rv = +ve control, Lanes 2 & 3: Sputum samples showing 123bp PCR product (no primer - dimer), Lane 4: CSF sample showing 123bp PCR product (no primer - dimer).

4.5.2. Results of PCR

Patients were grouped on the basis of their smear, Culture and Radiorespirometry results and PCR was then compared with each group.

Table 4.9: Comparative results of PCR with respect to smear, conventional culture and Radiorespirometry for Group A samples

		NO OF SAMPLES POSITIVE BY PCR	TOTAL
SMEAR	Smear positive n= 36	20 (55.5)	48 (48)
	Smear negative n= 64	28 (43.7)	
CULTURE	Culture positive n= 48	27 (56.2)	48 (48)
	Culture negative n= 52	21 (40.3)	
RADIO RESPIRO METRY	Radiorespirometry +ve n=39	23 (58.9)	48 (48)
	Radiorespirometry -ve n=61	25 (40.9)	

Figures in parenthesis indicate percentages

As observed in Table 4.9, of the 100 sputum samples collected from the Group A patients, a total of 48 tested positive by the *IS6110*-derived PCR. These included 20 samples that were positive by smear, 27 that were positive by conventional culture and 23 samples that were positive by radiorespirometry. Moreover, 28 (43.7%) of the 64 smear negative samples, 21 (40.3%) of the 52 culture negative samples and 25 (40.9%) of the 61 radiorespirometry negative samples were also found to be positive by PCR.

Table 4.10: Comparative results of PCR with respect to smear, conventional culture and Radiorespirometry for Group B samples

		NO OF SAMPLES POSITIVE BY PCR	TOTAL
SMEAR	Smear positive n= 2	2 (100)	12 (60)
	Smear negative n= 18	10 (55.5)	
CULTURE	Culture positive n= 5	5 (100)	12 (60)
	Culture negative n= 15	7 (46.6)	
RADIO RESPIRO METRY	Radiorespirometry +ve n= 4	4 (100)	12 (60)
	Radiorespirometry -ve n= 16	8 (50.0)	

Figures in parenthesis indicate percentages

As illustrated in Table 4.10, in the case of Group B, of the 20 CSF samples, 12 were found to be positive by PCR, of which 2 were smear positive, 5 were culture positive samples and 4 were radiorespirometry positive. In addition, 10 (55.5%) of the 18 smear negative samples, 7 (46.6%) of the 15 culture negative samples and 8 (50%) of the 16 radiorespirometry negative samples were also found to be positive by PCR.

None of the control sputum/CSF samples showed a 123bp PCR product on agarose gel electrophoresis, thus giving a specificity of 100% in both the groups.

4.6. DRUG SUSCEPTIBILITY PATTERN

Drug susceptibility testing by the proportion method was carried out for all of the 48 Group A and 4 Group B mycobacterial isolates that were obtained by the conventional culture method.

Of the 48 Group A clinical isolates, 30 (62.5 %) were found to be resistant to one or more first-line anti-TB drug. Resistance to Rifampicin (23/48 i.e. 47.9%) was seen to be most common, followed by Streptomycin (21/48 i.e. 43.7%), Isoniazid (20/48 i.e. 41.6%) and Ethambutol (10/48 i.e. 20.8%). On the other hand, all the 4 Group B mycobacterial isolates were found to be sensitive to all the first-line anti-TB drugs.

4.7. SEROPREVALENCE OF HIV

Serum samples collected from the entire Group A as well group B subjects were screened for the presence of anti-HIV antibodies using an SD HIV1/2 ELISA kit (PMC Medical, India). Ten (10%) of 100 Group A subjects tested positive for anti-HIV antibodies, while none of the Group B subjects were found to be positive for the same.

4.8. DETECTION OF ANTI-LAM ANTIBODIES

4.8.1. Purification Of Lipoarabinomannan Antigen

4.8.1.1. Isolation and partial purification of LAM

Isolation and partial purification was carried out as per the method described by Severn *et al* (289).

4.8.1.2. Calibration of Sephacryl S-200 column.

Six protein standards namely, Blue dextran, Albumin, Alcohol dehydrogenase & Carbonic Anhydrase, Amylase & Cytochrome C were passed through the Sephacryl S-200 column either alone or in combination as recommended by the manufacturer (Sigma, USA) and eluted with Tris deoxycholate buffer. The void volume of the column (V_o) and the elution volume (V_e) of the proteins were calculated (Fig 4.8 –Fig 4.12).

Fig 4.8: Calibration of Sephacryl S-200 column using Blue dextran

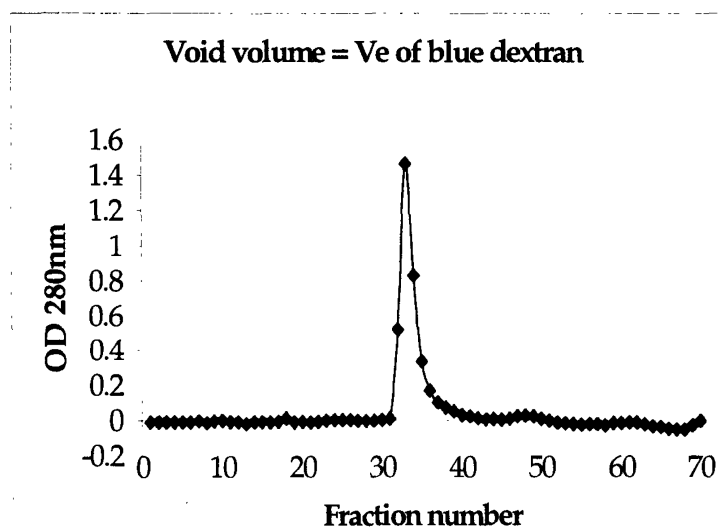


Fig 4.9: Calibration of Sephacryl S-200 column using Albumin

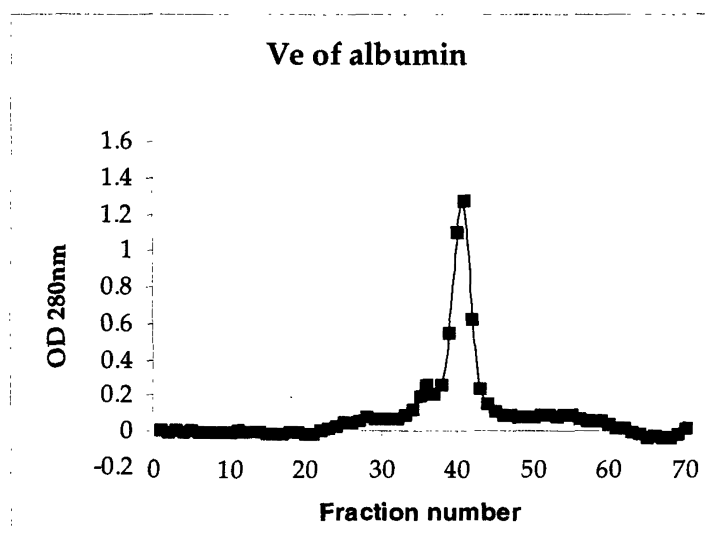


Fig 4.10: Calibration of Sephacryl S-200 column using Alcohol dehydrogenase and Carbonic anhydrase

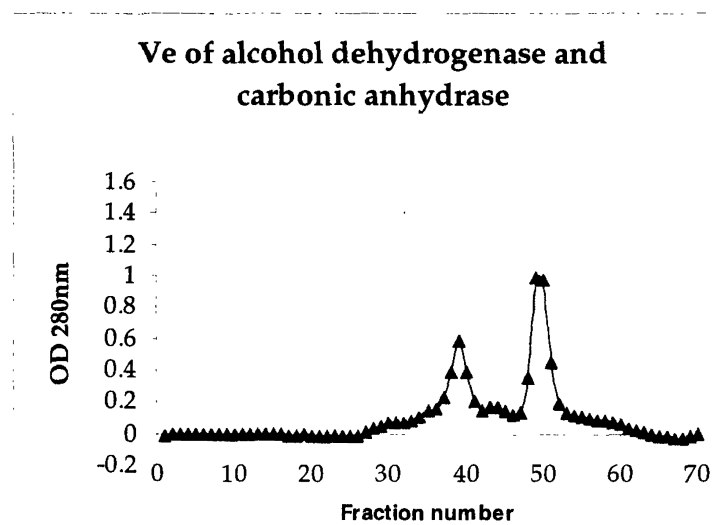


Fig 4.11: Calibration of Sephacryl S-200 column using Amylase and Cytochrome C

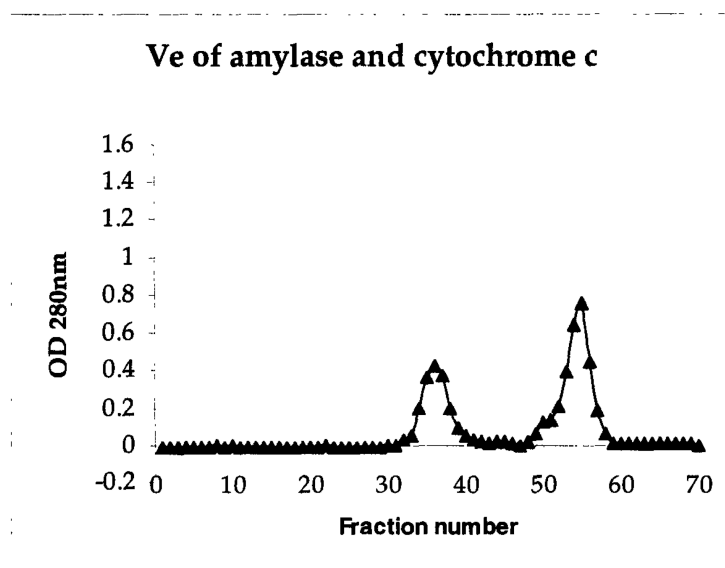
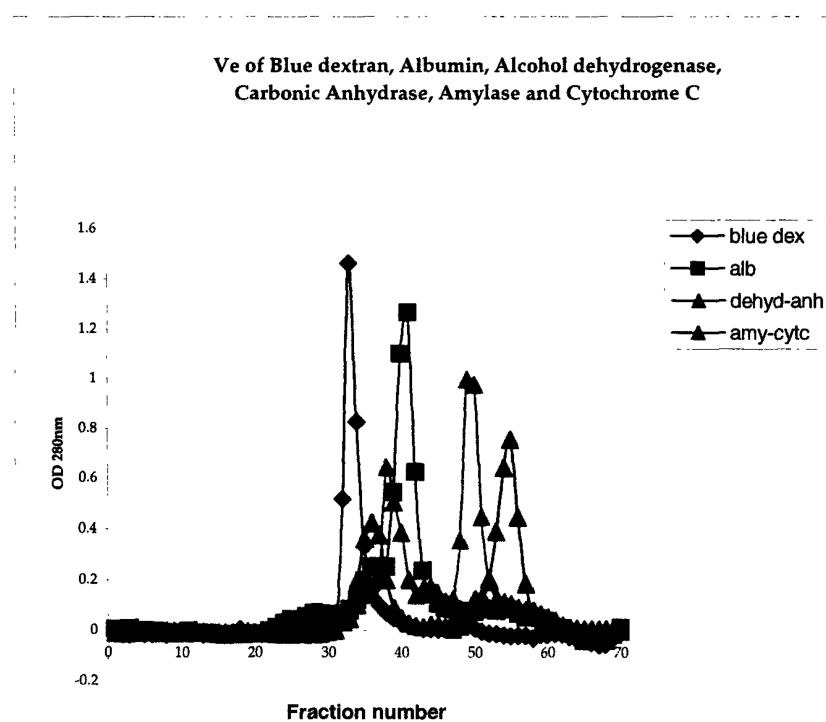


Fig 4.12: Calibration of Sephacryl S-200 column

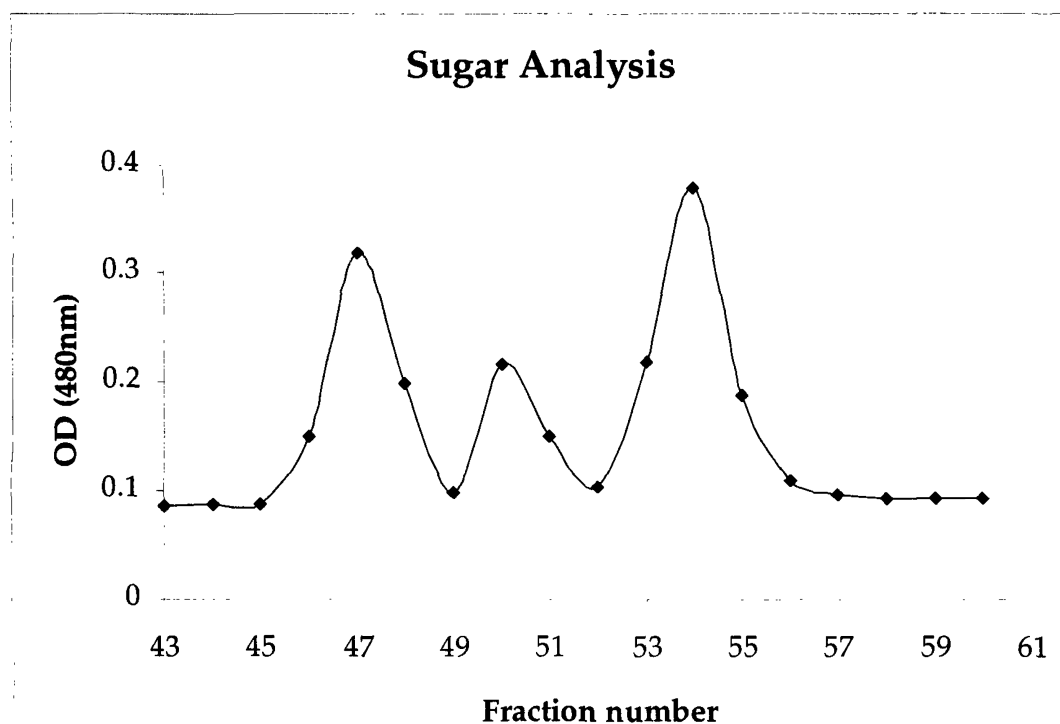


4.8.1.3. Purification of LAM from crude lipoglycan extract by gel filtration

A) Analysis of the fractions by colorimetric determination of sugars

The resolution of the lipoglycan species into their various classes was performed by running crude lipoglycan sample obtained after ultracentrifugation on a Sephacryl S-200 column using a disaggregating buffer containing deoxycholate. The collected fractions were analyzed for the presence of sugar. The neutral sugar assay exhibited three major peaks, which denoted lipoarabinomannan (first peak), lipomannan (second peak) and phosphatidylinositol (third peak) respectively (Fig 4.13).

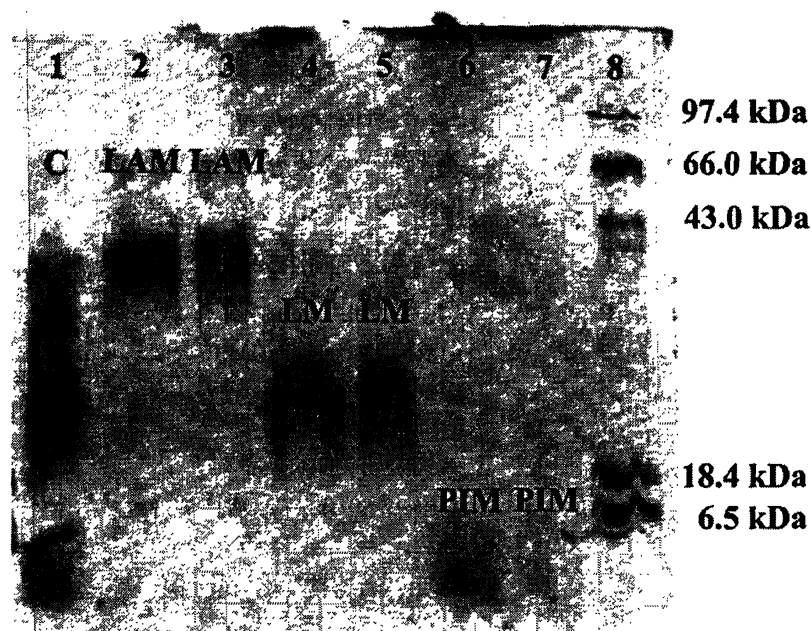
Fig 4.13: Analysis of fractions by colorimetric determination of sugars



B) Analysis of fractions By SDS-PAGE

The three molecules within the crude lipoglycan extract were further characterized by SDS-PAGE. Three broad bands were visible in the silver stained gel, corresponding to lipoarabinomannan (having the lowest electrophoretic mobility), lipomannan and phosphatidylinositolmannan (located in the gel running front). As shown in Fig 4.14, the diffuse nature of the bands was indicative of the molecular heterogeneity in the population of these compounds.

Fig 4.14: SDS-PAGE analysis of fractions



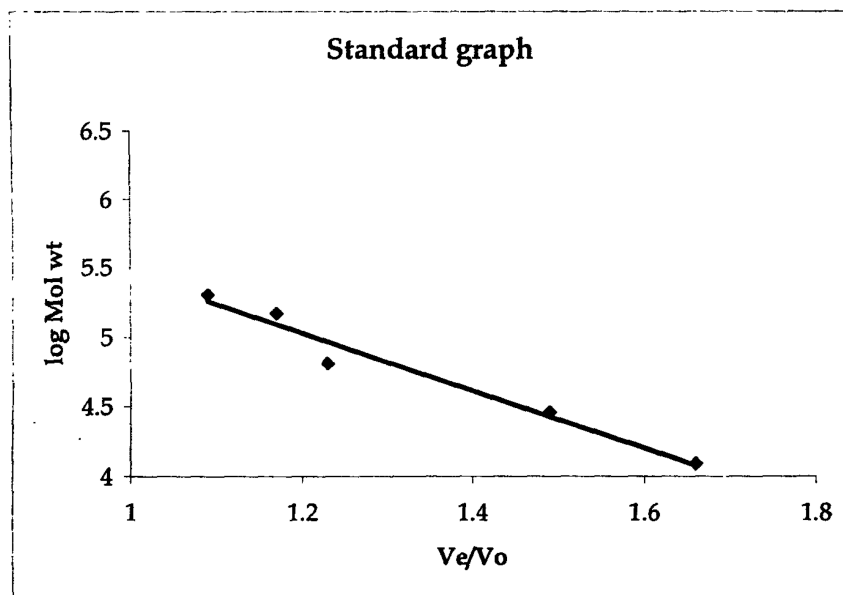
Lane 1: Crude lipoglycan extract (C) containing all three components namely lipoarabinomannan (LAM), lipomannan (LM) and phosphatidylinositolmannoside (PIM), Lanes 2 & 3: Fractions containing LAM, Lanes 4 & 5: Fractions containing LM, Lanes 6 & 7: Fractions containing PIM, Lane 8: Molecular weight marker for Silver Staining.

4.8.1.4. Determination of molecular weight of lipoarabinomannan by SDS/PAGE and Gel Filtration Chromatography

As shown in Fig 4.14, LAM appears as a diffuse band extending from 30-40kDa on a SDS-PAGE gel.

Molecular weight can also be determined by running protein standards on Sephacryl S-200, before running crude lipoglycan sample. On calculating elution volumes (V_e) of all standard proteins, void volume (V_o) of the column and drawing a graph of log molecular weight of the standards against V_e/V_o it was observed that the equation of the graph in Figure 4.15 was $y = -2.08x + 7.503$.

Fig 4.15: Determination of molecular weight



LAM being the most bulkier lipopolysaccharide of the three closely related antigens (i.e. LAM, LM and PIM), it gets eluted out of the column first followed by LM and PIM. The first antigen as determined colorimetrically was eluted in 46th fraction, 47th fraction and 48th fraction (later also confirmed by SDS-PAGE). On calculating V_e/V_o for all the three fractions and on substituting x value in the equation as V_e/V_o , log molecular weight (y) of the antigen was calculated. Molecular weight of the antigen was then back calculated from value y and it was confirmed that the molecular weight of the antigen is 30-40kDa.

4.8.1.5. Yield of LAM antigen

Two grams of heat-killed and lyophilized *M.tuberculosis* cells gave 30mg of lyophilized crude lipoglycan antigen. The yield of LAM antigen isolated from 15mg of crude lipoglycan antigen by Sephacryl S-200 was 1.0mg/ml.

4.8.2. Development and Standardization of ELISA for LAM antigen

Lysine (0.01%) in carbonate buffer (pH 9.6) was found to show a better blocking effect (was able to mask non-specific reactions) than different concentrations of Bovine Serum Albumin {1%-3%} in PBS (pH 7.4) and different concentrations of Bovine Serum Albumin {1%-3%} in carbonate buffer (pH 9.6). A 100 μ l volume of 5 μ g/ml LAM antigen, known positive sera and control sera, diluted 1:100 in dilution buffer and known positive CSF samples and control CSF sample diluted 1:20 with dilution buffer along with

a 1:5000 dilution of anti-human IgG, IgM and IgA HRP conjugate gave best results as determined by a checkerboard analysis. These optimal concentrations were used for further testing of the clinical samples.

4.8.3. Clinical evaluation of LAM ELISA kit

ELISA was carried out as per the protocol in Materials and Methods.

4.8.3.1. Antibody response to LAM antigen in controls and patients

Antibody response to LAM antigen in control group and patients was studied. In order to determine the level at which the test would have optimal sensitivity and specificity, statistical analysis at different cut-off levels such as Mean + 2SD and Mean + 3SD was calculated.

Table 4.11: Sensitivity and specificity at two different cut-off levels

Cut off at	Sensitivity in Group A n=100			Specificity in sera controls n=50		
	ELISA for IgG	ELISA for IgM	ELISA for IgA	ELISA for IgG	ELISA for IgM	ELISA for IgA
Mean + 2SD	97%	62%	99%	96%	96%	96%
Mean + 3SD	90%	46%	89%	98%	98%	98%

On the basis of clinical and radiological evidence, patients were included in this study. As shown in Table 4.11, on selecting Mean + 2SD as the cut off limit of negativity, 97% of the 100 patients in Group A tested positive for anti-LAM IgG antibodies while 62% and 99% of them tested positive for anti-LAM

IgM and anti-LAM IgA antibodies respectively (on comparison with clinical diagnosis).

At Mean + 2SD, except for 2 of the 30 controls comprising of students and technicians involved in tuberculosis research, others showed low absorbance. All of the 15 patients suffering from pneumonia, allergy and upper respiratory tract problems and 5 patients cured of tuberculosis 3-5 years back and having no clinical complaints (negative for Smear, Culture, PCR, Radiorespirometry) also showed lower absorbance for IgG, IgM and IgA antibodies against lipoarabinomannan by ELISA giving a specificity of 96%.

On selecting Mean + 3SD as the cut off limit of negativity, though the sensitivity (on comparison with clinical diagnosis) in Group A decreased marginally for all the three ELISA systems, only 1 student control gave a higher reading than the cut off limit of negativity giving a specificity of 98% (Table 4.11).

Table 4.12: Sensitivity and specificity at two different cut-off levels for CSF

Cut off at	Sensitivity in Group B n=20			Specificity in CSF controls n=20		
	ELISA for IgG	ELISA for IgM	ELISA for IgA	ELISA for IgG	ELISA for IgM	ELISA for IgA
Mean + 2SD	45%	20%	25%	90%	90%	90%
Mean + 3SD	45%	20%	25%	90%	90%	90%

As seen in Table 4.12, at both the cut -off limits for 20 CSF specimens in Group B, 45% of the patients tested positive for anti-LAM IgG antibodies while 20% and 25% of them tested positive for anti-LAM IgM and anti-LAM IgA antibodies respectively (on comparison with clinical diagnosis). Two of 20 CSF controls showed higher absorbance giving a specificity of 90% at both the cut off limits of negativity.

In a country like India where TB is endemic, to minimize the possibility of false positive results due to the presence of environmental mycobacteria and BCG-vaccination, all the further analysis (as explained in details in following sections) was carried out by selecting a higher cut off limit of negativity i.e. Mean + 3SD, whereby samples in Group A having an absorbance above 0.258 were considered positive for IgG and values above 0.145 and 0.131 were considered positive for IgM and IgA antibodies respectively. Similarly, samples in Group B showing an absorbance above 0.176 were considered positive for IgG and values above 0.122 and 0.109 were considered positive for IgM and IgA antibodies respectively.

4.8.3.2. Results of LAM based ELISA kit and its comparison with other diagnostic methods.

Patients were grouped according to their smear, culture, radiorespirometry and PCR status and antibody response against LAM antigen (at Mean + 3SD:

M+ 3SD) was compared with each group for Group A (Table 4.13) and Group B (Table 4.14).

Table 4.13: Antibody response to LAM and its comparison with all the other diagnostic tests in pulmonary tuberculosis (Group A).

Groups for P.TB (n = 100)	Total	No of samples positive (%) for antibodies against LAM (M+3SD)		
		ELISA for IgG	ELISA for IgM	ELISA for IgA
Smear positive	36	34 (94.4)	17 (47.2)	33 (91.6)
Smear negative	64	56 (87.5)	29 (45.3)	56 (87.5)
Culture positive	48	44 (91.6)	23 (47.9)	43 (89.5)
Culture negative	52	46 (88.4)	23 (44.2)	46 (88.4)
Radiorespirometry positive	39	36 (92.3)	18 (46.1)	36 (92.3)
Radiorespirometry negative	61	54 (88.5)	28 (45.9)	53 (86.8)
PCR positive	48	45 (93.7)	19 (39.5)	44 (91.6)
PCR negative	52	45 (86.5)	27 (51.9)	45 (86.5)
Clinically positive	100	90 (90)	46 (46)	89 (89)

Figures in parenthesis indicate percentages

As illustrated in Table 4.13, in the case of Group A, of the 36 smear positive samples, 34 (94.4%) tested positive for IgG, 33 (91.6%) for IgA and 17 (47.2%) for IgM antibodies, while of the 48 culture positive samples, 44 (91.6%) tested positive for IgG, 43 (89.5%) for IgA antibodies and 23 (47.9%) tested positive for IgM antibodies. Also, of the 39 radiorespirometry positive samples, 36

(92.3%) were positive for both IgG and IgA antibodies while 18 (46.1%) were positive for IgM antibodies. Similarly, of the 48 PCR positive samples, 45 (93.7%) tested positive for IgG, 44 (91.6%) tested positive for IgA antibodies and 19 (39.5%) tested positive for IgM antibodies. Statistical analysis of the Group A results, showed no significant difference in the efficiency of the ELISA for detecting antibodies in smear positive and smear negative cases ($p > 0.05$), culture positive and culture negative cases ($p > 0.05$), radiorespirometry positive and radiorespirometry negative cases ($p > 0.05$) as well as in PCR positive and PCR negative cases ($p > 0.05$).

Table 4.14: Antibody response to LAM and its comparison with all the other diagnostic tests in tuberculous meningitis (Group B).

Groups for CSF	Total no	No of samples positive (%) for antibodies against LAM (M+3SD)		
		ELISA for IgG	ELISA for IgM	ELISA for IgA
Smear positive	2	2 (100)	2 (100)	2 (100)
Smear negative	18	7 (38.8)	2 (11.1)	3 (16.6)
Culture positive	5	5 (100)	4 (80)	5 (100)
Culture negative	15	4 (26.6)	-	-
Radiorespirometry positive	4	4 (100)	4 (100)	4 (100)
Radiorespirometry negative	16	5 (31.2)	-	1 (6.2)
PCR positive	12	9 (75)	4 (33.3)	5 (41.6)
PCR negative	8	-	-	-
Clinically positive	20	9 (45)	4 (20)	5 (25)

Figures in parenthesis indicate percentages

As explained in Table 4.14, in the case of Group B, on comparing the smear, culture, radiorespirometry and PCR results with those of the antibody responses against the LAM antigen, it was observed that all of the smear positive and radiorespirometry positive samples, also tested positive for anti LAM IgG, IgM and IgA antibodies. Of the 5 conventional culture positive samples, 4 (80%) tested positive for IgM, while 5 (100%) tested positive for IgG and IgA antibodies. However, of the 12 PCR positive CSF samples, only 9 (75%) tested positive for anti-LAM IgG, while 4 (33.3%) and 5 (41.6%) of them tested positive for anti-LAM IgM and IgA, respectively.

Table 4.15: Antibody response to LAM antigen in special clinical cases

	Total	Number of samples (%) positive for antibodies against LAM (M +3SD)		
		ELISA for IgG	ELISA for IgM	ELISA for IgA
HIV	10	7 (70)	4 (40)	7 (70)
One/more Drug resistant	30	28 (93.3)	12 (40)	28 (93.3)

Figures in parenthesis indicate percentages

As seen in Table 4.15, antibody detection to LAM antigen have been compared in special clinical cases like 1) patients positive for antibodies against HIV and also clinically and radiologically positive for tuberculosis and 2) clinical isolates collected from patients either resistant to one or more first-line drugs for tuberculosis. Of the 10 patients positive for antibodies against HIV, 7 (70%) also tested positive for anti-LAM-IgG and anti-LAM IgA

antibodies, while only 4 (40%) tested positive for anti LAM-IgM. Also, of the 30 patients resistant to one or more first-line anti TB drugs, 28 (93.3%) tested positive for anti-LAM IgG and anti-LAM IgA, while only 12 (40%) tested positive for anti-LAM IgM antibodies.

Table 4.16: Antibody response to LAM antigen in pulmonary tuberculosis cases grouped on the basis of treatment status (Group A).

	Treatment	Total	Number of samples (%) positive for antibodies against LAM (M +3SD)		
			ELISA for IgG	ELISA for IgM	ELISA for IgA
Group I	Fresh	13	11 (84.6)	8 (61.5)	10 (76.9)
Group II	Relapse	18	16 (88.8)	6 (33.3)	16 (88.8)
Group III	2 - 6 months	17	15 (88.2)	8 (47.0)	15 (88.2)
Group IV	6months-1year	23	21 (91.3)	11 (47.8)	21 (91.3)
Group V	Above 1 year	29	27 (93.1)	13 (44.8)	27 (93.1)

Figures in parenthesis indicate percentages

As seen in Table 4.16, pulmonary tuberculosis cases have been grouped depending on the duration of treatment received as, Group I representing fresh cases, Group II representing relapse cases (patients reported to have been cured 7-25 yrs back) and not on treatment at the time of sputum and blood collection, Group III representing those that had received less than 6 months of treatment, Group IV representing patients under treatment for 6 months to a year and Group V representing patients treated for more than 1

year. As illustrated in the Table 4.16, percentage of positivity for the presence of IgG antibodies and IgA antibodies increased as the treatment lengthened. Though, the trend observed showed an increase in positivity rate, the increase seen was not statistically significant ($p > 0.05$). The detection rate for anti-lipoarabinomannan IgM antibodies was the least (33.3%) in relapse cases and the highest in fresh cases (61.5%).

Table 4.17: Antibody response to LAM antigen in tuberculous meningitis cases grouped on the basis of length of treatment (Group B).

	Treatment	Total	Number of samples (%) positive for antibodies against LAM (M+3SD)		
			ELISA for IgG	ELISA for IgM	ELISA for IgA
Group I	Fresh	9	4 (44.4)	2 (22.2)	2 (22.2)
Group II	On treatment	11	5 (45.4)	2 (18.1)	3 (27.2)

Figures in parenthesis indicate percentages

As seen in Table 4.17, tuberculous meningitis patients have been grouped depending on the treatment status of the patients; as Group I representing fresh cases without initiation of anti-TB treatment and Group II representing patients on treatment. In this case, the percentage of patients mounting IgG, IgM and IgA antibodies was similar with no significant difference ($p > 0.05$) among the fresh and treated cases.

Table 4.18: Antibody response to LAM antigen in pulmonary tuberculosis patients grouped on the basis of sex distribution (Group A).

	Total number	Number of samples positive (%) for antibodies against LAM (M+3SD)		
		ELISA for IgG	ELISA for IgM	ELISA for IgA
Males	66	63 (95.4)	32 (48.4)	62 (93.9)
Females	34	27 (79.4)	14 (41.1)	27 (79.4)

Figures in parenthesis indicate percentages

As illustrated in Table 4.18, the difference in the percentage of males and females that tested positive for IgG as well as IgA antibodies against lipoarabinomannan was significant in the case of Group A ($p < 0.05$). On the other hand, the IgM response observed in both the sexes was not statistically different ($p > 0.05$) and was found to be lower than the respective IgG and IgA responses.

Table 4.19: Antibody response to LAM antigen in pulmonary tuberculosis patients grouped on the basis of age distribution in males (Group A).

Age groups for males	Total	Number of samples positive (%) for antibodies against LAM (M+3SD)		
		ELISA for IgG	ELISA for IgM	ELISA for IgA
1yr-20yrs	6	5 (83.3)	3 (50)	4 (66.6)
21yrs-40yrs	38	37 (97.3)	21 (55.2)	37 (97.3)
41yrs-60yrs	18	18 (100)	7 (38.8)	18 (100)
Above 60yrs	4	3 (75)	1 (25)	3 (75)

Figures in parenthesis indicate percentages

When the age-wise analysis of the male population in Group A was carried out (Table 4.19), it was observed that anti-LAM IgG and anti-LAM IgA responses in the patients between the age group of 41 - 60 years was 100%, followed by the patients in the age group of 21- 40 years (97.3%). However, in the case of the IgM antibody response, patients in the age group between 21- 40 years gave the maximal response i.e. 55.2%.

Table 4.20: Antibody response to LAM antigen in pulmonary tuberculosis patients grouped on the basis of age distribution in females (Group A).

Age groups for females	Total	Number of samples positive (%) for antibodies against LAM (M+3SD)		
		ELISA for IgG	ELISA for IgM	ELISA for IgA
1yr-20yrs	8	5 (62.5)	6 (75)	5 (62.5)
21yrs-40yrs	21	18 (85.7)	7 (33.3)	18 (85.7)
41yrs-60yrs	5	4 (80)	1 (20)	4 (80)

Figures in parenthesis indicate percentages

Among the 34 female patients in Group A (Table 4.20), the age group of 21-40 years showed the maximal anti-LAM IgG and anti-LAM IgA antibody responses i.e. 85.7%, while patients in the age group of 1-20 years showed the maximal anti-LAM IgM response i.e. 75%.

Table 4.21: Antibody response to LAM antigen in tuberculous meningitis patients grouped on the basis of sex distribution (Group B).

	Total	Number of samples positive (%) for antibodies against LAM (M+3SD)		
		ELISA for IgG	ELISA for IgM	ELISA for IgA
Males	12	6 (50)	3 (25)	4 (33.3)
Females	8	3 (37.5)	1 (12.5)	1 (12.5)

Figures in parenthesis indicate percentages

As illustrated in Table 4.21, the trend observed among the tuberculous meningitis cases was similar to that seen earlier in the case of pulmonary tuberculosis cases, wherein the percentage of males that tested positive for anti- LAM IgG, anti-LAM IgM and anti-LAM IgA responses were higher than the percentage of females.

Table 4.22: Antibody response to LAM antigen in tuberculous meningitis patients grouped on the basis of age distribution in males (Group B).

Age groups for males	Total	Number of samples positive (%) for antibodies against LAM (M+3SD)		
		ELISA for IgG	ELISA for IgM	ELISA for IgA
1yr-10yrs	8	3 (37.5)	2 (25)	2 (25)
11yrs-20yrs	4	3 (75)	1 (25)	2 (50)

Figures in parenthesis indicate percentages

Table 4.23: Antibody response to LAM antigen in tuberculous meningitis patients grouped on the basis of age distribution in females (Group B).

Age groups for females	Total	Number of samples positive (%) for antibodies against LAM (M+3SD)		
		ELISA for IgG	ELISA for IgM	ELISA for IgA
1yr-10yrs	5	1 (20)	-	-
11yrs-20yrs	3	2 (66.6)	1 (33.3)	1 (33.3)

Figures in parenthesis indicate percentages

On analysis of age dependent humoral response against lipoarabinomannan in males (Table 4.22) and females (Table 4.23) in Group B, it was observed that the percentage of patients in the age group of 11-20 years (in males as well as females) mounted a better IgG, IgM and IgA response than the percentage of patients in the age group of 1-10 years.

DISCUSSION



Chapter 5.

5. DISCUSSION

The genus *Mycobacterium* consists of the members of the *Mycobacterium tuberculosis* complex and more than 80 species of non-tuberculous mycobacteria, including pathogenic, opportunistic and non-pathogenic species. The most important species of this genus is *M.tuberculosis*, the causative agent of tuberculosis.

Tuberculosis continues to be as it has been for centuries, one of the most prevalent infectious diseases of humans and is the leading cause of mortality from a single infectious disease worldwide (293). Death from tuberculosis comprises 25% of all the avoidable deaths in developing countries. In India, out of a total population of over 1 billion, each year about 2 million develop active disease and up to half a million die of TB (294). It implies that every minute, a death occurs due to tuberculosis in our country. It also imposes a cost burden on our economy in terms of current and future output losses because of premature deaths and ill health (295).

Pulmonary tuberculosis constitutes the major form of tuberculosis in clinical practice. Tuberculous meningitis is an important form of extrapulmonary tuberculosis in the Indian subcontinent with a significant mortality. Presenting symptoms are non-specific and the diagnosis may remain unsuspected until signs of meningeal irritation appear.

The present study included Group A, that consisted of 100 clinically and radiologically diagnosed pulmonary tuberculosis patients and Group B, that comprised of 20 clinically suspected cases of tuberculous meningitis.

The age incidence of tuberculosis depends on the overall prevalence of the disease in the community and in the country. Nevertheless, within any one country, the incidence may vary depending not only on factors such as socioeconomic status, but also perhaps genetic factors that determine the immune response of the individuals. In the present study amongst Group A patients, 82% of the population belonged to the age group of 21-60 yrs (Table 4.1). Similar observations have been made in a WHO document, which reported that of the 2 million people dying from TB annually, 75 percent are between the ages of 15 and 54 years (296). In an earlier study conducted by Harris *et al* (1997) 61% were adults aged 15-44 years (297). Hence, TB does not only affect individuals and families, but also affects socio-economic development of countries, as it generally afflicts the most economically active age segments of the population (296).

In countries where tuberculosis is endemic, tuberculous meningitis occurs predominantly in children. In recent years the effective development of vaccination programmes is believed to have reduced the incidence of the disease in childhood. However, it is still a major cause of death and serious neurological damage, especially among children in developing countries. In

the present study, majority of the cases in Group B belonged to the age group of 1-10 years i.e. 65% (Table 4.2). This is similar to the studies conducted by Auerbach (1951), Singh *et al* (1972) and Desai *et al* (2002) where the main group affected among TBM patients was in the first decade of life (298, 299, 300).

Tuberculosis cases have always been higher in males than in females. Hudelson (1996) reported that female patients worldwide are either undernotified or underdiagnosed due to reduced access to health care services and socio cultural factors (301) while Nair *et al* (1997) reasoned stigma & fear associated with the disease as one of the important factors for the lower notification rate in females (302). In the present study too, the percentage of males observed in the case of both pulmonary tuberculosis (66%) and tuberculous meningitis cases (60%) was higher than the percentage of females (Table 4.1 and Table 4.2). In an earlier study, Styblok (1991) reported that 40-50% of tuberculosis cases occur between the age group of 15-49, with rates being higher among males (303). One of the reasons, for higher TB prevalence and TB infection in men as compared to women could be the comparatively higher risk of exposure to the bacilli in men due to more active professional and social life styles as compared to their female counterparts.

Both socioeconomic and cultural factors appear to influence the infection rate for a disease like tuberculosis. Low socioeconomic status as evidenced by

factors like poverty, income and unemployment has frequently been linked to increased risk of tuberculosis, especially among those living in crowded households and congregate living facilities (304, 305). Rieder (1999) reported a clear association between risk of TB and malnutrition and crowding (306). Studies conducted both in high-income countries like USA, UK, Germany and Norway and in low-income countries like Vietnam, Mexico and Phillipines reveal significantly higher rates of TB in their respective poor populations (307, 308, 309). While TB is not exclusively a disease of the poor, deprivation associated with poverty increases the risk of infection and development of the disease. In the present study, in the case of Group A, 35% cases were illiterate, 21% had only received primary education, while 45% cases were unemployed (Table 4.4). A similar observation was made by Buskin *et al* (1994) who reported that relative to people with higher income, more stable conditions and post secondary education, people with lower socioeconomic status were more likely to develop tuberculosis (310).

Smoking and alcohol consumption have also been sited as possible risk factors for acquiring tuberculosis. Lowe (1956) reported that the rate of tuberculosis rises according to the number of cigarettes smoked (311). Lewis and Chamberlain (1963) reported an excess of drinkers among tuberculosis patients irrespective of the smoking habit (312). Buskin *et al* (1994) also reported that current and former smokers had a 30-50% higher risk of tuberculosis than that of non-smokers (310). In the present study in Group A,

27% were smokers, 15% were alcoholics and 2% also chewed pan (Table 4.4). Similar observations were also made in several studies conducted in many countries like Kenya, Phillipines and India (302, 313, 314).

A history of close contact with an open case of tuberculosis is often noted in tuberculosis patients. In the present study 8% of patients in Group A and 10% of patients in Group B had a history of contact with an open case of tuberculosis (Table 4.4). Nozaki *et al* (1996) also reported that 10% of their patients had a history of contact with an infectious case of pulmonary tuberculosis (315).

Several prospective studies have described the prevalence of HIV among patients with tuberculosis. Mohanty *et al* (1994), in Mumbai, reported a rise of HIV seroprevalence in tuberculosis patients from 2.32% in 1988 to 8.9% in 1992-93 to 10.15% in the year 1994 (316). In a later study, Rao *et al* (1996) reported 13.89% of HIV seroprevalence among tuberculosis patients (317). In the present study, 10% of the patients clinically diagnosed for pulmonary tuberculosis i.e. Group A also tested positive for antibodies against HIV. In a recent study conducted by Swaminathan *et al* (2000), the author reported that the HIV seroprevalence among TB patients in India ranges from 2-20% with an estimated 60% of HIV infected persons breaking down with active TB disease in their lifetime (318).

Rapid diagnosis of patients with active disease is essential for initiating prompt treatment and thereby preventing the spread of infection to the healthy population. There are two basic approaches for the diagnosis of tuberculosis. The direct approach includes detection of *Mycobacteria* or its products and the indirect approach includes measurements of humoral and cellular responses of the host against tuberculosis. Tests for diagnosis of tuberculosis vary in sensitivity, specificity, speed and cost.

Acid fast staining remains an inexpensive and rapid means for identifying highly infectious tuberculosis patients. It allows a quantitative estimation of the number of bacilli being excreted and therefore remains the cornerstone of infection control. The most common, classic and conventional acid fast staining method for detection of tubercle bacilli is Ziehl-Neelson staining. There are many methods available for the cultural isolation of mycobacteria; however, the most popular medium for cultivation of mycobacteria is the egg-based Lowenstein Jensen medium. Though, the definitive diagnosis of *M.tuberculosis* is hampered by the slow growth rate of the bacilli, the conventional culture technique for the isolation of tubercle bacilli remains the "Gold Standard" even today.

In the present study, of 100 sputum samples in Group A, collected from clinically diagnosed patients, 36 (36%) samples were detected by ZNCF staining and 48 (48%) samples were culture positive (Table 4.6). Tan MF *et al*

(1997) reported a similar sensitivity of 38% for microscopy when compared with clinical findings (319). Limited sensitivity of 40-60% for a combination of 3 smear examinations has also been reported in other studies (320, 321). Lower sensitivity of microscopy can be attributed to the initial low bacterial load in the test specimens, as it is known that 10^4 organisms/ml of sample is essential for microscopic detection of mycobacteria (322, 323).

Daginawala *et al* (1991) and Nguyen *et al* (1993) have reported low smear positivity rates of 1% and 4% from a total of 99 and 499 TBM cases respectively, using the ZNCF method (324, 325) while Radhakrishnan *et al* (1993) and Desai *et al* (1993) in their respective studies have reported a low culture positivity rate of 11.6% and 8.78% (326, 327). In the present study, 5 (25%) of 20 CSF samples in Group B, were positive by culture, which included 2 (10%) samples that were also positive for AFB by the ZNCF method (Table 4.6). These findings are similar to those by Hassan *et al* (1989), who reported a smear positivity of 10.3% by the ZNCF method and Rodrigues *et al* (1997) who reported a low sensitivity (21%) for the culture technique for CSF samples (328, 329). Several reasons may account for the low isolation rate of *M.tuberculosis* in patients with TBM. The CSF specimens are generally obtained through the lumbar route in which case it contains low concentrations of *M.tuberculosis* compared to ventricular or cisternal CSF. Secondly, *M.tuberculosis* organisms are embedded in the dense exudates at the

base of the brain and the exudates forms a barrier for the tubercle bacilli to circulate in lumbar CSF.

To combat TB epidemic, the RNTCP DOTS has been implemented as a part of public health programme. However most patients receive treatment from private physicians and hence remain outside the purview of the strategy. In 1991, Uplekar and Shepard reported that 100 physicians in Dharavi slums prescribed 80 different anti-TB regimens, most were both inappropriate and expensive (330). Inappropriate regimen and patient incompliance are the major reasons for the development of drug resistance. Two reference mycobacterial laboratories from private hospitals have reported a high prevalence of MDR-TB: 11% from 1991-95 and 58% from 1994-95 (331, 332). Almeida *et al* (2003) also have reported a higher percentage of drug resistant strains in Mumbai than in rural population (333). In the present study, 62.5% of the isolates from sputum samples were resistant to one or more first-line anti-TB drugs.

Bacteriologic culture of *M.tuberculosis* is definitive but takes 4-8 weeks to yield results. Because the genetic properties of mycobacteria are fixed, it is unlikely that new or better culture media would accelerate the appearance of colonies for naked eye examination. Various techniques, which rely upon radiolabeled and non-radiolabeled substances, have been employed in an effort to substantially reduce the time for identification and drug susceptibility testing.

Some of the important methods involving radiolabeled compounds include Bactec and Radiorespirometry that are based on the measurement of radiolabeled carbon dioxide generated from radiolabeled substrates, i.e. radiolabeled palmitate and radiolabeled acetate, respectively. The automated BACTEC 12B system has an ionisation chamber as a detecting device for monitoring $^{14}\text{CO}_2$ produced while radiorespirometry based on the principle of biphasic vial system employs a liquid scintillation counter for the measurement of $^{14}\text{CO}_2$.

Of 48 culture positive samples in Group A, 25 (52%) were positive by radiorespirometry (Table 4.7). Similarly, in the case of Group B, of 5 culture positive samples, 4 (80%) were positive by radiorespirometry (Table 4.8). Shah DH *et al* (1985) in their study had only selected smear positive sputum samples to demonstrate the supremacy of radiorespirometry over LJ media. They reported that few more samples were detected by conventional culture (86.9%) than radiorespirometry (85.7%) and the possible reasons could be either inhibition of CO_2 liberation from the media or the inability of few clinical strains to metabolize acetate (144). Shah DH *et al* (1985) also recommended a second decontamination procedure to eliminate any kind of contamination and to facilitate even layering of sputum sample on LJ media for radiometric purposes (144). In the present study, patients were selected on the basis of clinical diagnosis and not with reference to smear status of the patients. With already fewer mycobacterial cells in the sputum sample,

second decontamination procedure may have accounted to further loss of the viability of tubercle bacilli in the sample thus resulting in lower sensitivity of radiorespirometry when compared with conventional culture.

Of the 52 culture negative sputum samples from Group A (Table 4.7), 14 (26.9%) were positive by radiorespirometry. Fisher *et al* (1954) and Tsukamura (1990) have shown that *M.tuberculosis* follows arithmetic growth prior to the onset of logarithmic growth (334, 335). For visible colonies it is imperative that the inoculum be large enough so as to enable the bacteria to initiate the exponential growth. It is possible that if the initial inoculum is low, the bacteria may respire without forming visible colonies. This probably explains why culture negative samples were positive by radiorespirometry.

Analysis of 243 *M.tb* cultures isolated from 1994 to 1996 indicated that only about half of the isolates grew on all media used namely LJ, 7H11 agar, 7H11 broth and Bactec media and about 14% grew in Bactec broth only (336). In the present study also, only 25 sputum samples of Group A and 4 samples of Group B were positive by both radiorespirometry and conventional culture (Table 4.7 and Table 4.8).

In a study comparing radiometric technique i.e. Bactec with nonradiometric techniques i.e. MGIT and MB Redox it was found that both the recovery rates and time to detection in two manual liquid medium were less favorable than

in the Bactec 460 (336). A comparison of Bactec with conventional LJ media undertaken at the Tuberculosis Research Center showed that the rate of isolation of positive cultures was significantly faster in the case of Bactec, with 87% of the positives being obtained by day 7 and 96% by day 14 (337). In the present study, it was observed that Radiorespirometry detected the presence of bacilli in sputum/CSF samples significantly ($p < 0.01$) early as compared to the convention culture method (Fig 4.4 and Fig 4.5).

A variety of PCR methods have been developed for detection of specific sequences of *M.tuberculosis* and other mycobacteria. These PCR assays either target DNA or RNA and employ a conventional DNA based PCR, nested PCR or RT-PCR. Targets include insertion and repetitive elements, various protein encoding genes, ribosomal RNA etc. Developments in this area have been very rapid and a large number of PCR assays targeting different gene stretches of *M.tuberculosis* have been described. Indian laboratories have been active in the development of PCR methods for detection of *M.tuberculosis*. Different Indian investigators have used separate gene targets like MPB 64 (338), GC repeats (339), 38kDa (340), repetitive sequences (329), dev R (341), and TRC4 (342). PCR methods also differ in DNA isolation technique (343, 344, 345), number of amplification cycles (343, 345) and methods of amplicon detection (345, 346, 347). A variety of different protocols for all types of specimens have been tested using in-house methods, but a standard, general method remains to be established.

The only repetitive sequence-based target useful for nucleic acid amplification in tuberculosis is the insertion sequence *IS6110*. The latter is specific for the *M.tuberculosis* complex and generally occurs as 1-20 copies per cell, making it an ideal target for amplification, which is why *IS6110*-based nucleic acid amplification techniques are the systems, applied most frequently, in the case of in-house PCR protocols. Hence, in the present study also, *IS6110* was chosen as target gene for the PCR-based diagnosis.

In studies that assessed the sensitivity and specificity of PCR for sputum samples, in terms of its clinical correlation the results have varied considerably. An Indian study conducted by Rodrigues *et al* (1997) using the *IS6110* primers for DNA amplification has shown a sensitivity of 93% and a specificity of 81.5%, when clinical picture was taken into consideration (329). Clarridge *et al* (1993) in a study of more than 5000 samples, using an *IS6110* targeted PCR system reported a sensitivity of 94% for smear positive specimens and 62% for smear negative specimens (348). On the other hand, in an assessment of reliability and reproducibility between laboratories, all of which routinely used PCR and targeted *IS6110*, Noordhoek *et al* (1994) found that the sensitivity and the specificity for detecting 10^3 *M.bovis* BCG organisms varied between 2 and 90% and 3 and 77%, respectively (349). The results of this study showed that the nucleic acid amplification methods for the detection of *M.tuberculosis* might be unreliable, even in the case of laboratories that used routinely applied procedures.

The two categories of samples encountered in the present study were the culture negative and PCR positive samples (n= 21/52) and the culture positive and PCR negative samples (n=21/48) (Table 4.9). Rapid acceptance of amplification assays for the direct detection of *Mycobacterium tuberculosis* in clinical specimens has been hindered by multiple problems, including inhibition of PCR assays, with studies reporting rates of inhibition from as low as 3% to greater than 15% (153, 348, 350, 351). In our study, 27 of 48 culture positive sputum specimens from Group A were positive for PCR giving a sensitivity of 56.2% {on comparison with culture} (Table 4.9) while the remaining 21 (43.7%) samples of 48 culture positive specimens were negative for PCR (Table 4.9). Peirre *et al* (1991) and Soini *et al* (1992) reported insensitive PCR assays (63 and 55.9%) respectively, while Al Zahrani *et al* (1995) reported that although the Amplicor test had excellent specificity, it was less sensitive than culture (42% vs 73%) for diagnosis of minimal active pulmonary TB (352, 353, 354). Lim *et al* (2000) too in their study reported a sensitivity of 44% and a specificity of 99% (355).

Although heme compounds present in blood specimens have been identified as inhibitors of PCR amplification, the nature of endogenous PCR inhibitors present in respiratory samples has not been delineated (356). Extraction procedures employing multiple steps to purify DNA from respiratory samples appear to have lower rates of inhibition (357, 351) compared with those having fewer steps and involving simple methods (350). Yen *et al* (1993)

reported that inhibitors were found in 3.2% of sputum samples when the DNA was extracted by digestion with lysozyme, lysed by heat and a sodium dodecyl sulfate solution, followed by extraction with phenol-chloroform and ethanol precipitation (358). Nolte *et al* (1993) reported that 10% of specimens were inhibitory to PCR when samples were centrifuged and the pellets were lysed by detergent and heating and then centrifuged again (153). Using a simple extraction protocol of boiling and beating with glass beads for routinely processed respiratory sediments, Forbes *et al* (1993) initially estimated that 13% of specimens were inhibitory in a PCR assay. Subsequently, using an internal control in their reaction mixture, they prospectively analyzed 100 respiratory specimens and showed that 52% of those specimens had evidence of interfering substances (350). Unfortunately, reextraction of DNA with phenol-chloroform and ethanol precipitation did not consistently remove substances in clinical specimens that were inhibitory to the PCR (350). Kirschner *et al* (1996) reported that 21% of clinical specimens were inhibitory in their PCR assay using a commercially prepared kit i.e. Amplicor (154). Since unwanted substances can bind to the nucleic acids immediately after cell lysis, even improved nucleic acid purification methods cannot completely prevent this phenomenon. The elimination of inhibitory factors continues to be a challenge in the use and acceptance of PCR assays in a clinical diagnostic setting.

The other main reason for our insensitive PCR assay could also be the presence of either single or no copy of *IS6110* in the clinical mycobacterial strains as reported by some investigators (359, 360).

In our study, of the 52 culture negative sputum samples in Group A, 21 (40.3%) were positive by PCR (Table 4.9). Culture negative and PCR positive results can be deliberated either as false negative culture results or as false positive PCR results. Although sputum digestion, decontamination and concentration methods have increased the yield of *M.tuberculosis*, the organism may be killed if the process is improperly performed resulting in false negative culture results (362).

In a study evaluating utility of Amplicor PCR, Bennedsen *et al* (1996) credited false positive PCR reactions to noncultivable *M.tuberculosis* organisms that were either non-viable because of host defenses, treatment or overzealous decontamination (156). In the present study too, 69% of the patients in Group A were at different stages of treatment during sample collection. Bennedsen *et al* (1996) further raised a possibility that noncultivable *M.tuberculosis* organisms were being detected in patients with past or current primary pulmonary disease with no active tuberculosis (156). Since all the precautions were taken in the present study, the cause is unlikely to be related to crossover contamination.

The lack of PCR sensitivity has not been a major problem with respect to extrapulmonary specimens as the sensitivity of the assay has generally been higher than culture. PCR has been extensively employed for the detection of *M.tuberculosis*-specific sequences in CSF as well as biopsy specimens among cases of tubercular meningitis and tuberculomas of the brain. As compared to 5-20% positivity with demonstration of AFB and mycobacterial cultures, PCR has been found to be positive in 50-70% of specimens from cases having cardinal features as well as biochemical/cytological evidence of neurotuberculosis (342, 363). Bonnington *et al* (1998) reported that the Amplicor test had a sensitivity of 60% and a specificity of 100% for detecting cases of definite and probable tuberculous meningitis in patients treated for less than 10 days (364). In the present study, 12 of the 20 CSF samples tested gave a positive PCR (Group B), with a sensitivity of 60% as compared to clinical diagnosis and a sensitivity of 100% when compared to conventional culture (Table 4.10). Caws *et al* (2000) reported that their PCR method, which targeted *IS6110*, revealed a sensitivity of 35% when compared with the final clinical diagnosis of tuberculous meningitis, while culture yielded positive results in 17% of cases when compared with clinical diagnosis (365). Earlier, Delacourt *et al* (1995) and Smith *et al* (1996) reported CSF PCR sensitivities of 50% and 40% respectively, with respect to the clinical diagnosis while the culture results of their respective studies were relatively poor (366, 367). In our study also, culture results in the case of CSF samples were poor as compared to PCR (Table 4.10).

None of the sputum/CSF samples collected from healthy individuals, patients suffering from upper respiratory infections and non-TBM patients showed a 123bp PCR product on agarose gel electrophoresis, thus giving a specificity of 100% in both the control groups. Specificity of our PCR reaction is comparable to the best possible reports published in literature (319, 364).

The performance of nucleic acid amplification techniques depends also on factors like collection, volume and preparation of samples (368, 369). Other factors that influence the outcome of a PCR reaction include uneven distribution of mycobacteria in the sample; presence of mycobacteria at an extremely low density, presence of PCR inhibitors and inefficient classical lysis protocols (155). Earlier studies focused on testing different targets, using a wide range of different sample preparation and amplification protocols (150, 153, 348, 357, 361, 370). However, the overall outcome of these studies was that various difficulties arose in obtaining sensitivities and specificities comparable to those of conventional culture methods. Many significant issues need to be dealt with before applying PCR routinely in diagnostic laboratories.

In view of the limitations posed by other assays, continuous efforts have been made to develop simple and sensitive assays for serodiagnosis of tuberculosis. A simple and rapid assay that would improve case holding, empower health care workers at the peripheral level and decrease diagnostic confusion and

delay is the need of the hour. Recognizing these needs, serologic approaches to TB diagnosis have been tried on and off since the sero-agglutination work of Arloing (1898) (169).

A variety of methods have been employed for detection of antibodies. Early TB serology tests used PPD or other crude antigens and showed relatively poor specificities (371, 372). Till date, many in-house ELISA and RIA based techniques using semi-purified and purified antigens, (189, 373, 374, 375, 376, 377) with varying degrees of sensitivities and specificities have been reported. The availability of monoclonal antibodies, recombinant antigens and methods for the accurate application of controlled amounts of immunologic reagents to nitrocellulose or other solid matrices have enhanced the specificity and reproducibility of such tests (378). However, variable sensitivities observed, depending on the antigen employed, immunoglobulin class measured and geographic origin of the survey participants has limited its wide application (380, 381, 382). Genetic restriction of the immune response to mycobacteria (383) and/or differential characteristics among mycobacterial strains prevalent in distinct regions of the world, inducing different host responses, could also account for these differences. Heterogeneous antigen recognition may also occur because of production of different mycobacterial antigens at different stages of TB (191), differential bacillary load in the sputum (384), antituberculosis therapy as well as association between antibody titers against particular *M.tuberculosis* specific epitopes and certain HLA alleles (385).

One such immunodominant antigen that has been studied elaborately is Lipoarabinomannan (LAM). LAM is a major lipoglycan that is non-covalently attached to the plasma membrane through its phosphatidyl-*myo*-inositol anchor and extends to the exterior of the cell wall (229, 230). ManLAM exhibits a wide spectrum of immunomodulatory functions, which include inhibition of phagosome maturation, evasion of cytokine-mediated mechanisms of elimination and promotion of bacterial survival in macrophages (discussed in details in chapter 1.12). Due to the presence of repetitive D-arabinofuranose residues it has a direct B cell stimulatory capacity. In the present study, Man-LAM was isolated from virulent standard strain H₃₇Rv, but for convenience it would be referred to as lipoarabinomannan and/or LAM in the following discussion.

The amphipathic nature of Lipoarabinomannan makes it difficult to isolate and purify. Current isolation procedures involve repetitive solvent partitioning techniques (386, 230) or utilization of the aqueous phenol extraction procedure (387). Further purification is based on selective precipitation or chromatographic procedures involving hydrophobic interaction (387), ion-exchange (230), lectin affinity (240) or size-exclusion chromatography in the presence of detergents (388). The yields of LAM obtained are often low due to losses that occur due to the harsh nature or the complexity of the extraction procedures.

In order to improve the availability and purity of lipoarabinomannan for use in structural and biological studies, LAM in the present study was successfully prepared from *M.tuberculosis* (H₃₇Rv) in a three step procedure as described by Severn *et al* (289): 1) cellular disruption and extraction of lipophilic components from the membrane fragments by Triton X-114; 2) purification of the lipoglycans and removal of non-lipid contaminants by Proteinase K treatment and ultracentrifugation; and 3) chromatographic resolution of the lipoglycan species into various classes i.e. LAM, LM and PIM. Though, the solvent extraction techniques mentioned above are efficacious, they involve many lengthy and complex steps for obtaining a homogeneous preparation and hence in the present study, LAM was purified by the method described by Severn *et al* (1997) for rapid preparation with greater yields and high purity (289).

An ELISA for the detection of anti-LAM antibodies was developed and its clinical utility was evaluated. Though initial analysis was carried out both at Mean + 2SD as well as Mean + 3SD, a higher cut-off value was chosen to minimize the possibility of false positivity due to presence of environmental bacteria and BCG vaccination. At Mean + 3SD, the sensitivity of LAM based ELISA kit for the detection of IgG, IgM and IgA antibodies obtained in the present study in pulmonary tuberculosis cases (Group A) was 90%, 46% and 89% on comparison with clinical diagnosis (Table 4.11) and 91.6 %, 47.9% & 89.5% when compared to culture respectively (Table 4.13). Results obtained

were comparable to the results reported by Del Prete R *et al* (1998) and Chan *et al* (2000) (375, 380).

Del Prete R *et al* (1998) evaluated Mycodot kit for the presence of anti-LAM antibodies in 102 non-HIV patients with suspected active TB. Among the patients with microbiologically ascertained tuberculosis in their study, 89.2% (25/28) serum samples were found to be positive. The test was negative for the remaining 74 suspected cases and the subsequent follow-up of those patients excluded mycobacterial infection. Control subjects were also negative (375). Chan *et al* (2000) developed a visually detectable, colloidal gold-based serological assay to qualitatively detect IgG against the LAM antigen. In sera of active TB patients obtained from Bombay, 93% of the patients tested positive for anti-LAM IgG. The author also reported that majority of PPD negative individuals (37 of 39 i.e. 94.87%) among South-East Asian and Mexican immigrants obtained from Denver Metropolitan TB clinic, tested negative for anti-LAM IgG (380). In an earlier study by Sada *et al* (1990) LAM was purified from *M.tuberculosis* by anion-exchange and gel filtration chromatography and was used as a solid phase antigen to measure anti-LAM IgG antibodies in sera by ELISA (189). Results for the total patient population showed a specificity of 91% and a sensitivity of 72%. However, when the results were analyzed group-wise, it was observed that the sensitivity was the highest for miliary tuberculosis (82.3%), followed by pulmonary tuberculosis

(80.6%), tuberculous lymphadenitis (75%) and the least for pleural tuberculosis (42.8%).

In the present study, sensitivities obtained for the detection of IgG, IgM and IgA antibodies in tuberculous meningitis cases (Group B) were 45%, 20% and 25% respectively, when compared to the clinical diagnosis (Table 4.12) and 100% for the detection of both IgG and IgA antibodies while 80% for IgM antibodies when compared to culture (Table 4.14). This is similar to the study by Chandramukhi *et al* (1989) who reported presence of IgG antibodies to purified LAM in 42% of CSF cases by ELISA, while antibody responses to 38kDa and 14kDa proteins were found to a lesser degree than to the LAM antigen (192). In the same study, results for the measurement of levels of IgM antibody to LAM in CSF were disappointingly low. Chandramukhi *et al* carried out this study in collaboration with Brennan PJ (Colorado University) who isolated and purified LAM. In another study Patil *et al* (1996) using immunoblotting technique showed that the major immune response was to lipoarabinomannan as 93% (28 of 30) of CSF samples in their study showed immune reactivity (389). Brennan PJ of Colorado University gifted LAM to Patil *et al* for their study.

Julian *et al* (1997) reported that the Mycodot test (manufactured by Genelabs, Geneva, Switzerland) when employed in their study showed an excellent specificity of 100% (280). Moreover, LAM antibodies were not detected in

patients with non-tuberculous mycobacterial diseases. Similarly, in an earlier study by Da Costa *et al* (1993), HIV infected non-tuberculous individuals from Kenya showed no response to LAM (390). Despite previous exposure to *M.tuberculosis* in a large proportion of the endemic African population, it appears that in the absence of clinical tuberculosis the immune system is not exposed to LAM to a degree sufficient to result in detectable antibody production. The UK control group in the same study also did not show detectable responses to LAM (390). In the present study too, at Mean + 3SD cut off limit of negativity, the specificity of LAM based ELISA kit for the detection of IgG, IgM and IgA antibodies in Group A was 98% and in Group B was 90% (Table 4.11 and Table 4.12).

In our study on comparison with clinical diagnosis, at Mean +3SD cut off limit of negativity, the percentage of patients testing positive for IgG (90%) and IgA antibodies (89%) were higher than that for IgM (46%) among pulmonary tuberculosis cases (Group A) (Table 4.11) while in the case of tuberculous meningitis (Group B), maximum patients tested positive for IgG antibodies i.e. 45% (Table 4.12). Various studies have reported earlier that the percentage positivities of IgG and IgA antibodies were significantly higher in patients of pulmonary tuberculosis when compared with healthy subjects, while IgM was not significantly raised (362, 391, 392). Fahey and McKelvey (1965) reported significant increase of IgG concentration without significant change in IgA and IgM levels in Caucasian tuberculous patients (393). South *et al*

(1967) reported a predominant increase in IgA antibodies in their study and suggested the presence of high proportion of IgA producing immune cells in lungs to be responsible for the same (394). Jha *et al* (1974) suggested that the insignificant increase in IgM levels could be attributed to the fact that more than 80% of their cases had cavitory lesions signifying post-primary disease and that the patients exhibited a secondary response by synthesizing more IgG (392). Bhatia *et al* (2003) after reviewing many different serological tests reported that IgM levels for different antigens tested were so low that their reliable measurement would be difficult (362).

Of the 10 HIV-TB coinfectd patients in Group A, 7 (70%) were positive for anti-LAM IgG and anti-LAM IgA antibodies, while 4 (40%) were positive for anti-LAM IgM (Table 4.15). Di Pierri *et al* (1996) in his study observed that even in the absence of cavitory lesions, HIV infected TB patients can have very high bacillary loads in their lungs, and high antigenic stimulation may be responsible for the higher titers of antimycobacterial antibodies observed in these patients (395). However, low sensitivity of the Mycodot test (manufactured by Genelabs, Geneva, Switzerland) was observed in HIV-TB coinfectd group in studies reported by Boggian *et al* (1996) and Julian *et al* (1997) (280, 396). CD4 cell counts of the patients included in our study were not known, and hence a higher number of HIV-TB patients showing an antibody response in our study may be attributed to the asymptomatic phase in HIV patients wherein the immune system has yet to collapse.

In an Indian study performed at the National Institute of Communicable Diseases, New Delhi, on analysis of partially and fully treated cases of pulmonary tuberculosis (i.e. patients who had received at least six months of anti-tubercular treatment), quite a large number were found to be positive for anti A60 IgA (58.3%) and IgG (46.6%), though this was not the case with IgM antibodies (11.6% positivity) (397). Julian *et al* (1997) also reported that in a TB-HIV negative group, 100% of the patients with relapse TB were Mycodot positive as compared to 11.1% of fresh cases (280). In an earlier study Kaplan *et al* (1980) observed that the response to antigens in relapse TB patients was considerably higher (47). In our study too, it was observed that the percentage positivity for anti-LAM IgG in Group A, was the least in fresh cases when compared to relapse cases and patients under treatment (Table 4.16). In the case of Group B, however, the percentage of patients testing positive for anti-IgG antibodies in fresh and treated patients was somewhat similar (Table 4.17). The possible reason could be that in the case of patients under treatment in Group B, included in our study, the duration of treatment was less than a month.

In our study, the levels of IgG and IgA in Group A were significantly more in males than in females, whereas IgM showed no significant relation with sex (Table 4.18). In Group B also, the percentage of males showing an antibody response (anti-IgG, anti-IgM and anti-IgA response) to LAM was higher than females (Table 4.21). Our observations are different from Alarcon-Segovia and

Fishbein (1971) who observed no significant difference in the levels of any of the three immunoglobulins in relation to sex among patients of pulmonary tuberculosis (398). Jain VK *et al* (1984) reported significantly higher levels of IgA in females, whereas IgG and IgM levels in their study showed no significant relation with sex (399).

On analysis of the age distribution in our study population, it was observed that in the case of Group A, the percentage of males and females in the age groups between 21 to 60 years mounted a better anti-IgG and anti-IgA immune response as compared to the more younger and older age groups i.e. those below 20 yrs and above 60 yrs of age (Table 4.19, Table 4.20). Also, in the case of Group B, the percentage of subjects in the age group of 11-20 yrs could mount a higher immune response than the younger age group of 1-10yrs (Table 4.22 and Table 4.23). In an earlier study, Bothamley G *et al* (1988) reported that antibody titers in children were lower than in adults (400).

In a study with A60 antigen, in patients with smear negative but culture positive pulmonary tuberculosis, measurement of both IgM and IgG by ELISA revealed a sensitivity of 68% and a specificity of 100% (401). Clinical evaluation of the Andelisa IgG kit, which uses the A60 antigen, gave a sensitivity of 48.3% and a specificity of 92%, while the Kreatech IgA kit, which employs the KP90 antigen derived from *M.tuberculosis* showed a sensitivity of 49.7% and a specificity of 84% (402). An immunoblotting study reported that

anti-38kDa protein antibodies were present in only 60% of the cavitary TB patients in their study, while they were rarely present in noncavitary TB patients (403, 404). In another study, serum of smear-positive pulmonary tuberculosis showed good sensitivity for anti-38kDa antibodies (191), whereas in smear negative cases antibodies to 14kDa and 30-40kDa components were more sensitive than anti-38kDa antibodies (404).

Use of the 19kDa antigen for the detection of antibodies in CSF of patients with tuberculous meningitis could clearly differentiate the patient group from the control group in studies with a European population, but no such differentiation was observed in the study with an Indian population (405).

On evaluation of Immunochromatographic kit employing five recombinant mycobacterial proteins (AMRAD-ICT, Sydney, Australia), it was observed that the kit detected an antibody response in 64.2% of smear positive and 46.3% of smear negative TB patients and that the specificity was 100% in healthy control subjects (406).

Sousa *et al* (1998) had observed higher anti-LAM antibodies than anti-38kDa antibodies in tuberculosis patients (407). Imaz *et al* (2004) evaluated Pathozyme Myco G, Pathozyme Myco M, Pathozyme Myco A and Pathozyme TB complex plus (all kits manufactured by Omega Diagnostics, Scotland) in his study and noted that Pathozyme Myco G alone or in combination with

Pathozyme TB complex plus, would be a complementary diagnostic tool in patients with suspected TB long before culture results are available. The sensitivities ranged from 29% (Pathozyme Myco M) to 82% (Pathozyme Myco G) in smear-positive patients and from 29% (Pathozyme TB complex plus) to 49% (Pathozyme Myco G) in smear negative patients. The specificities of the assays varied from 93% (Pathozyme Myco M) to 100% (Pathozyme Myco G and Pathozyme TB complex plus). Pathozyme TB complex plus detects IgG antibodies against recombinant forms of 38kDa and 16kDa while Pathozyme Myco G and Pathozyme Myco M detects IgG and IgM antibodies against LAM and recombinant 38kDa respectively (408).

Okuda *et al* (2004) compared Amplicor with Multiple Antigen ELISA kits in his study (409) and showed that a single serodiagnostic method is not satisfactory, but that combined results from multiple serodiagnostic tests (ELISA kits to detect antibodies against LAM {manufactured by Mossman associates, Blackstone}, A60 {manufactured by ANDA Biologicals, France} and TBGL {manufactured by Kyowa Medex Co. Ltd, Japan}) were very effective, for detecting active pulmonary TB at high rate, even in non-infectious patients who were smear, culture and PCR negative. The percentage of patients positive in all three tests were 58.8% for smear positive active pulmonary TB and 71.4% for chronic active pulmonary TB. When the results were evaluated in combination, the sensitivity increased to 91.5% in patients with active pulmonary TB and to 80.6% in smear and culture negative patients. It was

also shown that no improvement in sensitivity was seen when PCR was added to the battery of three diagnostic tests.

As discussed above, detection of anti-LAM antibodies have been evaluated in different countries like Mexico, Italy, Spain, Tanzania and US. Few foreign laboratories have collaborated with Indian laboratories and have included Indian patients while evaluating antibody responses against LAM (380, 389). Three important inferences can be drawn from these reports. Firstly, the specificity of the test as reported by various authors was excellent. Secondly, though a wide range of sensitivities were reported among HIV negative TB patients, the sensitivity was generally lower in HIV positive TB patients. Thirdly, a lower sensitivity was also observed while detecting anti- LAM antibodies using Mycodot test (Genelabs, Geneva, Switzerland) when compared to dot blot or ELISA techniques. This must be due to longer incubation periods in the case of ELISA (1 - 2 hours) as compared to the Mycodot (20 minutes).

In the present study, sensitivities and specificities of the three ELISA kits for the detection of anti-LAM IgG, anti-LAM IgM and anti-LAM IgA antibodies were comparable to the best studies reported elsewhere in the world.

In conclusion, detection of anti-LAM antibodies using the ELISA technique can greatly facilitate the diagnosis of TB in patients because it is a rapid,

inexpensive and reasonably reliable test for detecting active TB. It may well be of great value in developing countries like India where its sensitivity is likely to be higher due to a very high prevalence of tuberculosis. LAM based ELISA kit can also be a very useful supportive test in the RNTCP (Revised National Tuberculosis Control Programme), as a large number of cases of pulmonary tuberculosis found negative either by smear examination or radiography can be detected by this ELISA technique.



SUMMARY

Chapter 6.

6. SUMMARY

The present study was undertaken to develop and evaluate a Lipoarabinomannan based ELISA for the detection of anti-TB antibodies in pulmonary tuberculosis and tuberculous meningitis patients and to compare its clinical efficacy with alternate diagnostic methods like smear, conventional culture, Radiorespirometry and PCR. The study included 100 clinically and radiologically diagnosed pulmonary tuberculosis patients (Group A) and 20 clinically suspected cases of tuberculous meningitis (Group B).

In the case of Group A, 35% of the patients were illiterate and 45% were unemployed due to recurrent ill health. A higher percentage of smokers (27%) and alcoholics (15%) were also seen in Group A. Various factors as mentioned above, indicative of low socio-economic status and substance abuse, could be linked to increased risk for acquiring tuberculosis.

Lipoarabinomannan was purified and an ELISA for the detection of anti-LAM antibodies in serum/CSF was developed and its results were compared with those of smear, conventional culture, Radiorespirometry and PCR.

The percentage of samples positive by smear, conventional culture, Radiorespirometry, PCR and ELISA (positive for either IgG, IgM or IgA antibodies at Mean + 3SD) in Group A was 36%, 48%, 39%, 48% and 90%

respectively. Similarly, in Group B percentage of positivity for smear, conventional culture, Radiorespirometry, PCR and ELISA (positive for either IgG, IgM or IgA antibodies at Mean + 3SD) was 10%, 25%, 20%, 60% and 45% respectively.

Statistical analysis of the results of the Group A samples, showed no significant difference in the efficiency of the ELISA for detecting antibodies in smear positive and smear negative cases ($p > 0.05$), culture positive and culture negative cases ($p > 0.05$), Radiorespirometry positive and Radiorespirometry negative cases ($p > 0.05$) as well as in PCR positive and PCR negative cases ($p > 0.05$). The data thus indicates that, a positive case of tuberculosis missed out either, because of low sensitivity of smear, overzealous decontamination in culture, inhibition of carbon-dioxide liberation in Radiorespirometry or inhibition of the PCR reaction in sputum samples, can be detected by this simple ELISA method.

The number of samples positive by ELISA (9/20 i.e. 45%) in Group B was marginally less than the number of samples positive by PCR (12/20 i.e. 60%), when compared to clinical diagnosis. However, all the samples positive by Smear, Culture and Radiorespirometry also tested positive for anti-LAM antibodies.

Moreover, the in-house ELISA method could detect anti-LAM antibodies in 70% of the HIV positive cases in Group A, 93.3% of the drug resistant cases in Group A, 84.6% of the fresh cases in Group A and 44.4% of the fresh cases in Group B.

The specificity of the test for the detection of anti-LAM antibodies in pulmonary tuberculosis cases was 98% and in tuberculous meningitis cases was 90%.

Consideration of the role of new TB diagnostics should start with the recognition that more than one diagnostic test is needed, and with the acknowledgement that case detection needs to be made more patient friendly. Current algorithms are designed to minimize cost to public health systems, but little thought is given to the patient cost of diagnosis, multiple health center visits, time lost from work, morbidity attributable to late diagnosis, etc.

Diagnostic tools, which reduce patient cost and increase convenience, are likely to increase patient willingness to pay for testing and to reduce delays in seeking diagnosis. Sustained public expenditure on diagnostics on other hand will depend on evidence of demonstrable public health value. For this, research is needed to support models predicting the impact of early diagnosis on morbidity, mortality, disease transmission and ultimately on TB control. In the present study, it has been shown that the simple and rapid ELISA

technique using the immunodominant Lipoarabinomannan antigen can be looked upon as one part of the overall effort to improve TB services and facilitate TB control.

APPENDIX

APPENDIX

A.1 Homogenizing and decontaminating agent

4% NaOH	50ml
2.94% Tri-Sodium Citrate	50ml
N-acetyl-L-cysteine	0.5g

A.2 Phosphate Buffered saline (0.05M, pH7.4)

$\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$	8.9g
NaCl	9.0g

Dissolve the above ingredients in water, adjust the pH to 7.4 and then make up the volume to 1000ml.

A.3 Ziehl Neelson Carbol Fuchsin

ZNCF stain

Basic Fuchsin	1.0g
Phenol	5.0g
Absolute alcohol	10ml
Distilled water	100ml

Dissolve Basic fuchsin in absolute alcohol and then add the solution of phenol in water. Filter the stain before use.

Acid Alcohol

Sodium chloride	20.0g
Concentrated hydrochloric acid	20ml

Distilled water	500ml
Absolute alcohol	1500ml

Dissolve sodium chloride in distilled water and add concentrated hydrochloric acid, mix thoroughly and then add absolute alcohol.

Counter stain

Methylene blue	1.0g
Distilled water	100ml

Dissolve the stain thoroughly and filter after 24 hours and then use.

A.4 Lowenstein-Jensen Medium

Mineral Salt Solution

Potassium dihydrogen phosphate	2.4g
Magnesium sulfate	0.24g
Magnesium citrate	0.6g
Asparagine	3.6g
Glycerol	12ml
Distilled water	600ml

Dissolve the ingredients except glycerol by heating and then add glycerol.

Autoclave at 121°C for 20 min.

Malachite Green Solution

Malachite Green	2.0g
Distilled water	100ml

Dissolve by heating and autoclave at 121°C for 20 min.

Homogenized whole egg solution

Clean about 20-22 hen's eggs, 3-4 days old by scrubbing with hand brush in 5% soap solution and allow to stand for 30 minutes. Rinse thoroughly in running water. Soak these eggs in 70% alcohol for 15 minutes. Break these eggs into sterile flask containing glass beads under aseptic condition and homogenized by shaking vigorously. Filter through 4 layers of sterile gauze to obtain 100 ml of sterile solution.

Complete medium

Mineral salt Solution	600ml
Malachite green solution	20ml
Homogenized egg solution	1000ml

Prepare under aseptic condition. Mix well and dispense 8 ml medium into 20x150mm sterile screw capped culture tubes aseptically. Slant the tubes and inspissate at 85°C for 50 minutes. Store the tubes at 4°C until use.

A.5 Reagents for Niacin test

4% Aniline

Aniline	4ml
Absolute alcohol	96ml

Store at 4°C in amber colored bottle

10% Aqueous cyanogen bromide

Cyanogen bromide	5.0g
Distilled water	50ml

Note: Cyanogen bromide is a tear gas and therefore prepared in a well-ventilated safety cabinet using moistened mask to cover mouth and nose. Discard niacin tubes into disinfectant made alkaline by addition of NaOH to prevent formation of hydrocyanic acid under acidic conditions.

A.6 Buffer for nitrate test

NaNO ₃	0.085g
KH ₂ PO ₄	0.117g
Na ₂ HPO ₄ .12H ₂ O	0.485g

A.7 PNB

Weigh out 0.884 gm of p-nitro benzoic acid and add to 60 ml of distilled water containing 5.5ml of NaOH. Heat to 60°C and stir until dissolved. Make volume upto 90ml with distilled water and neutralize excess NaOH with 1N HCl using a pH meter. Make upto 100ml with distilled water to give a full concentration of 10,000 µg/ml. This solution may be sterilized by membrane filtration or autoclaving and 5 ml added to every 100ml of LJ medium prior inspissation.

A.8 Youman's medium

Asparagine	0.5gm
KH ₂ PO ₄	0.05g
Glycerol	2.0ml
Distilled water	100ml

Note: Dissolve the ingredients in the water in the above order, ensuring that one is dissolved before the next is added. Adjust the pH to 7.0 with 4% sodium hydroxide and add magnesium citrate 0.15 gm. Autoclave at 121°C for 20 mins.

A.9. Tris Buffered Saline (0.05M, pH 7.5)

Tris	6.05g
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NaCl	9.0g
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Dissolve the above ingredients in water, adjust the pH to 7.5 and then make up the volume to 1000ml.

BIBLIOGRAPHY

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1. Grange JM. (edns.). Tuberculosis. In: Parker MT, Collier LH. (eds.). Topley and Wilson's Principles of Bacteriology, Virology and Immunology. 8th edn, Vol 3. London: Edward Arnold Ltd. 1990; 94-121.
2. Trudeau EL. Environment in its relation to the progress of bacterial invasion in tuberculosis. *Am J Sci* 1887; 94: 118-123.
3. Dubos R, Dubos J. The white plague. Boston: Little Brown and Co. 1952.
4. Anderson P. TB vaccines: progress and problems. *Trends Immunol* 2002; 22: 160-168.
5. Frieden TR, Fujiwara PI, Washko RM, Hamburg MA. Tuberculosis in New York city- turning the tide. *N Engl J Med* 1995; 333: 229-233.
6. World Health organization. Global tuberculosis control: surveillance, planning, Finance. WHO/CDS/2002.295. WHO, Geneva, Switzerland. 2002.
7. Waaler HT. Tuberculosis and poverty. *Int J Tubercle Lung Dis* 2002; 6: 745-746.
8. Iseman MM. Evolution of drug resistant tuberculosis: a tale of two species. *Proc Natl Acad Sci USA* 1994; 91: 2428-2429.
9. Timpe A, Runyon EH. The relationship of atypical acid-fast bacteria to human disease. *J Lab Clin Med* 1954; 44: 202-209.
10. Wayne LG. Dynamics of submerged growth of *M.tuberculosis* under aerobic and microaerophilic conditions. *Am Rev Respir Dis* 1976; 114: 807-811.

11. Clark-Curtiss JE. (edns.). Genome structure of mycobacteria. In: McFadden J. (edns.). Molecular Biology of the Mycobacteria. Section 4. Academic Press, London. 1990; 77-96.
12. Groenen PMA, Bunschoten AE, van Soolingen D, van Embden JDA. Nature of DNA polymorphism in the direct repeat cluster of *Mycobacterium tuberculosis*; application for strain differentiation by a novel typing method. *Mol Microbiol* 1993; 10: 1057-1065.
13. Riley RL, Mills CC, O'Grady F, et al. Infectiousness of air from a tuberculosis ward: Ultraviolet irradiation of infected air: Comparative infectiousness of different patients. *Am Rev Respir Dis* 1962; 85: 511-525.
14. Lurie MB. Resistance to tuberculosis: Experimental studies in Native and Acquired Defensive Mechanisms. Cambridge, MA, Harvard University Press: 1964.
15. Dannenberg AM Jr. Delayed-type hypersensitivity and Cell-mediated immunity in the pathogenesis of tuberculosis. *Immunol Today* 1991; 12: 228-233.
16. Dannenberg AM Jr. Immunopathogenesis of pulmonary tuberculosis. *Hosp Pract* 1993; 28: 33-40, 51-58.
- Dannenberg AM Jr, Rook GAW. (edns.). Pathogenesis of pulmonary tuberculosis: An interplay of tissue damaging and macrophage activating immune responses-dual mechanisms that control bacillary multiplication. In: Bloom BR. (edns.). Tuberculosis; Pathogenesis,

- Protection and Control. Washington, DC, American Society for Microbiology. 1994; 459-483.
18. Allison MJ, Zappasodi P, Lurie MB. Host parasite relationships in natively resistant and susceptible rabbits on quantitative inhalation of tubercle bacilli. *Am Rev Respir Dis* 1962; 85: 553-569.
 19. Shima K, Dannenberg AM Jr, Ando M, *et al.* Macrophage accumulation, division, maturation, and digestive and microbicidal capacities in tuberculous lesions: I. Studies involving their incorporation of tritiated thymidine and their content of lysosomal enzymes and bacilli. *Am J Pathol* 1972; 89: 273-277.
 20. Kaufmann SHE. CD8⁺ T lymphocytes in intracellular microbial infections. *Immunol Today* 1988; 9: 168-74.
 21. Boom WH, Wallis RS, Chervenak KA. Human mycobacterium tuberculosis -reactive CD4⁺ T cell clones: Heterogeneity in antigen recognition, cytokine production and cytotoxicity for mononuclear phagocytes. *Infect Immun* 1991; 59: 2737-43.
 22. Lowrie DB. Is macrophage death on the field of battle essential to victory, or a tactical weakness in immunity against tuberculosis? *Clin Exp Immunol* 1990; 80: 301-03.
 23. Filley EA, Rook GAW. Effect of mycobacteria on sensitivity to the cytotoxic effects of tumor necrosis factor. *Infect Immun* 1991; 59: 2567-72.
 24. Rook GAW, Al-Attayah R. Cytokines and Koch phenomenon. *Tubercle* 1991; 72: 13-20.

25. Liew FY, Cox FEG. Nonspecific defense mechanism: The role of nitric oxide. *Immunoparasitol Today* Combined issue of *Immunol Today* 1991; 12(3) and *Parasitol Today* 1991; 7(3): A17-A21.
26. Klebanoff SJ. (edns.). Phagocytic cells: Products of oxygen metabolism. In: Gallin JI, Goldstein IM, Synderman R. (edns). *Inflammation: Basic principles and Clinical Correlates*. New York, Raven Press. 1988; 391-444.
27. Poole JCF, Florey HW. (edns.). Chronic inflammation and tuberculosis. In: Florey HW. (edns.). *General Pathology*. 4th ed, Philadelphia, WB Saunders. 1970; 1183-1224.
28. Nathan C. Mechanisms and modulations of macrophage activation. *Behring Inst Mitt* 1991; 88: 200-207.
29. Dannenberg AM Jr, Sugimoto M. Liquefaction of caseous foci in tuberculosis (Editorial). *Am Rev Respir Dis* 1976; 113: 257-259.
30. Yuan D Koh CY, Wilder JA. Interactions between B-lymphocytes and NK cells. *FASEB J* 1994; 8: 1012-1018.
31. Mosmann TR, Sad S. The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunol Today* 1996; 17: 138-146.
32. Tormey VJ, Faul J, Leonard C, *et al.* T cell cytokines may control the balance of functionally distinct macrophage populations. *Immunology* 1997; 90: 463-469.
33. Orme IM, Miller ES, Roberts AD, Furney SK, Griffin JP, Dubos KM, Chi D, Brennan PJ. T lymphocytes mediating protection and cellular

cytolysis during the course of *Mycobacterium tuberculosis* infection. *J Immunol* 1992; 148: 189-196.

34. Romagnani S. Lymphokine production by human T cells in disease states. *Ann Rev Immunol* 1994; 12: 227-257.
35. Berke G. Unlocking the secret of CTL and NK cells. *Immunol Today* 1995; 16: 343-346.
36. Boismenu R, Havran WL. An innate view of γ/δ T cells. *Curr Opin Immunol* 1997; 9: 57-63.
37. Tsuyuguchi I. Regulation of the human immune response in tuberculosis. *Infect Agents Dis* 1996; 5: 82-97.
38. Warren HS. NK cell proliferation and inflammation. *Immunol Cell Biol* 1996; 74: 473-480.
39. Gumperz JE, Parham P. The enigma of natural killer cell. *Nature* 1995; 378: 245-248.
40. Trinchieri G. Cytokines acting on or secreted by macrophages during intracellular infection. *Curr Opin Immunol* 1997; 9: 17-23.
41. Scott P, Tricheri G. The role of natural killer cells in host-parasite interactions. *Curr Opin Immunol* 1995; 7: 34-40.
42. Trinchieri G. Interleukin 12 and interferon gamma: Do they always go together? *Am. J Pathol* 1995; 147: 1534-1538.
43. Chaparas SD. Immunity in tuberculosis. *Bull WHO* 1982; 60: 447-462.
44. Chaparas SD. The immunology of mycobacterial infections. *CRC critical review in microbiology* 1982; 139-197.

45. Grange JM. (edns.). The humoral immune response in tuberculosis: its nature, biological role and diagnostic usefulness. In: Fon W, Crosset J, Styblo K. (edns.). *Advances in Tuberculosis Research*. Publishers-S. Karger, Basel. 1984; 21: 1-78.
46. Bardana EJ, McClathy JK, Farr RS, Minden P. Universal occurrence of antibodies to tubercle bacilli in sera from non-tuberculous and tuberculous lymphadenitis. *Clin Exp Immunol* 1973; 13: 65-77.
47. Kaplan MH, Chase MW. Antibodies to mycobacteria in human tuberculosis I: Development of antibodies before and after antimicrobial therapy. *J Infect Dis* 1980; 142: 825-834.
48. Marodi L, *et al.* Enhancement of macrophage candidacidal activity of interferon gamma: increased phagocytosis, killing, and calcium signal mediated by a decreased number of mannose receptors. *J Clin Invest* 1993; 91: 2596-2601.
49. Manz-Keinke H, Plattner H, Schlepper-Schafer J. Lung surfactant protein A enhances serum independent phagocytosis of bacteria by alveolar macrophages. *Eur J Cell Biol* 1992; 57: 95-100.
50. Peterson PK, Anderson WR, Ulevitch RJ, Tobias PS, *et al.* CD14 receptor mediated uptake of non-opsonized *M.tuberculosis* by human microglia. *Infect Immun* 1995; 63: 1598-1602.
51. Frehel C, Rastogi N. *Mycobacterium leprae* surface components intervene in the early phagosome-lysosome fusion inhibition event. *Infect Immun* 1987; 55: 2916-2921.

52. D'Arcy Hart P, Young MR, Jordan MM, Geisow MJ. Chemical inhibitors of phagosome-lysosome fusion in cultured macrophages also inhibit salutatory lysosomal movements. A combined microscopic and computer study. *J Exp Med* 1983; 158: 477-492.
53. Fratti RA, Chua J, Vergne I, Deretic V. *Mycobacterium tuberculosis* glycosylated phosphatidylinositol causes phagosome maturation arrest. *Proc Natl Acad Sci USA* 2003; 100: 5437-5442.
54. Pancholi P, Mirza A, Bharadwaj N, Steinman RM. Sequestration from immune CD4⁺ T cells of mycobacteria growing in human macrophages. *Science* 1993; 260: 984-986.
55. Nathan C, Xie QW. Nitric oxide synthases: roles, tolls and controls. *Cell* 1994; 78: 915-918.
56. Gazzinelli R, Oswald I, James S, Sher A. IL-10 inhibits parasite killing and nitrogen oxide production by IFN-gamma activated macrophages. *J Immunol* 1992; 148: 1792-1796.
57. Ozwald I, Gazzinelli R, Sher A, James S. IL-10 synergizes with IL-4 and transforming growth factor beta to inhibit macrophage cytotoxic activity. *J Immunol* 1992; 148: 3578-3582.
58. Flesch I, Kaufmann S. Mechanisms involved in mycobacterial growth inhibition by gamma interferon activated bone marrow macrophages: role of reactive nitrogen intermediates. *Infect Immun* 1991; 59: 3213-3218.
59. Stamler J, Singel D, Loscalzo J. Biochemistry of nitric oxide and its redox-activated forms. *Science* 1992; 258: 1898-1902.

60. Chan J, Fan X, Hunter SW, Brennan PJ, Bloom BR. Lipoarabinomannan, a possible virulence factor involved in persistence of *Mycobacterium tuberculosis* within macrophages. *Infect Immun* 1991; 59: 1755-1761.
61. Wright SD, Silverstein SC. Receptors for C3b and C3bi promote phagocytosis but not the release of toxic oxygen from human phagocytes. *J Exp Med* 1983; 158: 2016-2023.
62. Orme IM. Immunity to mycobacteria. *Curr Opin Immunol* 1993; 5: 497-502.
63. Denis M, Ghadirian E. GM-CSF restricts growth of tubercle bacilli in human macrophages. *Immunol Lett* 1990; 24: 203-206.
64. Gazzinelli R, Hieny S, Wynn T, Wolf S, Sher A. IL-12 is required for the T-lymphocyte independent induction of interferon gamma by an intracellular parasite and induces resistance in T-cell deficient hosts. *Proc Natl Acad Sci USA* 1993; 90: 6115-6119.
65. Caux C, Liu Y-J, Banchereau J. Recent advances in the study of dendritic cells and follicular dendritic cells. *Immunol Today* 1995; 16: 2-4.
66. Boom WH, Chervenak KA, Mincek MA, Ellner JJ. Role of the mononuclear phagocyte as an antigen-presenting cell for human $\gamma\delta$ T cells activated by live *Mycobacterium tuberculosis*. *Infect Immun* 1992; 60: 3480.
67. Haldane JBS. Disease and evolution. *Ric Sci Suppl* 1949; 19: 68-76.
68. Comstock GW. Tuberculosis in twins: a re-analysis of the Proffit study. *Am Rev Respir Dis* 1978; 117: 621-624.

69. Ellner JJ. The immune response in human tuberculosis-Implications for Tuberculosis Control. *J Infec Dis* 1997; 176: 1351-1359.
70. Bellamy R, Hill AVS. Genetic susceptibility to mycobacteria and other infectious pathogens in humans. *Curr Opin Immunol* 1998; 10: 483-487.
71. Davies PDO. A possible link between vitamin D deficiency and impaired host defense to *Mycobacterium tuberculosis*. *Tubercle* 1985; 66: 301-306.
72. Rook GAW, Steele J, Fraher L, Barker S, Karmali R. Vitamin D3, gamma interferon, and control of *Mycobacterium tuberculosis* by human monocytes. *Immunology* 1986; 57: 159-163.
73. Bellamy R, Ruwende C, McAdam KPWJ, Thurst M, *et al.* Mannose binding protein deficiency is not associated with malaria, hepatitis B carriage nor tuberculosis in Africans. *Q J Med* 1998; 91: 13-18.
74. Vidal SM, Malo D, Vogan K, Gross P. Natural resistance to infection with intracellular parasites: isolation of a candidate gene for *Bcg*. *Cell* 1993; 73: 469-485.
75. Cellier M, Govoni G, Vidal S, *et al.* Human natural resistance associated macrophage protein: cDNA cloning, chromosomal mapping, genomic organization and tissue specific expression. *J Exp Med* 1994; 180: 1741-1752.
76. Bellamy R, Ruwende C, Corrah T, Whittle HC. Variations in the *NRAMP1* gene and susceptibility to tuberculosis in West Africans. *N Eng J Med* 1998; 338: 640-644.

77. Fenten MJ, Vermeulen MW. Immunopathology of tuberculosis: Roles of macrophages and monocytes. *Infect Immun* 1996; 64: 683-690.
78. Wilianson RJ, Llewelyn M, Toossi Z, *et al.* Influence of vitamin D deficiency and vitamin D receptor polymorphisms on tuberculosis among Gujarati Asians in west London: a case control study. *Lancet* 2000; 355: 618-621.
79. Keane J, Gershon S, Wise RP, *et al.* Tuberculosis associated with infliximab, a tumor-necrosis factor alpha-neutralizing agent. *N Eng J Med* 2001; 345: 1098-1104.
80. Shafer RW, Edin BR. Tuberculosis in patients infected with human immunodeficiency virus: perspective on the past decade. *Clin Infect Dis* 1996; 22: 683-704.
81. Rieder HL, Snider DE Jr, Cauthen GM. Extrapulmonary tuberculosis in the United States. *Am Rev Respir Dis* 1990; 141: 347-351.
82. Roper WH, Waring JJ. Primary serofibrinous pleural effusion in military personnel. *Am Rev Tuberc* 1955; 71: 616-634.
83. Berenguer J, Moreno S, Laguna F, *et al.* Tuberculous meningitis in patients infected with the human immunodeficiency virus. *N Eng J Med* 1992; 326: 668-672.
84. World Health Organization, Global Programme on AIDS. The current global situation of the HIV/AIDS pandemic. *Wkly Epidemiol Rec* 1994; 69: 191-192.

85. Daley CL, Small PM, Schector GF, *et al.* An outbreak of tuberculosis with accelerated progression of tuberculosis with human immunodeficiency virus. An analysis using RFLP. *N Engl J Med* 1989; 320: 545-550.
86. Ellner JJ, Hinman AR, Dooley SW, *et al.* Tuberculosis symposium: Emerging problems and promise. *J Infect Dis* 1993; 168: 537-551.
87. Mukadi Y, Perriens JH, StLouis ME, *et al.* Spectrum of immunodeficiency in HIV-1-infected patients with pulmonary tuberculosis in Zaire. *Lancet* 1993; 342: 143-146.
88. Clerici M, Shearer GM. A Th1-TH2 switch is a critical step in the etiology of HIV infection. *Immunol Today* 1993; 14: 107-111.
89. Shattock RJ, Friedland JS, Griffin GE. Modulation of HIV transcription in and release from human monocytic cells following phagocytosis of *Mycobacterium tuberculosis*. *Res Virol* 1993; 144: 7-12.
90. Matsuyama T, Kobayashi N, Yamamoto N. Cytokines and HIV infection: is AIDS a tumour necrosis factor disease? *AIDS* 1991; 5: 1405-1417.
91. Wallis RS, Vjecha M, Amir-Tahmasseb M, *et al.* Influence of tuberculosis on human immunodeficiency virus (HIV-1): enhanced cytokine expression and elevated beta-2-microglobulin in HIV-1-associated tuberculosis. *J Infec Dis* 1993; 167: 43-48.
92. Toossi Z, Sierra-Madero JG, Blinkhorn RA, Metder MA, Rich EA. Enhanced susceptibility of blood monocytes from patients with pulmonary tuberculosis to productive infection with human immunodeficiency virus type 1. *J Exp Med* 1993; 117: 1511-1516.

93. Hart PD' A, Sutherland I. BCG and vole bacillus vaccines in the prevention of tuberculosis in adolescence and early adult life: Final report to the Medical Research Council. *Br Med J* 1977; 2: 293-95.
94. Comstock GW, Livesay VT, Woolpert SF. Evaluation of BCG vaccination among Puerto Rican children. *Am J Public Health* 1974; 64: 283-291.
95. Tripathy SP. Fifteen year follow up of the Indian BCG prevention trial. *Bull Int Union Tubec* 1987; 62(3): 69-72.
96. Rodrigues LC, Diwan VK, Wheeler JG. Protective effect of BCG against tuberculous meningitis and military tuberculosis: a meta analysis. *Int J Epidemiol* 1993; 22: 1154-1158.
97. Fifteen year follow up of trial of BCG vaccines in south India for Tuberculosis prevention. TRC, ICMR, Chennai. *Indian J Med Res* 1999; 110: 56-69.
98. Comstock GW. Field trials of tuberculous vaccines: How could we have done them better? *Controlled Clin Trials* 1994; 15: 247-76.
99. Behr PM, Small PM. A historical and molecular phylogeny of BCG strains. *Vaccine* 1999; 17: 915-922.
100. International Union against Tuberculosis: Phenotypes of BCG-vaccines seed lot strains: Results of an international cooperative study. *Tubercle* 1978; 59: 139-142.
101. Behr PM. BCG-different strains, different vaccines? *Lancet Infect Dis* 2002; 2: 86-92.

102. Behr MA, *et al.* Comparative genomics of BCG vaccines by whole genome DNA microarray. *Science* 1999; 284: 1520-1523.
103. Gordon SV, *et al.* Identification of variable regions in the genomes of tubercle bacilli using bacterial artificial chromosome arrays. *Mol Microbiol* 1999; 32: 643-655.
104. Skjot RL, *et al.* Comparative evaluation of low molecular mass proteins from *Mycobacterium tuberculosis* identifies members of ESAT-6 family as immunodominant T-cell antigens. *Infect Immun* 2000; 68; 214-220.
105. Palmer CE, Long MW. Effects of infection with atypical mycobacteria on BCG vaccination and tuberculosis. *Am Rev Respir Dis* 1996; 94: 553-568.
106. Rook GAW, *et al.* The effect of two distinct forms of cell mediated response to mycobacteria. *Tubercle* 1981; 62: 63-68.
107. Stead WW, Lofgren JP, Senner JW, Reddick WT. Racial differences in susceptibility to infection with *M.tuberculosis*. *N Engl J Med* 1990; 322: 422-427.
108. Stead WW. Genetics and resistance to tuberculosis. Could resistance be enhanced by genetic engineering? *Ann Intern Med* 1992; 116: 937-941.
109. Cole ST, Brosch R, Parkhill J, Garnier T, *et al.* Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* 1998; 393: 537- 544.
110. Sutherland I, Lindgren I. The protective effect of BCG vaccination as indicated by autopsy studies. *Tubercle* 1979; 60: 225-231.

111. Orme IM, McMurray DN, Belisle JT. Tuberculosis vaccine development: recent progress. *Trends in Microbiology* 2001; 9: 115-118.
112. Grosset J. Bacteriologic basis of short-course chemotherapy for tuberculosis. *Clin Chest Med* 1980; 1: 231-243.
113. Zhang Y, Young D. Molecular mechanisms of isoniazid: A drug at the front line of tuberculosis control. *Trends Microbiol* 1993; 1: 109-113.
114. Eng R, Padberg F, Smith S, *et al.* Bactericidal effects of antibiotics on slow growing and non-growing bacteria. *Antimicrob Agents Chemother* 1991; 35: 1824-1828.
115. McDermott W, Tompsett R. Activation of pyrazinamide and nicotinamide in acid environment in vitro. *Am Rev Tuberc* 1954; 70: 748.
116. Dickinson J, Aber V, Mitchison D. Bactericidal activity of streptomycin, isoniazid, rifampin, ethambutol, and pyrazinamide alone and in combination against *M.tuberculosis*. *Am Rev Respir Dis* 1977; 116: 627.
117. Takamaya K, Kilburn J. Inhibition of synthesis of arabinogalactan by ethambutol in *Mycobacterium smegmatis*. *Antimicrob Agents Chemother* 1989; 33: 1493-1499.
118. American Thoracic Society, Centers for Disease Control and Prevention, Infectious Diseases Society of America. Treatment of tuberculosis. *Am J Respir Crit Care Med* 2003; 167: 603-662.
119. Joint Tuberculosis Committee of the British Thoracic Society, Chemotherapy and management of tuberculosis in the United Kingdom: recommendations 1998. *Thorax* 1998; 53: 536-548.

120. World Health Organization Global Tuberculosis Programme. Treatment of tuberculosis: guidelines for national programmes, 3rd edition. (WHO/CDS/TB/2003, 13). Geneva: WHO, 2003.
121. Okwara A, Whalen C, Byekwaso F, *et al.* Randomised trial of thioacetazone and rifampicin containing regimens for pulmonary tuberculosis in HIV infected Ugandans. *Lancet* 1994; 344: 1323-1328.
122. Burman WJ, Gallicano K, Peloquin C. Therapeutic implications of drug interactions in the treatment of human immunodeficiency virus related tuberculosis. *Clin Infect Dis* 1999; 28: 419-430.
123. Sbarbaro J. The patient-physician relationship: Compliance revisited. *Ann Allergy* 1990; 64: 325-332.
124. Komaroff A. The practitioner and the compliant patient. *Am J Public Health* 1976; 66: 833-835.
125. Comstock GW. How much isoniazid is needed for prevention of tuberculosis among immunocompetent adults? *Int J Tuberc Lung Dis* 1999; 3: 847-850.
126. American Thoracic Society, Centers for Disease Control and Prevention: Treatment of tuberculosis and tuberculosis infection in adults and children. *Am J Respir Crit Care Med* 1994; 149: 1359-74.
127. Ratledge C. (edns.). Nutrition, growth and metabolism. In: Ratledge C, Stanford J. (edns.). *The Biology of the Mycobacteria*. London. Academic Press. 1982; 1: 185-271.

128. Churukian CJ. Demonstration of mycobacteria- a brief review with special emphasis on fluorochrome staining. *J Histotechnology* 1991; 14: 117-121.
129. Shaw JB, Wynn-Williams N. Infectivity of pulmonary tuberculosis in relation to sputum status. *Am Rev Tuberc* 1954; 69: 724-732.
130. Krasnow I, Wayne LG. Comparison of methods for tuberculosis bacteriology. *Appl Microbiol* 1969; 18: 915-917.
131. American Thoracic Society: Diagnostic standards and classification of tuberculosis. *Am Rev Respir Dis* 1990; 142: 725-35.
132. Menzies R, Vissandjee B. Effect of Bacille Calmette-Guerin vaccination on tuberculin reactivity. *Am Rev Respir Dis* 1992; 145: 621-25.
133. Rooney JJ, Crocco JA, Kramer S, *et al.* Further observations on tuberculin reactions in active tuberculosis. *Am J Med* 1976; 60: 517-522.
134. Rieder HL, Cauther GM, Block AB, *et al.* Tuberculosis and acquired immunodeficiency syndrome-Florida. *Arch Intern Med* 1989; 149: 1268-1273.
135. Khan MA, Kovnat DM, Bachus B, *et al.* Clinical and roentgenographic spectrum of pulmonary tuberculosis in adults. *Am J Med* 1977; 62: 31-38.
136. Huseby JS, Hudson LD. Miliary tuberculosis and adult respiratory distress syndrome. *Ann Intern Med* 1976; 85: 609-611.
137. Pitchenik AE, Robinson HA. The radiographic appearance of tuberculosis in patients with the acquired immunodeficiency syndrome (AIDS) and preAIDS. *Am Rev Respir Dis* 1985; 131: 393-396.

138. Glasser RM, Walker RI, Herion JC. The significance of the blood in patients with tuberculosis. *Arch Intern Med* 1970; 125: 691-695.
139. Maartens G, Wilcox PA, Benator SR. Miliary tuberculosis; rapid diagnosis, hematologic abnormalities and outcome in 109 treated adults. *Am J Med* 1990; 89: 291-296.
140. Light RW, Erozan YS, Ball WC. Cells in pleural fluid: their value in differential diagnosis. *Arch Intern Med* 1973; 132: 854-860.
141. Murtagh JJ, Mathukutty J, Kimani AP, Bai X, Metchock B. Detection and speciation of mycobacteria by immunomagnetic and bioluminescent techniques. In: 1995 International Conference of the American Thoracic Society.
142. Middlebrook G, Reggadio Z, Tigert WD. Automatable radiometric detection of growth of *Mycobacterium tuberculosis* in selective media. *Am Rev Respir Dis* 1977; 115: 1066-1069.
143. Buddemeyer EU. Liquid scintillation vial for cumulative and continuous radiometric measurement of invitro metabolism. *Appl Microbiol* 1974; 28: 177-180.
144. Shah DH, Devdhar MN, Ganatra RD, Kale PN, Viridi SS, Deshmukh MD. Modified Rapid Radiometric Method for detection of *Mycobacterium tuberculosis* from sputum samples. *Int J Nucl Med Biol* 1985; 12: 333-335.
145. Sandin RL. Polymerase chain reaction and other amplification techniques in mycobacteriology. *Clin Lab Med* 1996; 6(3): 617-39.

146. Shinnick TM, Good RC. Diagnostic Mycobacteriology Laboratory Practices. *Clin Infec Dis* 1995; 21: 291-299.
147. Kramer FR, Tyagi S, Guerra CE, Lomeli H, *et al.* Q-beta amplification assays. In: Vaheri A, Tilton RC, Balows A. (edns.). Rapid methods and automation in microbiology and immunology. Berlin. Springer Verlag Ltd. 1991; 17-22.
148. Jacobs WR, Barletta RG, Udani R, *et al.* Rapid assessment of drug susceptibilities of *Mycobacterium tuberculosis* by means of luciferase reporter phages. *Science* 1993; 260: 819-822.
149. Mullis KB, Faloona FA. Specific synthesis of DNA invitro via a polymerized catalyzed chain reaction. *Methods Enzymol* 1987; 155: 335-350.
150. Jonas V, Alden MJ, Curry JL, *et al.* Detection and identification of *Mycobacterium tuberculosis* directly from sputum sediments by amplification of rRNA. *J Clin Microbiol* 1993; 31: 2410-2416.
151. Walker GT, Fraiser MS, Schram JL, Little MC, Nadeau JG, Malinowski DP. Strand displacement amplification - an isothermal, invitro DNA amplification technique. *Nucleic Acids Res* 1992; 20: 1691-1696.
152. Iovannisci DM, Winn-Deen ES. Ligation amplification and fluorescence detection of *Mycobacterium tuberculosis* DNA. *Mol Cell Probes* 1993; 7: 35-43.

153. Nolte FS, Metchock B, McGowan JE, *et al.* Direct detection of *Mycobacterium tuberculosis* in sputum by polymerase chain reaction and DNA hybridization. *J Clin Microbiol* 1993; 31: 1777-1782.
154. Kirschner P, Rosenau J, Springer B, Teschner K, Feldmann K, *et al.* Diagnosis of mycobacterial infections by nucleic acid amplification: 18 month prospective study. *J Clin Microbiol* 1996; 34: 304-312.
155. Roth A, Schaberg T, Mauch H. Molecular diagnosis of tuberculosis: current clinical validity and future perspectives. *Eur Respir J* 1997; 10: 1877-1891.
156. Bennedsen J, Thomsen VO, Pfyffer GE, *et al.* Utility of PCR in diagnosing pulmonary tuberculosis. *J Clin Microbiol* 1996; 34: 1407-1411.
157. Carpentier E, Drouillard B, Dailloux M, *et al.* Diagnosis of tuberculosis by Amplicor *Mycobacterium tuberculosis* test. *J Clin Microbiol* 1995; 33: 3106-3110.
158. Noordhoek GT, vanEmbden JA, Kolk AHJ. Reliability of nucleic acid amplification for detection of *Mycobacterium tuberculosis*: An international collaborative quality control study among 30 laboratories. *J Clin Microbiol* 1996; 34(10): 2522-25
159. Rogall T, Flohr T, Bottgr EC. Differentiation of mycobacterium species by direct sequencing of amplified DNA. *J Gen Microbiol* 1990; 136: 1915-1920.
160. Troesch A, Nguyen H, Miyada CG, *et al.* *Mycobacterium* species identification and rifampin resistance testing with high density DNA probe. *J Clin Microbiol* 1999; 37: 49-55.

161. Metchock B, Diem L. Algorithm for the use of nucleic acid probes for identifying *M.tuberculosis* from Bactec 12 B bottles. *J Clin Microbiol* 1995; 33: 1934-37.
162. Bird BR, Denniston MM, Huebner RE, *et al.* Changing practices in mycobacteriology: A follow up survey of state and institutional public health laboratories. *J Clin Microbiol* 1996; 34: 554-59.
163. Tisdall PA, Roberts GD, Anhalt JP. Identification of clinical isolates of mycobacteria with gas-liquid chromatography. *J Clin Microbiol* 1979; 10(4): 506-14.
164. Craven RB, Brooks JB, Edman DC, Converse JD, Greenlee J, *et al.* Rapid diagnosis of lymphocytic meningitis by frequency pulsed electron capture GLC: differentiation of tuberculous, cryptococcal and viral meningitis. *J Clin Invest* 1977; 6: 27-32.
165. Glickman SE, Kilburn SO, Butler WR, *et al.* Rapid identification of mycolic acid patterns of mycobacteria by high performance liquid chromatography using pattern recognition software and a Mycobacterium library *J Clin Microbiol* 1994; 32: 740-49.
166. Ribera E, Martinez Vazquez JM, Icona I, Segura RM, Pascuas C. Activity of adenosine deaminase in CSF for the diagnosis and follow up of tuberculous meningitis in adults. *J Infec Dis* 1987; 155: 603-607.
167. Ena J, Crespo MJ, Valls V, deSalamanca RE. Adenosine deaminase activity in CSF- a useful tool for meningeal tuberculosis in patients with AIDS. *J Infec Dis* 1988; 158: 896.

168. Petterson T, Ojala K, Weber TH. Adenosine deaminase in diagnosis of pleural effusions. *Acta Med Scand* 1984; 215: 299-304.
169. Arloing S. Agglutination de bacille de la tuberculose vraie. *CR Acad Sci* 1898; 126: 1398-1400.
170. Nassau E, Parsons ER, Johnson GD. The detection of antibodies to *Mycobacterium tuberculosis* by microplate enzyme-linked immunosorbent assay (ELISA). *Tubercle* 1976; 57: 67-70.
171. Daniel TM, Debanne SM. The serodiagnosis of tuberculosis and other mycobacterial diseases by enzyme-linked immunosorbent assay. *Am Rev Respir Dis* 1987; 135: 1137-1151.
172. Ziess CR, Kalish SB, Erlich KS, *et al.* IgG antibody to PPD by enzyme linked immunosorbent assay in the diagnosis of pulmonary tuberculosis. *Am Rev Respir Dis* 1984; 130: 845-848.
173. Sada E, *et al.* Detection of mycobacterial antigens in CSF of patients with TBM by ELISA. *Lancet* 1983; 2: 651-652.
174. Chandramukhi A, *et al.* Detection of Mycobacterial antigen and antibodies in the CSF of patients with tuberculous meningitis. *J Med Microbiol* 1985; 20: 239-247.
175. Kadival GV, Mazarcelo TBMS, Chaparas SD. Sensitivity and specificity of enzyme linked immunosorbent assay in the detection of antigen in tuberculous meningitis. *J Clin Microbiol* 1986; 23: 901-904.
176. Radhakrishnan VV, Sehgal S, Mathai A. Corelation between culture of *Mycobacterium tuberculosis* and detection of mycobacterial antigens in

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CSF of patients with tuberculous meningitis. *J Med Microbiol* 1990; 33: 223-226.

177. Krambovitis E, Harris M, Hughes DTD. Improved serodiagnosis of tuberculosis by two-assay test. *J Clin Pathol* 1986; 39: 779-785.
178. Wadee AA, Boting L, Reddy SG. Antigen capture assay for detection of a 43kDa tuberculosis antigen. *J Clin Microbiol* 1990; 28: 2786-2791.
179. Radhakrishnan VV, Mathai A. ELISA to detect *Mycobacterium tuberculosis* antigen 5 and mycobacterial antibody in CSF of patients with tubercular meningitis. *J Clin Lab* 1991; 5: 233-237.
180. Sada E, Amguitar D, Torres M. Lipoarabinomannan antigenemia in patients with AIDS and tuberculosis. Presented at the 31st Interscience Conference on antimicrobial agents and chemotherapy. Chicago. 1991.
181. Daniel TM, McDonough JA, Huebner RE. Absence of IgM or IgG antibody response to *Mycobacterium tuberculosis* 30kDa antigen after primary tuberculosis infection (letter). *J Infect Dis* 1991; 164: 821.
182. Chan SL, Reggiardo Z, Daniel TM, Girling DJ, Mitchison DA. Serodiagnosis of tuberculosis using an ELISA with antigen 5 and a hemagglutination assay with glycolipid antigens. Results in patients with newly diagnosed pulmonary tuberculosis ranging in extent of disease from minimal to extensive. *Am Rev Respir Dis* 1990; 142: 385-390.
183. Daniel TM, Debanne SM, van der KF. Enzyme linked immunosorbent assay using *Mycobacterium tuberculosis* antigen 5 and PPD for the serodiagnosis of tuberculosis. *Chest* 1985; 88: 388-392.

184. Hernandez R, Munoz O, Guiscafne H. Sensitive enzyme immunoassay for early diagnosis of tuberculous meningitis. *J Clin Microbiol* 1984; 20: 533-535.
185. Stanford JL, Grange JM. The meaning and structure of species as applied to mycobacteria. *Tubercle* 1974; 55: 143-152.
186. Reggiardo Z, Vazquez E. Comparison of enzyme linked immunosorbent assay and haemagglutination test using mycobacterial glycolipids. *J Clin Microbiol* 1981; 13: 1007-1009.
187. Strobel AB, *et al.* Serologic diagnosis of bone and joint tuberculosis by ELISA assay. *J Infect Dis* 1982; 146: 280-283.
188. Benjamin RG, Daniel TM. Serodiagnosis of tuberculosis using ELISA of antibody to *Mycobacterium tuberculosis* antigen 5. *Am Rev Respir Dis* 1982; 126: 1011-1016.
189. Sada EM, Brennan PJ, Herrera T, *et al.* Evaluation of Lipoarabinomannan for serological diagnosis of tuberculosis. *J Clin Microbiol* 1990; 28(12): 2587-2590.
190. Krambovitis E. Detection of antibodies to *Mycobacterium tuberculosis* plasma membrane antigen by ELISA. *J Med Microbiol* 1986; 21: 257-264.
191. Jakkett PS, Bothamley GH, Batra HV, Mistry A, *et al.* Specificity of antibodies to immunodominant Mycobacterial antigens in pulmonary tuberculosis. *J Clin Microbiol* 1988; 26(11): 2313-2318.

192. Chandramukhi A, Bothamley GH, Brennan PJ, Ivanyi J, *et al.* Levels of antibody to defined antigens of *Mycobacterium tuberculosis* in tuberculous meningitis. *J Clin Microbiol* 1989; 27(5): 821-825.
193. Mathai A, Radhakrishnan VV, Thomas M. Rapid diagnosis of tuberculous meningitis with a dot enzyme immunoassay to detect antibody in CSF. *Eur J Clin Microbiol Infect Dis* 1991; 10: 440-443.
194. Samuel AM, *et al.* Evaluation of tubercular antigen and antitubercular antibodies in pleural and ascitic effusions. *Ind J Med Res* 1984; 80: 563-565.
195. Daniel TM, Janicki BW. Mycobacterial antigens- a review of their isolation, chemistry and immunological properties. *Microbiol Rev* 1978; 42: 84-113.
196. Goren MB. Immunoreactive substances of mycobacteria. *Am Rev Respir Dis* 1982; 125(Suppl): 50-69.
197. Daniel TM, Anderson PA. The isolation by immunosorbent affinity chromatography and physicochemical characterization of *Mycobacterium tuberculosis* antigen 5. *Am Rev Respir Dis* 1978; 117: 533-539.
198. Wiker HG, Harboe M, Bennedson J, *et al.* The antigens of *Mycobacterium tuberculosis*, H₃₇Rv, studied by crossed immunoelectrophoresis. Comparison with a reference system for *Mycobacterium bovis*, BCG. *Scand J Immunol* 1988; 27: 223-239.
199. Salata RA, Sanson AJ, Malhotra IJ, Wiker HG, Harboe M, *et al.* Purification and characterization of the 30kDa native antigen of

- Mycobacterium tuberculosis* and characterization of six monoclonal antibodies reactive with a major epitope of this antigen. *J Lab Clin Med* 1991; 118: 589-598.
200. Anderson AB, Hansen EB. Cloning of *lysA* gene from *M.tuberculosis*. *Gene* 1993; 124: 105-109.
201. Anderson AB, Anderson P, Ljungqvist L. Structure and function of a 40,000 molecular weight protein of *Mycobacterium tuberculosis*. *Infect Immun* 1992a; 60: 2317-2323.
202. Shinnick TM. The 65-kDa antigen of *Mycobacterium tuberculosis*. *J Bacteriology* 1987; 169: 1080-88.
203. Thole JER, Hindesson P, de Bruyn J, Cremers F, van der ZJ, de Cock H, *et al.* Antigenic relatedness of a strongly immunogenic 65kDa mycobacterial protein with a similarly sized ubiquitous bacterial common antigen. *Microbial Pathogen* 1988; 4: 71-83.
204. Anderson P, Askgaard D, Ljungqvist L, Bennedsen J, Heron I. Proteins released from *M.tuberculosis* during growth. *Infect Immun* 1991b; 59: 1905-1910.
205. Orme IM, Miller ES, Roberts AD, Furney SK, Griffin JP, Dobos KM, Chi D, Rivoire B, Brennan PJ. T lymphocytes mediating protection and cytotoxicity during the course of *Mycobacterium tuberculosis* infection. *J Immunol* 1992; 148: 189-196.

206. Abou-Zeid C, Smith I, Grange JM, Ratliff TL, Rook GAW. The secreted antigens of *Mycobacterium tuberculosis* and their relationship to those recognized by the available antibodies. *J Gen Microbiol* 1988; 134: 531-538.
207. Wiker HG, Harboe M, Nagai S. A localization index for distinction between extracellular and intracellular antigens of *Mycobacterium tuberculosis*. *J Gen Microbiol* 1991; 137: 875-884.
208. Anderson AB, Hansen EB. Structure and mapping of antigenic domains of protein b, A 38kDa molecular weight protein of *Mycobacterium tuberculosis*. *Infect Immun* 1989; 57: 2481-2488.
209. Haslov K, Anderson AB, Ljungquist L, Bentzon M. Comparison of the immunological activity of five defined antigens of *Mycobacterium tuberculosis* in seven inbred guinea pig strains. The 38kDa antigen is immunodominant. *Scand J Immunol* 1990; 31: 503-514.
210. Anderson AB, Ljungquist L, Olsen M. Evidence that protein antigen b of *Mycobacterium tuberculosis* is involved in phosphate metabolism. *J Gen Microbiol* 1990; 136: 477-480.
211. Daniel TM, Ellner JJ, Todd LS, McCoy DW, Payne VDN, Anderson PA, Bhe FT. Immunobiology and species distribution of *Mycobacterium tuberculosis* antigen 5. *Infect Immun* 1979; 24: 77-82.
212. Kadival GV, Chaparas SD, Hussong D. Characterization of serologic and cell-mediated reactivity of a 38kDa antigen isolated from *Mycobacterium tuberculosis*. *J Immunol* 1987; 139: 2447-2451.

213. Vordermeier HM, Harris DP, Mehrotra PK, Roman E, Elsaghier A, Ivanyi J. *M.tuberculosis* complex specific T cell stimulation and DTH reactions induced with a peptide from the 38kDa protein. *Scand J Immunol* 1992; 35: 711-718.
214. Anderson AB, Yuan ZL, Haslov K, Bennedson J. Interspecies reactivity of five monoclonal antibodies to *Mycobacterium tuberculosis* as examined by immunoblotting and enzyme linked immunosorbent assay. *J Clin Microbiol* 1986; 23: 446-451.
215. Fridovic I. The biology of oxygen radicals. *Science* 1978; 201: 875-880.
216. Wiker HG, Harboe M, Lea TE. Purification and characterization of two protein antigens from the heterogeneous BCG85 complex in *Mycobacterium bovis* BCG. *Int Arch Allergy Appl Immunol* 1986; 81: 298-306.
217. Wiker HG, Harboe M, Nagai S, Patarroyo ME, Ramirez C, Cruz N. MPB59, a widely cross-reacting protein of *Mycobacterium bovis* BCG. *Int Arch Allergy Appl Immunol* 1986; 81: 307-314.
218. Wiker HG, Sletten K, Nagai S, Harboe M. Evidence for three separate genes encoding the proteins of the mycobacterial 85 complex. *Infect Immun* 1990; 58: 272-274.
219. Yoneda M, Fukui Y. Isolation, purification and characterization of extracellular antigens of *Mycobacterium tuberculosis*. *Am Rev Respir Dis* 1965; 92(Suppl): 9-18.

220. Daniel TM, Ferguson LE. Purification and characterization of two proteins from culture filtrates of *Mycobacterium tuberculosis* H37Ra strain. *Infect Immun* 1970; 1: 164-168.
221. Janicki BW, Chaparas SD, Daniel TM, Kubica GP, *et al.* A reference system for antigens of *Mycobacterium tuberculosis*. *Am Rev Respir Dis* 1972; 104: 602-604.
222. Sada E, Ferguson LE, Daniel TM. An ELISA for the serodiagnosis of tuberculosis using a 30kDa antigen of *Mycobacterium tuberculosis*. *J Infect Dis* 1990b; 162: 928-931.
223. Havlir DV, Wallis RS, Boom WH, *et al.* Human immune response to *Mycobacterium tuberculosis* antigens. *Infect Immun* 1991; 59: 665-670.
224. Borremans M, De WL, Volkaert G, *et al.* Cloning, sequence determination, and expression of a 32-kDa protein gene of *Mycobacterium tuberculosis*. *Infect Immun* 1989; 57: 3123-3130.
225. Flynn JL, Chan J. Immunology of tuberculosis. *Annu Rev Immunol* 2001; 19: 93-129.
226. Misaki A, Seto N, Azuma I. Structural and immunological properties of D-arabino-D-galactans isolated from cell walls of *Mycobacterium* species. *J Biochem* 1974; 76: 15-27.
227. Wahl SM, Wahl LB, McCarthy JB, Chedid L, Mergenhagen, SE. Macrophage activation by mycobacterial water-soluble compounds and synthetic muramyl dipeptide. *J Immunol* 1979; 122: 2226-2231.

228. Kleinhenz ME, Ellner JJ, Spagnuolo PJ, Daniel TM. Suppression of lymphocyte responses by tuberculous plasma and mycobacterial arabinogalactan. Monocyte dependence and indomethacin reversibility. *J Clin Invest* 1981; 68: 153-162.
229. Hunter SW, Fujiwara T, Brennan PJ. Structure and antigenicity of the major specific glycolipid antigen *Mycobacterium leprae*. *J Biol Chem* 1982; 257: 15072-15078.
230. Hunter SW, Gaylord H, Brennan PJ. Structure and antigenicity of the phosphorylated lipopolysaccharide antigens from the leprosy and tubercle bacilli. *J Biol Chem* 1986; 261: 12345-12351.
231. Daniel TM. The antigenicity in guinea pigs and monkeys of three mycobacterial polysaccharides purified by affinity chromatography with conavalin A. *Am Rev Respir Dis* 1975; 111: 787-793.
232. Chan J, Fujiwara T, Brennan PJ, McNeil M, *et al.* Microbial glycolipids: possible virulence factors that scavenge oxygen radicals. *Proc Natl Acad Sci USA* 1989; 86: 2453-2457.
233. Besra GS, Brennan PJ. The mycobacterial cell wall: biosynthesis of arabinogalactan and lipoarabinomannan. *Biochem Soc Trans* 1997; 25: 845-850.
234. Nigou J, Gilleron M, Puzo G. Lipoarabinomannan: from structure to biosynthesis. *Biochimie* 2003; 85: 153-166.
235. Guerardel Y, Maes E, Ellass E, Leroy Y, *et al.* Structural study of lipomannan and lipoarabinomannan from *Mycobacterium chelonae*.

Presence of unusual components with alpha 1,3-mannopyranose side-chains. *J Biol Chem* 2002; 277: 30635-30648.

236. Chatterjee D, Bozic CM, McNeil M, Brennan PJ. Structural features of the arabinan component of lipoarabinomannan of *Mycobacterium tuberculosis*. *J Biol Chem* 1991; 266: 9652-9660.
237. Chatterjee D, Khoo KH, McNeil M, *et al*. Structural definition of the non-reducing termini of mannose-capped LAM from *Mycobacterium tuberculosis* through selective enzyme degradation and fast atom bombardment-mass spectrometry. *Glycobiology* 1993; 3: 497-506.
238. Nigou J, Gilleron M, Cahuzac B, Bounery JD, Herold M, *et al*. The phosphatidyl-myo-inositol anchor of the lipoarabinomannan from *M.bovis* bacillus Calmette Guerin. Heterogeneity, structure and role in the regulation of cytokine secretion. *J Biol Chem* 1997; 272: 23094-23103.
239. Khoo KH, Tang JB, Chatterjee D. Variation I mannose capped terminal arabinan motifs of lipoarabinomannan from clinical isolates of *Mycobacterium tuberculosis* and *Mycobacterium avium* complex. *J Biol Chem* 2001; 276: 3863-3871.
240. Khoo KH, Dell A, Morris HR, Brennan PJ, Chatterjee D. Inositol phosphate capping of the nonreducing termini of lipoarabinomannan from rapidly growing strains of *Mycobacterium*. *J Biol Chem* 1995; 270: 12380-12389.
241. McNeil MR, Brennan PJ. Structure, function and biogenesis of the cell envelope of mycobacteria in relation to bacterial physiology,

- pathogenesis, and drug resistance; some thoughts and possibilities arising from recent structural information. *Res Microbiol* 1991; 142: 451-463.
242. Rastogi N. Recent observations concerning structure and function relationships in the mycobacterial cell envelope: elaboration of a model in terms of mycobacterial pathogenicity, virulence and drug resistance. *Res Microbiol* 1991; 142: 464-476.
243. Kordulakova J, Gilleron M, Mikusova K, Puzo G, Brennan PJ, Gicquel B, Jackson M. Definition of the first mannosylation step in phosphatidylinositol mannosides synthesis. Pim A is essential for growth of mycobacteria. *J Biol Chem* 2002; 277: 31335-31344.
244. Jackson M, Crick DC, Brennan PJ. Phosphatidylinositol is an essential phospholipid of mycobacteria. *J Biol Chem* 2000; 275: 30092-30099.
245. Kordulakova J, Gilleron M, Puzo G, Brennan PJ, Gicquel B, Mikusova K, Jackson M. Identification of the required acyltransferase step in the biosynthesis of the phosphatidylinositol mannosides of *Mycobacterium* species. *J Biol Chem* 2003; 278: 36285-36295.
246. Schaeffer ML, Khoo KH, Besra GS, Chatterjee D, Brennan PJ, *et al.* The *pimB* gene of *M.tuberculosis* encodes a mannosyltransferase involved in lipoarabinomannan biosynthesis. *J Biol Chem* 1999; 274: 31625-31631.
247. Kremer L, Gurucha SS, Bifani P, Morris HR, *et al.* Characterization of a putative alpha-mannosyltransferase involved in phosphatidylinositol trimannoside biosynthesis in *M.tuberculosis*. *Biochem J* 2002; 363: 437-447.

248. Besra GS, Morehouse CB, Rittner CM, Brennan PJ. Biosynthesis of mycobacterial lipoarabinomannan. *J Biol Chem* 1997; 272: 18460-18466.
249. Gurcha SS, Baulard AR, Kremer L, Locht C, Moody DB, *et al.* Ppm1, a novel polyprenol monophosphomannose synthase from *Mycobacterium tuberculosis*. *Biochem J* 2002; 365: 441-450.
250. Brennan PJ. Structure, function and biogenesis of the cell wall of *Mycobacterium tuberculosis*. *Tuberculosis* 2003; 83: 91-97.
251. Wolucka BA, McNeil MR, de Hoffmann E, Brennan PJ. Recognition of the lipid intermediate for arabinogalactan/arabinomannan biosynthesis and its relation to the mode of action of ethambutol on mycobacteria. *J Biol Chem* 1994; 269: 23328-23335.
252. Barnes PF, Chatterjee D, Abrams JS, Lu S, Wang E, *et al.* Cytokine production induced by *Mycobacterium tuberculosis* lipoarabinomannan. Relationship to chemical structure. *J Immunol* 1992; 149: 541-547.
253. Vignal C, Guerardel Y, Kremer L, Masson M, Mazurier J, Elas E. Lipomannans, but not lipoarabinomannans, purified from *Mycobacterium chelonae* and *Mycobacterium kansasii* induce TNF-alpha and IL-8 secretion by a CD-14 Toll-like receptor 2-dependent mechanism. *J Immunol* 2003; 171: 2014-2023.
254. Dao DN, Kremer L, Guerardel Y, Molano A, Jacobs WR, *et al.* *Mycobacterium tuberculosis* lipomannan induces apoptosis and IL-12 production in macrophages. *Infect Immun* 2004; 72: 2067-2074.

255. Sibley LD, Hunter SW, Brennan PJ, Krahenbuhl JL. Mycobacterial lipoarabinomannan inhibits gamma-interferon mediated activation of macrophages. *Infect Immun* 1988; 56: 1232-1236.
256. Pai RK, Convery M, Hamilton TA, Harding CV. Inhibition of IFN-gamma induced class II transactivator expression by a 19kDa lipoprotein from *Mycobacterium tuberculosis*: a potential mechanism for immune evasion. *J Immunol* 2003; 171: 175-184.
257. Ting AM, Kim AC, Cattamanchi A, Ernst JD. *Mycobacterium tuberculosis* inhibits IFN-gamma transcriptional responses without inhibiting activation of STAT1. *J Immunol* 1999; 163: 3898-3906.
258. Rojas M, Garcia LF, Nigou J, Puzo G, Olivier M. Mannosylated lipoarabinomannan antagonizes *Mycobacterium tuberculosis* induced macrophage apoptosis by altering Ca²⁺ dependent cell signaling. *J Infect Dis* 2000; 183: 240-251.
259. Nigou J, Zelle-Rieser C, Gilleron M, Puzo G. Mannosylated lipoarabinomannans inhibit IL-12 production by human dendritic cells: evidence for a negative signal delivered through the mannose receptor. *J Immunol* 2001; 166: 7477-7485.
260. Kaene J, Remold HG, Kornfeld H. Virulent *Mycobacterium tuberculosis* strains evade apoptosis of infected alveolar macrophages. *J Immunol* 2000; 164: 2016-2020.
261. Hickman SP, Chan J, Salgame P. *Mycobacterium tuberculosis* induces differential cytokine production from dendritic cells and macrophages

- with divergent effects on naïve T cell polarization. *J Immunol* 2002; 168: 4636-4642.
262. Roach TA, Barton CH, Chatterjee D, Blackwell JM. Macrophage Activation: Lipoarabinomannan from Avirulent and Virulent Strains of *Mycobacterium tuberculosis* differentially induces the Early genes *c-fos*, *KC*, *JE* and *TNF- α* . *J Immunol* 1993; 150: 1886-1896.
 263. Armstrong JA, Hart PD. Response of cultured macrophages to *Mycobacterium tuberculosis* with observations on fusion of lysosomes with phagosomes. *J Exp Med* 1971; 134: 713-740.
 264. Kopp E, Medzhitov R. Recognition of microbial infection by Toll-like receptors. *Curr Opin Immunol* 2003; 15: 396-401.
 265. Heldwein KA, Fenton MJ. The role of Toll-like receptors in immunity against mycobacterial infection. *Microbes Infect* 2002; 4: 937-944.
 266. Geijtenbeek TB, Van Vliet SJ, Koppel EA, *et al.* Mycobacteria target DC-SIGN to suppress dendritic cell function. *J Exp Med* 2003; 197: 7-17.
 267. Strohmeier GR, Fenton MJ. Roles of lipoarabinomannan in the pathogenesis of tuberculosis. *Microbes Infect* 1999; 1: 709-717.
 268. Malik ZA, Denning GM, Kusner DJ. Inhibition of Ca (2+) signalling by *Mycobacterium tuberculosis* is associated with reduced phagosome-lysosome fusion and increased survival within human macrophages. *J Exp Med* 2000; 191: 287-302.
 269. Malik ZA, Iyer SS, Kusner DJ. *Mycobacterium tuberculosis* phagosomes exhibit altered calmodulin dependent signal transduction: contribution

- to inhibition of phagosome-lysosome fusion and intracellular survival in human macrophages. *J Immunol* 2001; 166: 3392-3401.
270. Vergne I, Chua J, Deretic V. *Mycobacterium tuberculosis* phagosome maturation arrest: selective targeting of PI3P-dependent membrane trafficking. *Traffic* 2003; 4: 600-606.
 271. Nair JS, DaFonseca CJ, Tjenberg A, *et al.* Requirement of Ca²⁺ and CaMKII for Stat1 Ser-727 phosphorylation in response to IFN- γ . *Proc Natl Acad Sci USA* 2002; 99: 5971-5976.
 272. Maiti D, Bhattacharya A, Basu J. Lipoarabinomannan from *Mycobacterium tuberculosis* promotes macrophage survival by phosphorylating BAD through a phosphatidylinositol 3-kinase/Akt pathway. *J Biol Chem* 2001; 276: 329-333.
 273. Prigozy TI, Sieling PA, Clemens D, Stewart PL, *et al.* The mannose receptor delivers lipoglycan antigens to endosomes for presentation to T cells by CD1b molecules. *Immunity* 1997; 6: 187-197.
 274. Schlesinger LS, Hull SR, Kaufman TM. Binding of the terminal mannosyl units of lipoarabinomannan from a virulent strain of *Mycobacterium tuberculosis* to human macrophages. *J Immunol* 1994; 152: 4070-4079.
 275. Stokes R, Speert D. Lipoarabinomannan inhibits nonopsonic binding of *Mycobacterium tuberculosis* to murine macrophages. *J Immunol* 1995; 155: 1361-69.

276. Sieling P, Chatterjee D, Porcelli S, Prigozy T, Bloom B, *et al.* CD1 restricted T cell recognition of microbial lipoglycan antigens. *Science* 1995; 269: 227-230.
277. Fairhurst RM, Wang C, Seiling PA, Modlin RL, Braun J. CD1 restricted T cells and resistance to polysaccharide encapsulated bacteria. *Immunol Today* 1998; 19: 257-259.
278. Dahl K, Shiratsuchi H, Hamilton B, Ellner J, Toossi Z. Selective induction of transforming growth factor beta in human monocytes by lipoarabinomannan of *Mycobacterium tuberculosis*. *Infect Immun* 1996; 64: 399-405.
279. Takeuchi M, Alard P, Streilein J. TGF beta promotes immune deviation by altering accessory signals of antigen presenting cells. *J Immunol* 1998, 160: 1589-1597.
280. Julian E, Matas L, Ausina V, Luquin M. Detection of Lipoarabinomannan antibodies in patients with newly acquired tuberculosis and patients with relapse tuberculosis. *J Clin Microbiol* 1997; 35: 2663-64.
281. Kubica GP, Dye WE, Cohn ML, Middlebrook G. Sputum digestion and decontamination with N-acetyl-L-cysteine -Sodium hydroxide for culture of mycobacteria. *Am Rev Respir Dis* 1963; 87: 775-779.
282. Sonnenwirth AC. (edns.). Stains and staining procedure. In: Sonnenwirth AC, Jarett L. (edns.). *Gradwohl's clinical laboratory methods and diagnosis*, 8th edition. 1980; 2: 1378-1390.

283. Kubica GP, David HL. (edns.). The mycobacteria. In: Sonnenwirth AC, Jarett L. (edns.). Gradwohl's clinical laboratory methods and diagnosis. 8th edition. 1980; 2: 1693-1730.
284. Konno K. New chemical method to differentiate human type tubercle bacilli from other mycobacteria. *Science* 1956; 124:958.
285. Virtanen S. A study of nitrate reduction of mycobacteria. *Acta Tuber Scand* 1960; 48: (Suppl) 1-11.
286. Tsukamara M, Tsukamara S. Differentiation of *Mycobacterium tuberculosis* and *M.bovis* by PNB susceptibility. *Tubercle* 1964; 45: 64-65.
287. Canetti G, Wallace F, Khomenko A, Mahler HT, Menon NK, Mitchison DA, Rist N. Advances in Techniques of Testing Mycobacterial Drug Sensitivity, and the use of sensitivity tests in tuberculosis control programmes. *Bull Org Mond Sanc* 1969; 41: 21-43.
288. Soolingen DV, Hermans PWM, Haas PEW, Soll DR, Embden JDA. Occurrence and stability of Insertion Sequences in *Mycobacterium tuberculosis* Complex strains: Evaluation of an Insertion Sequence-dependent DNA Polymorphism as a tool in the epidemiology of tuberculosis. *J Clin Microbiol* 1991; 29: 2578-2586.
289. Severn WB, Jones AM, Kittelberger R, de Lisle GW, Atkinson PH. Improved procedure for the isolation and purification of lipoarabinomannan from *Mycobacterium bovis* strain AN5. *J Microbiol Methods* 1997; 28: 123-130.

290. Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric method for the determination of sugars and related substances. *Anal Chem* 1956; 28: 350-356.
291. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; 227: 680-685.
292. Kittleberger R, Hilbink F. Sensitive silver staining detection for bacterial lipopolysaccharides in polyacrylamide gels. *J Biochem Biophys Methods* 1993; 26: 81-86.
293. Bloom BR, Murray JL. Tuberculosis: commentary on a re-emergent killer. *Science* 1992; 257: 1055-1064.
294. World Health Organization. Prevalence and Incidence of tuberculosis in India: a Comprehensive Review, 1997. WHO/TB/97.231, 1998.
295. Progress towards tuberculosis control-India 2001, MMWR 51: 229, 2002.
296. World Health Organization. 2002. The Global plan to stop tuberculosis. Stop TB, Geneva.
297. Harris AD, Parry C, Nyongonya-Mbewe L, *et al.* The pattern of tuberculosis in Queen Elizabeth Central Hospital, Blantyre, Malawi: 1986-1995. *Int J Tuberc Lung Dis* 1997; 1: 346-351.
298. Auerbach O. Tuberculous meningitis: Correlation of therapeutic results with the pathogenesis and pathologic changes. I. General considerations and pathogenesis *Am Rev Tuberc* 1951; 64: 408-418.
299. Singh B, Tandon PN. Critical surveillance of the tuberculosis of nervous system as seen in India. Publication of proceedings of Symposium on

- Tuberculosis of the nervous system, Mumbai: organized by WHO. 1972: 3-7.
300. Desai MM, Pal RB. Polymerase chain reaction for the rapid diagnosis of tuberculous meningitis. *Ind J Med Sci* 2002; 56: 546-552.
 301. Hudelson P. Gender differentials in tuberculosis: the role of socio economic and cultural factors. *Tuberc Lung Dis* 1996; 77: 391-400.
 302. Nair DM, George A, Chacko KT. Tuberculosis in Bombay: new insights from poor urban patients. *Health Policy and Planning* 1997; 12: 77-85.
 303. Styblok EDA. (edns.). The impact of infection with HIV on tuberculosis. In: Mitchell DM. (edns.). Recent advances respiratory medicine. London: Churchill Livingstone Publications. 1991; 5: 147-162.
 304. Comstock GW, Cauthen GM. (edns.). Epidemiology of tuberculosis. In: Reichman LB, Hershfield ES. (edns.). Tuberculosis: A comprehensive international approach. New York: Marcel Dekkar Inc Publications. 1993; 23-48.
 305. Mangtani P, Jolley DJ, Watson JM, Rodrigues L. Socioeconomic deprivation and notification rates for tuberculosis in London during 1991-1992. *BMJ* 1995; 310: 963-966.
 306. Reider HL. Epidemiological basis of tuberculosis control. Paris, International Union against Tuberculosis and Lung Disease, 1999.
 307. Davies RPO, Tocque K, Bellies A, Rimmington T, *et al*. Historical declines in tuberculosis in England and Wales: improving social conditions or natural selection? *Int J Tuberc Lung Dis* 1999; 3: 1051-1054.

308. Barnes PF. Tuberculosis among the inner city poor. *Int J Tuberc Lung Dis* 1998; 2: 41-45.
309. Tupasi TE, Radhakrishna S, Quelapio M, Villa ML, *et al.* Tuberculosis in the urban poor settlements in the Phillipines. *Int J Tuberc Lung Dis* 2000; 4: 4-11.
310. Buskin SE, Gale JL, Weiss NS, Nolan CM. Tuberculosis risk factors in adults in King Country, Washington. 1988 through 1990. *Am J Pub Health* 1994; 84: 1750-1756.
311. Lowe CR. An association between smoking and respiratory tuberculosis. *BMJ* 1956; 2: 1081-1086.
312. Lewis JG, Chamberlain DA. Alcohol consumption and smoking habits in male patients with pulmonary tuberculosis. *Brit J Prev Soc Med* 1963; 17: 149-152.
313. Liefoghe R. A Kenyan Community's perception of tuberculosis. *Trop Med and Int Health* 1997; 2: 809-21.
314. Auer C. Health seeking and perceived causes of tuberculosis among patients in Manila, Philippines. *Trop Med and Int Health* 2000; 5: 648-656.
315. Nozaki H, Koto A, Amano T, Tanahashi N, Tanaka K, *et al.* Clinical features of 10 cases of tuberculosis meningitis with special reference to patient's delay and doctor's delay. *Kekkaku* 1996; 71: 239-244.
316. Mohanty KC, Nair S, Sahasrabudhe T. Changing trend of HIV infection in patients with respiration disease in Bombay area since 1988. *Ind J Tuberc* 1994; 41: 147-150.

317. Rao SR, Amarnath SK. HIV infections in Pondicherry. *Ind J Med Virol* 1996; 14: 43-47.
318. Swaminathan S, Ramachandran R, Baskaran G, Paramasivan CN, *et al.* Risk of development of tuberculosis in HIV-infected patients. *Int J Tuberc Lung Dis* 2000; 4: 839.
319. Tan MF, NG WC, Chan SH, Tan WC. Comparative usefulness of PCR in the detection of *M.tuberculosis* in different clinical specimens. *J Med Microbiol* 1997; 46: 164-169.
320. Kraznow I, Wayne LG. Comparison of methods for tuberculosis bacteriology. *Appl Microbiol* 1969; 18: 915-917.
321. Lockman S, Hone NM, Kenyon TA, *et al.* Etiology of pulmonary infections in predominantly HIV-infected adults with suspected tuberculosis, Botswana. Botswana Respiratory Diseases Working Group. *Int J Tuberc Lung Dis* 2003; 7: 714-23.
322. Boddingtonhaus B, Rogall T, Flohr T, Blocker H, Bottger EC. Detection and identification of Mycobacteria by amplification of rRNA. *J Clin Microbiol* 1990; 28: 1751-59.
323. Manjunath N, Shankar P, Rajan L, Bhargava A, Saluja S, *et al.* Evaluation of a Polymerase chain reaction for the diagnosis of tuberculosis. *Tubercle* 1991; 72: 21-27.
324. Dagainawala FK, Banker DD. Rapid cultivation of Mycobacterium tuberculosis in Veeraraghvan's Liquid Medium. *Ind J Med Sci* 1991; 45: 293-306.

325. Nguyen LN, Kox LF, Pham LD, Kuijper S, *et al.* The potential contribution of the diagnosis of tuberculous meningitis. *Arch Neurol* 1993; 53: 771-776.
326. Radhakrishnan VV, Mathai A. Correlation between the isolation of *Mycobacterium tuberculosis* and estimation of mycobacterial antigen in cisternal, ventricular and lumbar cerebrospinal fluids of patients with tuberculous meningitis. *Ind J Pathol Microbiol* 1993; 36: 348-355.
327. Desai T, Gogate A, Deodhar L, Toddywalla S. Enzyme-linked immunosorbent assay for antigen detection in tuberculous meningitis. *Ind J Pathol Microbiol* 1993; 36: 348-355.
328. Hassan EM, Ezzat HO, Saleh LH, Girgis NI, *et al.* Diagnosis of tuberculous meningitis by Immunofluorescence and Enzyme Immunoassay. *J Egypt Public Health Assoc* 1989; 6: 45-54.
329. Rodrigues C, Nukala R, Menon S, *et al.* Amplification of *IS6110* in rapid detection of *Mycobacterium tuberculosis*. *J Med Microbiol* 1997; 15: 167-171.
330. Uplekar MW, Shepard DS. Treatment of tuberculosis by private general practitioners in India. *Tubercle* 1991; 72: 284-290.
331. Udwardia ZF, Hakimiyan A, Rodrigues C, *et al.* A profile of drug resistant tuberculosis in Bombay. *Chest* 1996; 110: 228.
332. Chowgule RV, Deodhar L. Pattern of secondary acquired resistance to antituberculosis drugs in Mumbai, India. 1991-95. *Ind J Chest Dis Allied Sci* 1998; 40: 23-31.

333. Almeida D, Rodrigues C, Udwadia ZF, Lalvani A, *et al.* Incidence of multi-drug resistance in urban and rural India and implications for prevention. *Clin Infec Dis* 2003; 36: 152-154.
334. Fisher MW, Kerchheimer WF. Studies on the growth of mycobacteria I. The occurrence of arithmetic linear growth. *Am Rev Tuberc* 1954; 66: 758-761.
335. Tsukamura, M. Growth Physiology of Mycobacteria in Modified Dubos Liquid Medium. *Microbiol Immunol* 1990; 34: 995-1003.
336. Heifets L, Linder T, Sanchez D, Spencer D, *et al.* Two liquid systems, MGIT and MB Redox Tube for *Mycobacterium tuberculosis* from Sputum specimens. *J Clin Microbiol* 2000; 38: 1227-1230.
337. Venkataraman P, Herbert D, Paramasivan CN. Evaluation of the BACTEC radiometric method in the early diagnosis of tuberculosis. *Ind J Med Res* 1998; 108: 120-127.
338. Shankar P, Manjunath N, Mohan KK, Prasad K, *et al.* Rapid diagnosis of tubercular meningitis by Polymerase chain reaction. *Lancet* 1991; 337: 3-7.
339. Verma A, Dasgupta N, Agrawal AN, Pande JN, *et al.* Utility of a GC-rich repetitive sequence in the diagnosis of tuberculosis pleural effusion by PCR. *Indian J Biochem Biophys* 1995; 32: 429-436.
340. Kadival GV, D'Souza CD, Kulkarni SP, Samuel AM. A highly specific Polymerase chain reaction for detection of *M.tuberculosis*. *Indian J Tuberc* 1996; 43: 151-154.

341. Singh KK, Nair MD, Radhakrishnan K, Tyagi JS. Utility of PCR assay in diagnosis of En-Plaque tuberculoma of the brain. *J Clin Microbiol* 1999; 37: 467-470.
342. Narayanan S, Parandaman V, Narayanan PR, Venkatesan P, *et al.* Evaluation of PCR using TRC-4 and IS6110 primers in detection of tuberculous meningitis. *J Clin Microbiol* 2001; 39: 2006-2008.
343. Miyazaki Y, Koga H, Kohno S, Kaku M. Nested polymerase chain reaction for detection of *Mycobacterium tuberculosis* in clinical samples. *J Clin Microbiol* 1993; 31: 2228-2232.
344. Buck GE, O'Hara LC, Summersgill JT. Rapid, simple method for treating clinical specimens containing *Mycobacterium tuberculosis* to remove DNA for polymerase chain reaction. *J Clin Microbiol* 1992; 30: 1331-1334.
345. Wilson SM, McNerney R, Nye PM, Godfrey-Faussett PD, Stoker NG, *et al.* Progress toward a simplified polymerase chain reaction and its application to diagnosis of tuberculosis. *J Clin Microbiol* 1993; 31: 776-782.
346. Kolk AHJ, Schuitema ARJ, Kuijper S, *et al.* Detection of *Mycobacterium tuberculosis* in clinical samples by using polymerase chain reaction and a nonradioactive detection system. *J Clin Microbiol* 1992; 30: 2567-2575.
347. Shawar RM, El-Zaatari FAK, Nataraj A, Clarridge JE. Detection of *Mycobacterium tuberculosis* in clinical samples by two-step polymerase chain reaction and nonisotopic hybridization methods. *J Clin Microbiol* 1993; 31: 61-65.

348. Clarridge JE, Shawar RM, Shinnick TM, Plikaytis BB. Large-scale use of polymerase chain reaction for detection of *Mycobacterium tuberculosis* in a routine mycobacteriology laboratory. *J Clin Microbiol* 1993; 31: 2049-2056.
349. Noordhoek GT, Kolk AHJ, Bjune G, Catty D, *et al.* Sensitivity and specificity of PCR for detection of *Mycobacterium tuberculosis*: a Blind comparison study among Seven Laboratories. *J Clin Microbiol* 1994; 32: 277-284.
350. Forbes BA, Hicks KES. Direct detection of *Mycobacterium tuberculosis* in respiratory specimens in a clinical laboratory by polymerase chain reaction. *J Clin Microbiol* 1993; 31: 1688-1694.
351. Kox LFF, Rhienthong D, Miranda AM, *et al.* A more reliable PCR for detection of *Mycobacterium tuberculosis* in clinical samples. *J Clin Microbiol* 1994; 32: 672-678.
352. Pierre C, Lecossier D, Boussougant Y, *et al.* Use of reamplification protocol improves sensitivity of detection of *Mycobacterium tuberculosis* in clinical samples by amplification of DNA. *J Clin Microbiol* 1991; 29: 712-717.
353. Soini H, Skurnik M, Lippo K, *et al.* Detection and identification of mycobacteria by amplification of a segment of the gene coding for the 32kDa protein. *J Clin Microbiol* 1992; 30: 2025-2028.
354. Al Zahrani K, Al Jahdali H, Poirier L, Rene P, Gennaro ML, Menzies D. Accuracy and utility of a commercial test based on Polymerase chain

- reaction for detecting *Mycobacterium tuberculosis* in respiratory specimens. *Am J Respir Crit Care Med* 1995; 151: 1872-1877.
355. Lim TK, Gough A, Chin N, Kumarsinghe G. Relationship between estimated pretest probability and accuracy of automated *Mycobacterium tuberculosis* assay in smear negative pulmonary tuberculosis. *Chest* 2000; 118: 574-575.
356. Akane A, Matsubara K, Nakamura H, *et al.* Identification of heme compound copurified with DNA from bloodstains, a major inhibitor of PCR amplification. *J Forensic Sci* 1994; 2: 362-372.
357. Eisenach KD, Siford MD, Cave MD, *et al.* Detection of *Mycobacterium tuberculosis* in sputum samples using Polymerase chain reaction. *Am Rev Respir Dis* 1991; 144: 1160-1163.
358. Yuen KY, Chan KS, Chan CM, *et al.* Use of PCR in routine diagnosis of treated and untreated pulmonary tuberculosis. *J Clin Pathol* 1993; 46: 318-322.
359. Sahadevan R, Narayanan S, Paramasivan CN, Prabhakar R, Narayanan PR. Restriction fragment Length Polymorphism typing in clinical isolates of *Mycobacterium tuberculosis* from patients with pulmonary tuberculosis in Madras, India, by use for direct probe. *J Clin Microbiol* 1995; 33: 3037-3039.
360. Soolingen DV, Haas PEW, Hermans PWM, Groenen PMA, Embden JDA. Comparison of various repetitive DNA elements as genetic markers for

strain differentiation and epidemiology of *Mycobacterium tuberculosis*. *J Clin Microbiol* 1993; 31: 1987-1995.

361. Pao CC, Benedict TS, You JB, Maa JS, *et al*. Detection and identification of *Mycobacterium tuberculosis* by DNA amplification. *J Clin Microbiol* 1990; 28: 1877- 1880.
362. Bhatia AS, Kumar S, Harinath BC. Immunodiagnosis of tuberculosis: An update. *Ind J Clin Biochem* 2003; 18: 1-5.
363. Lin JJ, Harn HJ, Hsuy YD, Tsao NL, Lee HS, *et al*. Rapid diagnosis of tuberculous meningitis by polymerase chain reaction assay of cerebrospinal fluid. *J Neurol* 1995; 242: 147-152.
364. Bonnington A, Strang JIG, Klapper PE, *et al*. Use of Roche AMPLICOR *Mycobacterium tuberculosis* PCR in early diagnosis of tuberculous meningitis. *J Clin Microbiol* 1998; 36: 1251-1254.
365. Caws M, Wilson SM, Clough C, Drobniewski F. Role of *IS6110* targeted PCR, culture, biochemical and immunological criteria for diagnosis of tuberculous meningitis. *J Clin Microbiol* 2000; 38: 3150-55.
366. Delacourt C, Poveda DJ, Chureau C, Beydon N, *et al*. Use of Polymerase chain reaction for improved clinical diagnosis of tuberculosis in children. *J Pediatr* 1995; 126: 703-709.
367. Smith KC, Starke JR, Eisenach, *et al*. Detection of *Mycobacterium tuberculosis* in clinical specimens from children using a Polymerase chain reaction. *Pediatrics* 1996; 97: 155-160.

368. Pfyffer GE, Kissling P, Jahn EM, Welsher H, Salfinger M, Weber R. Diagnostic performance of Amplified MTD Test with Cerebrospinal fluid, other nonrespiratory and respiratory specimens. *J Clin Microbiol* 1996; 34: 834-841.
369. Ehlers S, Ignatius R, Renath T, Hahn H. Diagnosis of extrapulmonary tuberculosis by Gen-Probe Amplified *Mycobacterium tuberculosis* Direct Test. *J Clin Microbiol* 1996; 34: 2275-2279.
370. Soini H, Bottger EC, Viljanen MK. Identification of mycobacteria by PCR-based sequence determination of 32kDa protein gene. *J Clin Microbiol* 1994; 32: 2944-2947.
371. Granje JM, Gibson J, Batty A. The specificity of the humoral immune response soluble mycobacterial antigens in tuberculosis. *Tubercle* 1980; 61: 153-156.
372. Struass E, Wu N. Radioimmunoassay of the tuberculoprotein derived from *Mycobacterium tuberculosis*. *Proc Natl Acad Sci USA* 1980; 77: 4301-4304.
373. Maekura R, Nakagawa M, Nakamura Y, Hiraga T, Yamamura Y, *et al.* Clinical evaluation of rapid serodiagnosis of pulmonary TB by ELISA with cord factor as antigen purified from *Mycobacterium tuberculosis*. *Am Rev Respir Dis* 1993; 148: 997-1001.
374. Harboe M, Wiker HG. The 38-kDa protein of *Mycobacterium tuberculosis*. *Chest* 1992; 100: 1689-1693.

375. Del Prete R, Picca V, Mosca A, Alagni MD, Miragliotta G. Detection of anti-LAM antibodies for the diagnosis of active tuberculosis. *Int J Tuberc Lung Dis* 1998; 2: 160-163.
376. Gupta S, Kumari S, Banwalikar JN, Gupta SK. Diagnosis utility of the estimation of mycobacterial antigen A60 specific immunoglobulins IgM, IgA and IgG in the sera of cases of adult human tuberculosis. *Tuberc Lung Dis* 1995; 76: 418-424.
377. Kadival GV, Kameswaran M, Ray MK. Radioimmunoassay antibody detection in pulmonary tuberculosis. *Ind J Tub* 2000; 47: 97-100.
378. Hewitt J, Coates ARM, Mitchison DA, *et al.* The use of murine monoclonal antibodies without the purification of antigen in the serodiagnosis of tuberculosis. *J Immunol Methods* 1982; 55: 205-211.
380. Chan ED, Reves R, Belisle JT, Brennan PJ, Hahn WE. Diagnosis of tuberculosis by a visually detectable immunoassay for lipoarabinomannan. *Am J Respir Crit Care Med* 2000; 161: 1713-1719.
381. Lawn SD, Frimpong EH, Nyarko E. Evaluation of a commercial immunodiagnostic kit incorporating lipoarabinomannan in serodiagnosis of pulmonary tuberculosis. *Ghana Trop Med Int Health* 1997; 2: 978-981.
382. Bothamley GH. Serological diagnosis of tuberculosis. *Eur Respir J* 1995; 8: 676s-688s.

383. de Vries RRP, Ottenhoff THM, van Schooten WCA. Human Leukocyte antigens (HLA) and mycobacterial disease. *Springer Scmin Immunopathol* 1988; 10: 305-318.
384. Lyashchenko K, Colangeli R, Houde M, Menzies D, *et al.* Heterogeneous antibody responses in tuberculosis. *Infec Immun* 1998; 66: 3936-3940.
385. Bothamly GH, Bech JS, Schreuder GMT, D'Amaro J, *et al.* Association of tuberculosis and *M. tuberculosis*-specific antibody levels with HLA. *J Infect Dis* 1989; 159: 549-555.
386. Lee YC, Ballou CE. Structural studies on the myo-inositol mannosides from the glycolipids of *Mycobacterium tuberculosis* and *Mycobacterium phlei*. *J Biol Chem* 1964; 239: 1316-1327.
387. Leopold K, Fischer W. Molecular analysis of the lipoglycans of *Mycobacterium tuberculosis*. *Anal Biochem* 1993; 208: 57-64.
388. Prinzis S, Chatterjee D, Brennan PJ. Structure and antigenicity of lipoarabinomannan from *Mycobacterium bovis* BCG. *J Gen Microbiol* 1993; 139: 2649-2658.
389. Patil S, Gourie Devi M, Chaudhari JR, Chandramukhi A. Identification of Antibody responses to *Mycobacterium tuberculosis* antigens in the CSF of Tuberculosis Meningitis patients by Western Blotting. *Clin Immunol Immunopathol* 1996; 81: 35-40.
390. Da Costa CTK, Khanolkar S, Eliot AM, Wasunna KMA, McAdam KPW. Immunoglobulin G subclass responses to mycobacterial

lipoarabinomannan in HIV infected and non-infected patients with tuberculosis. *Clin Exp Immunol* 1993; 91: 25-29.

391. Skvor J, Trnka L, Kugukovova Z. Immunoprofile studies in patients with pulmonary tuberculosis. *Scand J Resp Dis* 1979; 60: 168-171.
392. Jha VK, Bajpai BK, Gupta RM. Levels of serum immunoglobulins in pulmonary tuberculosis patients *Ind J Chest Dis* 1974; 16: 361-367.
393. Fahey JL, McKelvey EM. Quantitative determination of serum immunoglobulins in antibody agar plates. *J Immunol* 1965; 94: 84-90.
394. South MA, Warwic WJ, Wolheim FA, Good RA. The IgA system III. IgA levels in serum and saliva of paediatric patients. *J Paediatr* 1967; 71: 645-653.
395. DiPierri G, Cazzadori A, Vento S, Malena M, *et al.* Comparative histopathological study of pulmonary tuberculosis in human immunodeficiency virus infected and non-infected patients. *Tuber Lung Dis* 1996; 77: 244-249.
396. Boggian K, Fierz W, Vernazza PL, *et al.* Infrequent detection of lipoarabinomannan antibodies in human immunodeficiency virus associated mycobacterial disease. *J Clin Microbiol* 1996; 34: 1854-1855.
397. Gupta S, Bhatia R, Datta KK. Serodiagnosis of tuberculosis. *J Commun Dis* 1995; 27: 208-214.
398. Alarcon Segovia D, Fishbein E. Serum immunoglobulins in pulmonary tuberculosis. *Chest* 1971; 60: 133-136.

399. Jain VK, Bishnoi HS, Beniwal OP, Misra SN. Immunoglobulin profile in pulmonary tuberculosis *J Postgraduate Med* 1984; 30: 80-84.
400. Bothamley G, Udani P, Rudd R, *et al.* Humoral response to defined epitopes of tubercle bacilli in adult pulmonary and child tuberculosis. *Eur J Clin Microbiol Infect Dis* 1988; 7: 639-645.
401. Charpin H, Herbault H, Gevaudan MJ, Saadjian M, *et al.* Value of ELISA using A60 antigen in the diagnosis of active pulmonary tuberculosis. *Am Rev Respir Dis* 1990; 142: 380-384.
402. Chandrasekharan S, Chauhan MM, Parimala N. Serodiagnosis of pulmonary tuberculosis and evaluation of two ELISA kits. *Indian J Tuberc* 1996; 43: 159-162.
403. Samanich K, Belisle JT, Laal S. Homogeneity of antibody responses in tuberculosis patients. *Infect Immun* 2001; 69: 4600-4609.
404. Bothamly GH, Rudd R, Festensein F, Ivanyi J. Clinical value of the measurement of *Mycobacterium tuberculosis* specific antibody in pulmonary tuberculosis. *Thorax* 1992; 47: 270-275.
405. Bothamley G, Batra H, Ramesh V, *et al.* Serodiagnosis value of 19kDa antigen of *M.tuberculosis* in Indian patients. *Eur J Clin Microbiol Infect Dis* 1992; 11: 912-915.
406. Perkins MD, Conde MB, Martins M, Kritski AL. Serologic diagnosis of tuberculosis using a simple commercial mutant antigen assay. *Chest* 2003; 123: 107-112.

407. Sousa AO, Henry S, Maroja FM, Lee FK, *et al.* IgG subclass distribution of antibody response to protein and polysaccharide mycobacterial antigens in leprosy and tuberculosis patients. *Clin Exp Immunol* 1998; 111: 48-55.
408. Imaz MS, Comini MA, Zebini E, Sequeira MD, Latini O, *et al.* Evaluation of Commercial enzyme-linked immunosorbent assay kits for detection of tuberculosis in Argentinian Population. *J Clin Microbiol* 2004; 42: 884-887.
409. Okuda Y, Maekura R, Hirotani A, Kitada S, Yoshimura K, *et al.* Rapid serodiagnosis of active pulmonary *Mycobacterium tuberculosis* by analysis of results from Multiple Antigen specific tests. *J Clin Microbiol* 2004; 42: 1136-1141.

SYNOPSIS

**SYNOPSIS OF THE THESIS TO BE SUBMITTED
TO THE UNIVERSITY OF MUMBAI
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
IN MICROBIOLOGY**

TITLE OF THE THESIS : PURIFICATION AND APPLICATION OF
LIPOARABINOMANNAN ANTIGEN AS A
DIAGNOSTIC TOOL AND ITS COMPARISON
WITH PCR, RADIORESPIROMETRY AND
OTHER CONVENTIONAL METHODS FOR
DIAGNOSIS OF TUBERCULOSIS.

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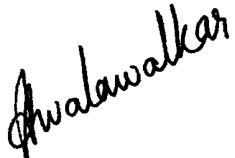
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SYNOPSIS


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1. INTRODUCTION

Tuberculosis (TB) is the leading cause of death from a single infectious agent. The current pandemic of HIV infection has contributed substantially to the recent increase in the worldwide incidence of tuberculosis (1,2). Worsening socioeconomic parameters, homelessness (3), and the rise of MDR-TB has further deteriorated this problem (4,5).

To cap this menace, World Health Organization (WHO) has been promoting "Direct Observed Therapy, short course i.e. DOTs" since 1996 in the national tuberculosis control programme of various countries, one aspect of which is case detection using sputum smear microscopy for patients with suspected TB (6). However, smear microscopy has limited sensitivity of 40-60% for a combination of 3 smear examinations (7,8). This may be still lower for those with HIV coinfection (9). Reflecting this lack of sensitivity, along with deficiencies in case reporting, currently less than 20% of the roughly 8 million predicted annual cases of tuberculosis are identified as smear-positive (10).

Chest X-ray is commonly used for the diagnosis of active TB but it has poor sensitivity, specificity and reproducibility (11). Lower sensitivity of tuberculin skin test in immunocompromised patients & HIV patients (12) and its decreased specificity because of cross-reactivity with BCG vaccine and environmental mycobacteria has further resulted in its failure to diagnose latent TB infection (13).

Bacteriologic culture of *M.tuberculosis* is definitive but takes 4-8 weeks to yield results. Although sputum digestion, decontamination and concentration methods have increased the yield of *M.tuberculosis*, the organism may be killed if the process is improperly performed resulting in false negative culture methods (14). False-positive cultures have also been reported; in a review of 12 studies that assessed more than 100 patients and used DNA fingerprinting, the median false-positive rate was 2.9% (range 0-33%) (15).

Various techniques, which rely upon radiolabeled substances e.g. Radiorespirometry and BACTEC, have been employed in an effort to substantially reduce the time for identification and drug susceptibility testing. However, the stringent regulations involved while working with radioactive materials, necessity of expensive apparatus for the detection of radioactive gas, and other expensive materials does not encourage routine use of these techniques.

During recent years various PCR assays have been developed for the rapid identification of *M.tuberculosis*. These methods differ from each other in choice of target sequence (16, 17, 18, 19) as well as DNA isolation technique (19, 20,21), number of amplification cycles (19,21) and methods of amplicon detection (18, 21, 22). A variety of different protocols for all types of specimens have been tested using in-house methods, but a standardized general method remains to be established. PCR is also often associated with

false positive reactions because of the amplification of contaminating DNA (23,24) and false negative results due to presence of inhibitors in clinical samples (25). Further, failure to yield a consensus opinion on laboratory diagnosis after application of PCR on the same set of samples has floated skepticism about reproducibility and specificity of this technique in diagnosis of tuberculosis (24).

Serodiagnosis offers a fair advantage over the problems faced with other diagnostic tests and therefore has attracted the attention of a number of laboratories all over the globe. Arloing described the first serodiagnostic test for tuberculosis in 1898 (26), but since then progress in serodiagnosis has been slow. The situation dramatically changed with the introduction of ELISA by Engvall and Perlman in 1972. Nassau and coworkers applied ELISA to the serodiagnosis of tuberculosis in 1976 achieving a sensitivity of 57% and a specificity of 98% (27). ELISA based serodiagnosis tests to detect antibodies are simple, inexpensive and are a potentially practical tool for the diagnosis of active pulmonary TB.

Till date, many in-house ELISA and RIA based techniques using semi-purified and purified antigens, (28, 29, 30, 31, 32, 33) and commercially available ELISA kits (34) with varying degree of sensitivity and specificity have been reported. Variability in sensitivity observed depending on antigen employed, immunoglobulin class measured and geographic origin of the survey participants has limited its wide application (35, 36, 37).

One such immunodominant antigen studied elaborately is Lipoarabinomannan (LAM). LAM is a major lipoglycan that is non-covalently attached to the plasma membrane through its phosphatidyl-*myo*-inositol anchor and extends to the exterior of the cell wall (38, 39). In addition to the mannosyl-phosphatidyl-*myo*-inositol, LAM possesses a mannan core with a branched arabinan polymer and in some cases cap motifs decorate the termini of the branched arabinan (38, 39). The arabinan termini in the pathogenic strains *M.tuberculosis*, *M.leprae*, *M.kansasii* and *M.avium* are modified with caps consisting of a single mannose, a dimannoside or a tri-mannoside (40, 41) resulting in molecules designated ManLAM. In the fast-growing non-pathogenic species e.g. *M.smegmatis*, branches of the terminal arabinan are terminated by inositol phosphate caps, (42) characterizing the PILAM family while in *M.chelonae* it comprises of a LAM molecule devoid of both the manno-oligosaccharide and inositol phosphate caps i.e. AraLAM (43).

ManLAM exhibits a wide spectrum of immunomodulatory functions. Sibley *et al* (44) reported the inhibition of the interferon- γ response of macrophages by ManLAM. Furthermore, ManLAM from *M.tuberculosis* inhibited the *M.tuberculosis* infection induced apoptosis of macrophages and the secretion of IL-12 induced by LPS in dendritic cells and macrophages (45, 46). ManLAM has the capacity to avoid the triggering of several macrophage early response genes (c-fos, JE and TNF) that may be involved in cell anti-microbial activities (47). By avoiding macrophage activation, ManLAM may

act as a virulence factor that allows the organism to evade cytokine-mediated mechanisms of elimination. Latex beads coated with ManLAM inhibit phagosome-lysosome fusion (48) suggesting that ManLAM is an important mediator of the inhibition of phagosome maturation in the context of infection with live bacteria. ManLAM directly activates Bad/AKT signalling pathway and thus promotes bacterial survival (49). LAM is also associated with scavenging of potentially cytotoxic oxygen free radicals (50) and inhibition of protein kinase C activity (50). Due to the presence of repeating D-arabinofuranose residues it has a direct B cell stimulatory capacity.

The chief goal of this study was to prospectively evaluate under field conditions the programmatic use and accuracy of Lipoarabinomannan based ELISA kit in serodiagnosis of tuberculosis and to compare the efficacy of this kit with other available methods like Smear, Culture, PCR and Radiorespirometry.

2. AIMS AND OBJECTIVES

- To purify Lipoarabinomannan (Man-LAM) from mycobacterial (Std virulent *M.tuberculosis* strain: H₃₇Rv) cell wall.
- Development of an ELISA for the detection of anti-LAM antibodies using purified Lipoarabinomannan antigen.
- To demonstrate the suitability of Lipoarabinomannan ELISA kit for the detection of antibody in clinical specimens.
- To compare the results obtained from Lipoarabinomannan ELISA kit with other techniques like
 1. Smear examination of clinical samples by Acid Fast Staining
 2. Isolation and identification of *M.tuberculosis* by Culture & Biochemicals testing
 3. PCR (in-house PCR for *IS6110* gene target) and
 4. Radiorespirometry.

3. MATERIALS & METHODS

3.1 Purification Of Lipoarabinomannan Antigen (51)

3.1.1 Cell preparation and isolation of crude lipoglycan extract

Heat-killed and lyophilized *M.tuberculosis* H₃₇Rv cells were suspended in Tris Buffer Saline, sonicated and after the addition of MgCl₂, cells were digested with RNase and DNase at 37°C for 60 min and then at 60°C for additional 60 min. Triton X-114 was then added to a concentration of 8% (v/v) and the solution was stirred for 16 hours. After centrifugation, the supernatant was incubated at 37°C to induce phase separation. The detergent phases were combined and crude lipoglycan precipitated by the addition of cold ethanol was collected by centrifugation.

3.1.2 Preparation of crude Lipoarabinomannan (LAM) sample

The crude lipoglycan extract was dissolved in water and ultracentrifuged at 35,000g for 16 hours. The pellets were collected, dissolved in a minimal amount of water and treated with Proteinase K for 2 hr at 60°C. The solution was ultracentrifuged twice more, reconstituted in water and lyophilized.

3.1.3 Gel filtration chromatography

Crude LAM sample was prepared for column chromatography by resuspension in tris-deoxycholate buffer to a concentration of 10mg/ml. The sample was applied to a column (100 x 1.5 cm) of Sephacryl S-200 and eluted using the same buffer. The fractions were collected and analyzed

colorimetrically for neutral glycoses and by SDS-Page. The appropriate Lipoarabinomannan, Lipomannan and Phosphatidylinositolmannoside fractions were pooled, desalted by ultrafiltration in a centriplus concentrator using a 3000 mol weight cut-off membrane, resuspended in water and lyophilized.

3.2 Development and Standardization of ELISA using Lipoarabinomannan antigen

Different concentrations of LAM preparation (0.1µg/100µl - 8µg/100µl) was coated onto polyvinyl ELISA plates (Dynatech; UK) along with different concentrations of blocking agents [i.e. BSA {1%-3%} and lysine {0.01%-0.03%}], various dilutions of sera/CSF (1:20 - 1:200) and anti-human Ig HRP conjugate (1:1000 - 1:20,000) by checker-board method to determine the optimum concentrations of the reagents for ELISA.

3.3 Collection and Preparation of Sputum Samples, CSF samples

A) **Sputum samples** were collected from 100 patients with pulmonary tuberculosis who were clinically, radiologically positive and referred to the TB Hospital, Sewri, Mumbai. A proforma wherein details of each patient such as age, sex, signs and symptoms, X-ray result, family history, treatment etc were recorded. Out of 66 males and 34 females included in this study, 82% belonged to age group between 20 and 60yrs of age, 27% were either addicted to either tobacco/pan/cigarette/alcohol or all of the above, 8% of the cohort

had one of the family members previously treated for tuberculosis while most of them belonged to lower socioeconomic status.

A smear for AFB staining was prepared and later the sputum samples were digested and decontaminated using NaOH-NALC method. The pellets obtained were stored at -20°C for further use. For detection of anti-Lipoarabinomannan antibodies, blood samples were also collected from above-mentioned patients.

B) CSF samples were collected from 20 patients with tuberculous meningitis KEM Hospital, Mumbai. Similar to the pulmonary tuberculosis cases, details of patients admitted to KEM were also recorded in the proforma. The clinical criteria used for the diagnosis of tuberculosis meningitis were fever, neck stiffness, CT-scan results, abnormal CSF findings like elevated protein, lowered glucose and chloride levels and an increased lymphocyte count. The specimens were collected in sterile screw-capped containers and concentrated by centrifugation. The deposit was used for AFB staining and stored at -20°C for further use.

C) Control sera samples were collected from 50 individuals that consisted of students, lab technicians and people suffering from asthma, allergy and pneumonia. Control CSF samples were collected from 20 patients with complaints of viral meningitis, epilepsy and pyogenic meningitis.

3.4 Conventional Culture

A loopful of the processed sputum samples were streaked on two sterile Lowenstein Jensen slants and incubated at 37°C for 8 weeks or till appearance of colonies, whichever was early. Strains were identified by standard methods including catalase, niacin test, nitrate test and para-nitro benzoic acid test results.

3.5 Drug Susceptibility by Proportion Method (52)

Lowenstein Jensen medium slopes with drugs and without drugs were prepared. The results were read for the first time on the 28th day. If, according to the criteria indicated below, the result of the reading made on the 28th day was resistant, no further reading of the test for that drug was required; the strain was classified as resistant. If the result at the 28th day was sensitive, a second reading was made on the 42nd day: this provided the definitive result.

Criteria of resistance

Any strain showing a proportion of resistant bacilli equal or superior to that indicated below is classified as resistant to the corresponding drug:

	Drug concentration (µg/ml)	Critical proportion for resistance
Isoniazid	0.2µg/ml	1
Streptomycin	4µg/ml	10
Rifampicin	40µg/ml	1
Pyrazinamide	100µg/ml	10
Ethambutol	2µg/ml	10

3.6 Polymerase Chain Reaction

3.6.1 Extraction of DNA

DNA extraction was done according to the method described by Soolingen *et al.* (53). Briefly, 100µl sputum specimens were heated at 80°C for 60 min. After the treatment of lysozyme and SDS-Proteinase K mix, 100µl of 5M NaCl and 100µl of 10% N-cetyl-N,N,N-trimethyl ammonium bromide (CTAB) in 4.1%NaCl solution were added to the tubes and further incubated at 65°C. 750µl of Chloroform/isoamylalcohol mixture was added, centrifuged, supernatant was transferred to a fresh tube and DNA precipitated with 0.6vol of isopropanol for 30min at -20°C. DNA was pelleted, washed in 70%v/v ethanol in water and dried before dissolving in Tris-EDTA buffer.

3.6.2 Polymerase Chain Reaction

Primers flanking a 123bp fragment of *IS6110* locus: a highly repetitive insertion sequence of the *M.tuberculosis* genome, was obtained from Bangalore Genei (Forward Primer: 5'CCT GCG AGC GTA GGC GTC GG3' and Reverse Primer: 5'CTC GTC CAG CGC CGC TTC GG 3'). A 25µl PCR mix was prepared containing 5µl of the extracted DNA, 200µM each of dNTPs, 0.5µM primers, 1unit of Taq polymerase diluted in 1X PCR buffer. Amplification protocol constituted of an initial denaturation step of 95°C for 5min followed by 40 cycles of 1min at 95°C, 1min at 67°C, 1min at 72°C. At the end an extension of 5min at 72°C was also included.

3.6.3 Detection and Analysis of PCR products

Ten micro liter of the PCR product was resolved electrophoretically on 2% Agarose gel, presence or absence of stained 123bp amplicon was confirmed on a UV transilluminator.

3.7 **Radiorespirometry**

Radiorespirometry was done as described by Shah *et al* (54). Whatman filter paper No. 40 (4cmx4cm) was dipped in a mixture containing 12ml liquid scintillation cocktail {2,5-diphenyloxazole and 1-4-bis-2-(5-phenyloxazolyl)-benzene dissolved in dioxane} and 1ml of 2N methanolic NaOH, dried and placed within a 20ml standard scintillation vial to line half the circumference of the vial. Standard *M.tuberculosis* (H₃₇Rv) strain and samples were redigested by NALC-NaOH for radiometric purposes and the pH of the sediment was adjusted in the range 6.5-7.5 by adding phosphate buffer saline (PBS: 0.05M, pH 7.0) An inner sterile metabolic vial containing glycerol-free Lowenstein Jensen medium (1.8ml) was inspissated and aseptically topped with 100µl of 1.0 µCi of Na-14C-acetate (obtained from BRIT) and 100µl of redigested sputum samples or live or heat inactivated standard strain. The metabolic vials were then incubated at 37°C for 72 hrs prior transferring into the liquid scintillation vial lined with scintillation fluor impregnated paper. The vial assembly was done inside a bio-safety cabinet. The entire assembly was then incubated at 37°C and the ¹⁴CO₂ produced was monitored every alternate day in a liquid scintillation counter upto a period of 4 weeks.

3.8 ELISA for anti-HIV

SD HIV1/2 ELISA microplate 3.0 precoated with gp41, p24 and gp36 antigens was used. The further procedure was carried out as per the instructions provided by the manufacturer.

3.9 In-house ELISA for detecting anti-lipoarabinomannan antibodies in sera /CSF samples. (Prior standardization as described before helped in determining optimum concentrations of the reagents for ELISA.)

Polyvinyl Microtiter plates: (Dynatech, United Kingdom) were coated with Lipoarabinomannan antigen at the concentration of 5µg/ml in Carbonate-Bicarbonate buffer (pH 9.6) and incubated overnight at 37°C. All appropriate blanks like conjugate blank and Antigen Blank were also run at the same time. After five washes with phosphate buffered saline containing 2% Tween-20, the plates were blocked with 0.01% lysine in carbonate buffer for 2 hrs at 37°C. Hundred microliter of 1: 100 dilution of the test sera and the control sera and 1:20 dilution of the control and test CSF samples were incubated at 37°C for 2 hrs, washed five times with PBS-Tween, and incubated with anti-human IgG, IgM and IgA HRP conjugate (I: 5000) for 1 hr at 37°C. The plates were washed and 100µl of substrate was added per well. After 15 min at room temperature, the reaction was stopped by adding 50µl of 2NH₂SO₄. A₄₉₀ was read in an ELISA reader.

All the methods will be discussed in detail in the thesis

4 RESULTS & DISCUSSION

Purification of Lipoarabinomannan antigen

Lipoarabinomannan was isolated and purified as per the method described by Severn *et al.* Lipophilic compounds were extracted from disrupted, nuclease treated *Mycobacterium tuberculosis* cells by temperature induced phase separation in Triton X-114 solution.

Proteinase-K treatment followed by gel filtration (Sephacryl S-200) performed in deoxycholate buffer resulted in the isolation of different size classes of lipopolysaccharides corresponding to lipoarabinomannan (LAM), lipomannan (LM) and phosphatidylinositolmannan (PIM), the purity of which was confirmed by SDS-PAGE.

On an average, the yield of LAM antigen isolated by physicochemical method from crude sonicate of H₃₇Rv strain of *M.tuberculosis* was 0.8-1mg/ml.

Development of ELISA for Lipoarabinomannan antigen (LAM)

ELISA for lipoarabinomannan antigen was standardized by checkerboard method. It was observed that 100µl of 5µg/ml of purified Lipoarabinomannan antigen, 1:100 dilution of test sera and 1:20 dilution of CSF sample with 1:5000 dilution of antihuman Ig HRP conjugate were the optimum concentrations to be used for ELISA. It was also observed that 0.01% lysine was a better blocking agent than Bovine Serum Albumin as it not only decreased non-specific reactions when compared to BSA but also enhanced antigenicity of LAM.

Overall, of 50 sera controls 2 showed readings above Mean+2SD giving 96% specificity and of 20 control CSF samples 2 gave readings above Mean+2SD giving a specificity of 90%.

In pulmonary tuberculosis cases, patients were classified as Smear +ve and Culture +ve (n=32) , Smear -ve and Culture +ve (n=16) , Smear +ve and Culture -ve (n=4), Smear -ve and Culture -ve (n=48).

Niacin test, Nitrate test, Catalase test and susceptibility to PNB drug were performed on culture positive samples to confirm the presence of *M.tuberculosis*.

Thirty of forty-eight culture positive samples (62.5 %) were resistant to one or more first-line anti-TB drugs and 23 (47.91%) were multidrug-resistant TB (MDR-TB) i.e. MDR-TB isolates showed resistance to two or more first-line drugs. Resistance was most common to rifampicin followed by streptomycin and isoniazid.

Ten % of people were positive for HIV.

IS6110 gene target is specific for *M.tuberculosis* complex and generally occurs in 1-20 copies per cell, making it an ideal target for amplification and the one that is used most commonly in in-house PCR protocols. Primers used in the present study targeted *IS6110* gene to produce 123bp gene fragment, which was detected by agarose gel electrophoresis.

Radiorespirometry was carried out as per the method described by Shah *et al.* Na-acetate was supplied as the sole carbon source in the medium

and the growth index was determined by continuously monitoring $^{14}\text{CO}_2$ respired out by the bacteria. CPM counts above Blank+3SD were considered positive.

Table 1: Positivity of pulmonary tuberculosis samples by PCR, Radiorespirometry, ELISA for anti-LAM IgG, anti-LAM IgM & anti-LAM IgA (Mean + 2SD cut off limit of negativity)

	n	PCR	Radiorespirometry	Anti-LAM IgG	Anti-LAM IgM	Anti-LAM IgA
S+C+	32	18	18	31	22	32
S-C+	16	9	7	16	9	16
S+C-	4	2	1	4	3	4
S-C-	48	19	13	46	28	47
Total	100	48	39	97	62	99

As shown in table above, of 100 clinically positive samples, 48 were positive by PCR, 39 by radiorespirometry while 97 were positive by ELISA for anti-LAM IgG, 62 were positive for anti-LAM IgM and 99 were positive for anti-LAM IgA giving a sensitivity of 97%, 62% and 99% respectively.

Of 11 samples positive by all four methods: Smear, Culture, PCR and Radiorespirometry, all 11 were positive by ELISA for anti-LAM IgG and anti-LAM IgA and only 5 of them were positive by ELISA for anti-LAM IgM while of 22 samples negative by Smear, Culture, PCR and Radiorespirometry, 22 of

them were positive by ELISA for anti-LAM IgG, 21 were positive by anti-LAM IgA and only 13 of them were positive by anti-LAM IgM.

Twenty-one of 30 Drug Resistant -TB cultures were positive by PCR, 17 were positive by radiorespirometry, 29 were positive by ELISA for anti-LAM IgG, 30 were positive by anti-LAM IgA while only 19 of them were positive by anti-LAM IgM.

Two of ten HIV-TB coinfection were positive by smear, 4 were positive by culture, 5 were positive by PCR, 2 were positive by radiorespirometry, 9 were positive by ELISA for anti-LAM IgG, 10 were positive by anti-LAM IgA and 4 of them were positive by anti-LAM IgM.

In tuberculous meningitis of 20 CSF samples, 12 were positive by PCR, 2, 5 and 4 of them were positive by smear, culture and radiorespirometry respectively while 9, 5 and 4 of them were positive by ELISA for anti-LAM IgG, anti-LAM IgA and anti-LAM IgM respectively.

Thus, lipoarabinomannan antigen was purified from crude sonicate of Standard *M.tuberculosis* strain (H₃₇Rv). A simple and rapid ELISA test to detect anti-Lipoarabinomannan antibodies in sera and CSF samples of patients was established. Lysine showed a better blocking ability as compared to Bovine serum Albumin.

PCR inhibition, a recurrent problem reported in previous studies (25,55) was also observed in this study as not all smear positives and culture positives showed 123bp band on agarose gel. Inefficient DNA extraction by

CTAB method (25) or absence of gene target in some Indian strains may also have contributed for the same (56,57).

It was also observed that not all culture positives were positive by radiorespirometry. Low sensitivity of radiorespirometry can be attributed to inhibition of CO₂ liberation and occasional failure of few clinical strains to metabolize acetate incorporated into the medium (54). A second decontamination procedure was recommended to eliminate any kind of false positivity (54). However, little is known about the viability of bacilli that has undergone decontamination procedure twice.

ELISA developed for the detection of anti-lipoarabinomannan antibody as described above gave a specificity of 96% in sera samples and that of 90% in CSF samples. ELISA for anti-lipoarabinomannan IgG showed a sensitivity of 97% and ELISA for anti-lipoarabinomannan IgA showed a sensitivity of 99% in pulmonary tuberculosis cases. In tuberculosis meningitis cases, % positivity by PCR was maximal followed by ELISA for anti-LAM IgG.

In the present study, Lipoarabinomannan ELISA kit developed for the detection of anti-LAM antibodies in sera/CSF samples was found to be rapid, sensitive and specific test for the diagnosis of tuberculosis.

REFERENCES

1. Cantwell MF, Binkin NJ. Impact of HIV on tuberculosis in sub-Saharan Africa: a regional perspective. *Int J Tuberc Lung Dis* 1997; 1: 205-214.
2. Corbett EL, Watt CJ, Walker N, *et al.* The growing burden of tuberculosis: global trends and interactions with the HIV epidemic. *Arch Intern Med* 2003; 163: 1009-21.
3. Brudney K, Dobkin J. Resurgent tuberculosis in New York City-human immunodeficiency virus, homelessness, and the decline of tuberculosis control programmes. *Am Rev Respir Dis* 1991; 144: 745-749.
4. Pablos-Mendez A, Raviglione MC, Laszlo A, *et al.* Global surveillance for anti-tuberculosis drug resistance, 1994-1997. WHO- international Union against tuberculosis and lung disease working group on anti-tuberculosis drug resistance surveillance. *N Eng J Med* 1998; 338: 1641-1649.
5. Hemvani N, Chitnis DS, Bhatia GC, Sharma N. Drug resistance among tubercle bacilli from pulmonary tuberculosis cases in central India. *Ind J Med Sci* 2001; 55: 382-392.
6. World Health Organisation (WHO). Treatment of tuberculosis: guidelines for national programmes. Geneva: WHO. 1997.
7. Kraznow I, Wayne LG. Comparison of methods for tuberculosis bacteriology. *Appl Microbiol* 1969; 18: 915-917.

8. Lockman S, Hone NM, Kenyon TA, *et al.* Etiology of pulmonary infections in predominantly HIV-infected adults with suspected tuberculosis, Botswana. Botswana Respiratory Diseases Working Group. *Int J Tuberc Lung Dis* 2003; 7: 714-23.
9. Raviglione MC, Harries AD, Msiska R, Wilkinson D, Nunn P. Tuberculosis and HIV: current status in Africa. *AIDS* 1997; 11 (Suppl B): S115-23.
10. World Health Organization. Global Tuberculosis Control. WHO Report 2000. Geneva, Switzerland, document no WHO/CDS/TB/2000.275.
11. Toman K. Tuberculosis- Case Finding and Chemotherapy: Questions and Answers. WHO. Geneva. 1979.
12. Pesanti EL. The negative tuberculin test: tuberculin, HIV and anergy panels. *Am J Respir Crit Care Med* 1994; 149: 1699-1709.
13. Huebner RE, Schein MF, Bass JB Jr. The tuberculin skin test. *Clin Infect Dis* 1993; 17: 968-975.
14. Bhatia AS, Kumar S, Harinath BC. Immunodiagnosis of tuberculosis: An update. *Ind J Clin Biochem* 2003; 18: 1-5.
15. Burman WJ, Reves RR. Review of false positive cultures for *Mycobacterium tuberculosis* and recommendations for avoiding unnecessary treatment. *Clin Infect Dis* 2000; 31: 1390-95.

16. Eisenach KD, Caww MD, Bates JH, Crawford JT. Polymerase chain reaction amplification of a repetitive DNA sequence specific for *Mycobacterium tuberculosis*. *J Infect Dis* 1990; 161: 977-981.
17. Boddingtonhaus B, Rogall T, Flohr T, Blocker H, Bottger EC. Detection and identification of Mycobacteria by amplification of rRNA. *J Clin Microbiol* 1990; 28: 1751-59.
18. Kolk AHJ, Schuitema ARJ, Kuijper S, *et al.* Detection of *Mycobacterium tuberculosis* in clinical samples by using polymerase chain reaction and a nonradioactive detection system. *J Clin Microbiol* 1992; 30: 2567-2575.
19. Miyazaki Y, Koga H, Kohno S, Kaku M. Nested polymerase chain reaction for detection of *Mycobacterium tuberculosis* in clinical samples. *J Clin Microbiol* 1993; 31: 2228-2232.
20. Buck GE, O'Hara LC, Summersgill JT. Rapid, simple method for treating clinical specimens containing *Mycobacterium tuberculosis* to remove DNA for polymerase chain reaction. *J Clin Microbiol* 1992; 30: 1331-1334.
21. Wilson SM, McNeerney R, Nye PM, Godfrey-Faussett PD, Stoker NG, *et al.* Progress toward a simplified polymerase chain reaction and its application to diagnosis of tuberculosis. *J Clin Microbiol* 1993; 31: 776-782.
22. Shawar RM, El-Zaatari FAK, Nataraj A, Clarridge JE. Detection of *Mycobacterium tuberculosis* in clinical samples by two-step polymerase

- chain reaction and nonisotopic hybridization methods. *J Clin Microbiol* 1993; 31: 61-65.
23. Kaul K, Luke S, McGurn C, Snowden N, Monti C, Fry W. Amplification of Residual DNA Sequences in Sterile Bronchoscopes leading to False-positive PCR results. *J Clin Microbiol* 1996; 34: 1949-1951.
 24. Noordhoek GT, Kolk AHJ, Bjune G, Catty D, Dale JW, *et al.* Sensitivity and specificity of PCR for detection of *Mycobacterium tuberculosis*: a Blind comparison study among Seven Laboratories. *J Clin Microbiol* 1994; 32: 277-284.
 25. Honore-Bouakline S, Vincensini JP, Giacuzzo V, Lagrange PH, Hermann JL. Rapid diagnosis of extrapulmonary tuberculosis by PCR: impact of sample preparation and DNA extraction. *J Clin Microbiol* 2003; 41: 2323-2329.
 26. Arloing S. Agglutination de bacille de la tuberculose vrte. *C R Acad Sci* 1898; 126: 1398-1400.
 27. Nassau E, Parsons ER, Johnson GD. The detection of antibodies to *Mycobacterium tuberculosis* by microplate Elisa. *Tubercle* 1976; 57: 67-70.
 28. Sada E, Brennan PJ, Herrera T, Torres M. Evaluation of lipoarabinomannan for the serological diagnosis of tuberculosis. *J Clin Microbiol* 1990; 28: 2587-2590.
 29. Maekura R, Nakagawa M, Nakamura Y, Hiraga T, Yamamura Y, *et al.* Clinical evaluation of rapid serodiagnosis of pulmonary TB by ELISA

- with cord factor as antigen purified from *Mycobacterium tuberculosis*. *Am Rev Respir Dis* 1993; 148: 997-1001.
30. Harboe M, Wiker HG. The 38-kDa protein of *Mycobacterium tuberculosis*. *Chest* 1992; 100: 1689-1693.
 31. Del Prete R, Picca V, Mosca A, D'Alagni M, Miragliotta A. Detection of anti-lipoarabinomannan antibodies for the diagnosis of active tuberculosis. *Int J Tuberc Lung Dis* 1998; 2: 160-163.
 32. Gupta S, Kumari S, Banwalikar JN, Gupta SK. Diagnosis utility of the estimation of mycobacterial antigen A60 specific immunoglobulins IgM, IgA and IgG in the sera of cases of adult human tuberculosis. *Tuberc Lung Dis* 1995; 76: 418-424.
 33. Kadival GV, Kameswaran M, Ray MK. Radioimmunoassay antibody detection in pulmonary tuberculosis. *Ind J Tub* 2000; 47: 97-100.
 34. Imaz M, Comini MA, Zerbini E, Sequeira MD, Latini O. et al. Evaluation of commercial enzyme-linked immunosorbent assay kits for detection of tuberculosis in Argentinean population. *J Clin Microbiol* 2004; 42: 884-887.
 35. Chan ED, Reves R, Belisle JT, Brennan PJ, Hahn WE. Diagnosis of tuberculosis by a visually detectable immunoassay for lipoarabinomannan. *Am J Respir Crit Care Med* 2000; 161: 1713-1719.
 36. Lawn SD, Frimpong EH, Nyarko E. Evaluation of a commercial immunodiagnostic kit incorporating lipoarabinomannan in

- serodiagnosis of pulmonary tuberculosis. *Ghana Trop Med Int Health* 1997; 2: 978-981.
37. Bothamley GH. Serological diagnosis of tuberculosis. *Eur Respir J* 1995; 8: 676s-688s.
38. Besra GS, Brennan PJ. The mycobacterial cell wall: biosynthesis of arabinogalactan and lipoarabinomannan. *Biochem Soc Trans* 1997; 25: 845-850.
39. Nigou J, Gilleron M, Puzo G. Lipoarabinomannan: from structure to biosynthesis. *Biochimie* 2003; 85: 153-166.
40. Nigou J, Gilleron M, Cahuzac B, Bounery JD, Herold M, *et al.* The phosphatidyl-myo-inositol anchor of the lipoarabinomannan from *M.bovis* bacillus Calmette Guein. Heterogeneity, structure and role in the regulation of cytokine secretion. *J Biol Chem* 1997; 272: 23094-23103.
41. Khoo KH, Tang JB, Chatterjee D. Variation in mannose capped terminal arabinan motifs of lipoarabinomannans from clinical isolates of *Mycobacterium tuberculosis* and *Mycobacterium avium* complex. *J Biol Chem* 2001; 276: 3863-3871.
42. Khoo KH, Dell A, Morris HR, Brennan PJ, Chatterjee D. Inositol phosphate capping of the nonreducing termini of lipoarabinomannan from rapidly growing strains of *Mycobacterium*. *J Biol Chem* 1995; 270: 12380-12389.

43. Guerardel Y, Maes E, Ellass E, Leroy Y, *et al.* Structural study of lipomannan and lipoarabinomannan from *Mycobacterium chelonae*. Presence of unusual components with alpha 1,3-mannopyranose side-chains. *J Biol Chem* 2002; 277: 30635-30648.
44. Sibley LD, Hunter SW, Brennan PJ, Krahenbuhi JL. Mycobacterial lipoarabinomannan inhibits gamma-interferon mediated activation of macrophages. *Infect Immun* 1988; 56: 1232-1236.
45. Rojas M, Garcia LF, Nigou J, Puzo G, Olivier M. Mannosylated lipoarabinomannan antagonizes *Mycobacterium tuberculosis* induced macrophage apoptosis by altering Ca^{2+} dependent cell signaling. *J Infect Dis* 2000; 183: 240-251.
46. Nigou J, Zelle-Rieser C, Gilleron M, Puzo G. Mannosylated lipoarabinomannan inhibit IL-12 production by human dendritic cells: evidence for a negative signal delivered through the mannose receptor. *J Immunol* 2001; 166: 7477-7485.
47. Roach TA, Barton CH, Chatterjee D, Blackwell JM. Macrophage Activation: Lipoarabinomannan from avirulent and virulent Strains of *Mycobacterium tuberculosis* differentially induces the Early genes *c-fos*, *KC*, *JE* and *TNF- α* . *J Immunol* 1993; 150: 1886-1896.
48. Fratti RA, Chua J, Vergne I, Deretic V. *Mycobacterium tuberculosis* glycosylated phosphatidylinositol causes phagosome maturation arrest. *Proc Natl Acad Sci USA* 2003; 100: 5437-5442.

49. Maiti D, Bhattacharya A, Basu J. Lipoarabinomannan from *Mycobacterium tuberculosis* promotes macrophage survival by phosphorylating Bad through a phosphatidylinositol 3-kinase/Akt pathway. *J Biol Chem* 2001; 276: 329-333.
50. Chan J, Fan X, Hunter SW, Brennan PJ, Bloom BR. Lipoarabinomannan, a possible virulence factor involved in persistence of *Mycobacterium tuberculosis* within macrophages. *Infect Immun* 1991; 59: 1755-1761.
51. Severn WB, Jones AM, Kittelberger R, de Lisle GW, Atkinson PH. Improved procedure for the isolation and purification of lipoarabinomannan from *Mycobacterium bovis* strain AN5. *J Microbiol Methods* 1997; 28: 123-130.
52. Canetti G, Wallace F, Khomenko A, Mahler HT, Menon NK, Mitchison DA, Rist N. Advances in Techniques of Testing Mycobacterial Drug Sensitivity, and the use of sensitivity tests in tuberculosis control programmes. *Bull Org Mond Sanc* 1969; 41: 21-43.
53. Soolingen DV, Hermans PWM, Haas PEW, Soll DR, Embden JDA. Occurrence and stability of Insertion Sequences in *Mycobacterium tuberculosis* Complex strains: Evaluation of an Insertion Sequence-dependent DNA Polymorphism as a tool in the epidemiology of tuberculosis. *J Clin Microbiol* 1991; 29: 2578-2586.

54. Shah DH, Devdhar MN, Ganatra RD, Kale PN, Viridi SS. Deshmukh, M.D., Modified Rapid Radiometric Method for detection of *Mycobacterium tuberculosis* from sputum samples. *Int J Nucl Med Biol* 1985; 12: 333-335.
55. Clarridge JE, Shawar RM, Shinnick TM, Plikaytis BB. Large-scale use of polymerase chain reaction for detection of *Mycobacterium tuberculosis* in a routine mycobacteriology laboratory. *J Clin Microbiol* 1993; 31: 2049-2056.
56. Sahadevan R, Narayanan S, Paramasivan CN, Prabhakar R, Narayanan PR. Restriction fragment Length Polymorphism typing in clinical isolates of *Mycobacterium tuberculosis* from patients with pulmonary tuberculosis in Madras, India, by use for direct probe. *J Clin Microbiol* 1995; 33: 3037-3039.
57. Soolingen DV, Haas PEW, Hermans PWM, Groenen PMA, Embden JDA. Comparison of various repetitive DNA elements as genetic markers for strain differentiation and epidemiology of *Mycobacterium tuberculosis*. *J Clin Microbiol* 1993; 31: 1987-1995.

