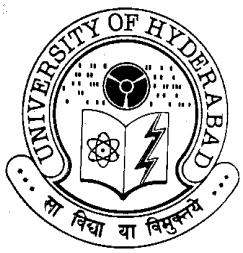
STUDIES ON THE IRON-REGULATED CULTURE FILTRATE PROTEINS OF *MYCOBACTERIUM TUBERCULOSIS*

Thesis submitted for the Degree of
Doctor of Philosophy

By

Sridevi Duggirala



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STATEMENT

I hereby declare that the work embodied in this dissertation is the result of the investigation carried out by me in the Department of Animal Sciences, School of Life Sciences, University of Hyderabad, Hyderabad, under the supervision of **Dr. Manjula Sritharan.**

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work described is based on the findings of other investigators. Any omission, which might have occurred by oversight or error, is regretted.

November 2008

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CERTIFICATE

Certified that the work embodied in this thesis entitled "**Studies in the iron-regulated culture filtrate proteins of *Mycobacterium tuberculosis*"** has been carried out by Ms. Sridevi Duggirala under my supervision and the same has not been submitted elsewhere for a degree.

Dr. Manjula Sritharan
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LIST OF PUBLICATIONS

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LIST OF ABBREVIATIONS

2D PAGE	Two dimensional Polyacrylamide gel electrophoresis
Ag	Antigen
ATP	Adenosine triphosphate
ATT	Anti-Tuberculosis-Treatment
BCG	Bacille Calmette Guérin
bp	base pair
CF-Ag pools	Culture filtrate antigen pools
CFPs	Culture filtrate proteins
Da	Dalton
DTH	Delayed-type hypersensitivity
DTT	Dithiothreitol
ELISA	Enzyme linked immuno-sorbent assay
ESAT-6	Early secretory antigenic target
IEF	Iso-electric focusing
IFN-γ	Interferon gamma
IL	Interleukin
IPG	Immobilized pH gradient
IREP	Iron-regulated envelope protein
kb	kilobase pair
kDa	kilo dalton
kV	kilo Volt
MS	Mass spectra
MTT	(3, (4, 5 dimethyl thiazol-2-yl) 2,5 diphenyl tetrazolium bromide)
NH	Normal healthy individuals
o / n	overnight
O.D	Optical density
ORF	Open reading frame
PBMC	Peripheral blood mononuclear cells

PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline with Tween 20
PCR	Polymerase chain reaction
PPD	Purified protein derivative
RT	Room temperature
SDS-PAGE	Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis
SN	Smear negative
SP	Smear positive
TBS	Tris buffered saline
TMB-H_2O_2	Tetramethyl benzidine-hydrogen peroxide
TST	Tuberculin skin testing
v / v	volume / volume
w / v	weight / volume

Introduction

Iron is an important micronutrient for all aerobic bacteria, including mycobacteria. However, they face conditions of iron limitation due to the poor solubility of the iron at biological pH. The mammalian host further limits the iron for pathogenic bacteria by holding the iron in a protein-bound form. Bacteria however, have adapted to low iron conditions by elaborating novel iron acquisition machinery. Both the siderophore-mediated and direct acquisition systems are well studied in a number of bacteria. Iron levels not only regulate the bacterial iron acquisition machinery but also control the expression of virulence determinants. Thus, the ability to acquire iron is one of the contributing factors to virulence.

Mycobacteria are unique in that they elaborate two kinds of siderophores, the intracellular mycobactin and the extracellular carboxymycobactin. The structure of mycobactin has been extensively studied in different mycobacterial species and the species-specific nature of the mycobactin has been used as a taxonomic marker. With the wealth of information from the genome analysis of *Mycobacterium tuberculosis*, the causative organism for tuberculosis, experimental approaches have made possible the understanding of the biosynthetic machinery of mycobactin. It is synthesized by the polyketide pathway, catalysed by proteins from the *mbt* cluster in the genome. The ability to synthesise mycobactin is considered a virulence determinant in *M. tuberculosis* as the *mbtB*-deficient mutant of *M. tuberculosis* failed to multiply inside macrophages. There is increasing evidence to show that this pathogen is subjected to conditions of iron limitation *in vivo* and hence understanding of the host-pathogen interactions with respect to iron acquisition will help in a significant manner towards control measures of tuberculosis.

Cell surface receptors for the uptake of ferri-siderophore are well characterized in a number of bacterial systems. In mycobacteria, the picture is not complete and the role of the iron-regulated envelope proteins (IREPs) is the focus of study by researchers interested in mycobacterial iron acquisition machinery. A 29 kDa protein is a ferri-exochelin receptor in *Mycobacterium smegmatis*. In *Mycobacterium tuberculosis*, a cell wall associated iron-regulated protein HupB, showing coordinate regulation with the

expression of the two siderophores mycobactin and carboxymycobactin is one of the areas of research interests in our lab. The clinical significance of the HupB was evident by the detection of anti-HupB antibodies in the serum of patients with tuberculosis.

Tuberculosis is a disease of major concern and concerted efforts are being made globally to develop new drugs against *M. tuberculosis* and / or an effective vaccine to control the disease. This is necessitated due to the large number of deaths each year, detection of increasing numbers of infected individuals and more important the development of multi-drug resistance. There is an urgent need for the identification of novel drug targets and detection of novel proteins as vaccine candidates.

The secretory proteins of *M. tuberculosis* probably play an important role in host-pathogen interactions and help in the establishment of infection and the development of the disease. It is well known that the cell mediated immunity play an important role in tuberculosis. There is a complex interplay of the immune cells via the host of cytokines produced by them in response to the release of the mycobacterial antigens by the macrophage. The proteins secreted by *M. tuberculosis* have been the focus of study and proteins like the ESAT-6, expressed only by *M. tuberculosis* has gained considerable importance in the light of its role as a virulence determinant and as a diagnostic antigen.

Thus the spent growth medium of *in vitro* cultures have been analysed and culture filtrate proteins (CFPs) have been studied extensively and explored as potential vaccine candidates. They include the secretory / excretory proteins released into the immediate environment by the growing mycobacteria. There is a wealth of information on the CFPs from *M. tuberculosis* and the immuno stimulatory effect of these proteins in experimental animals including mice, guinea pigs and cattle as well in human patients with the disease. Challenge studies have shown the immuno-protective effect of some of the culture filtrate proteins.

The CFP profile is influenced by several factors, including temperature and time of growth. In this study, the two major objectives include understanding the influence of iron levels on the CFP profile of *M. tuberculosis* and analysis of the immune potential of these CFPs in human patients with tuberculosis.

CHAPTER 1

REVIEW OF LITERATURE

1.1. Genus *Mycobacterium*

1.1.1. Classification. Mycobacteria belong to the Order *Actinomycetales*, Family *Mycobacteriaceae*. Robert Koch identified *Mycobacterium tuberculosis* as the causative organism of tuberculosis in 1882. It was referred to as Koch's bacillus till Lehmann and Neumann gave the generic name *Mycobacterium* (meaning fungus bacterium) due to the mould-like growth of the bacillus in liquid medium (Lehmann & Neumann, 1896).

Some mycobacteria are pigmented and based on the production of pigments, they can be classified as scotochromogens (produce yellow pigment in the dark) or photochromogens (produce an orange pigment in the light) or achromogens (do not produce any pigment). Based on the growth characteristics mycobacteria are grouped into fast growers and slow growers, the latter comprising mostly of the pathogenic mycobacteria. *Mycobacterium tuberculosis* and *M. leprae* are two important human pathogens that cause tuberculosis and leprosy respectively.

1.1.2. Features of mycobacteria

Bacteria of the genus *Mycobacterium* are aerobic, non-motile and non-sporulated rods. Their genome show high G + C content (61-71 %) and their cell wall shows unique features with notably high lipid content in their cell wall. *Mycobacterium* and other closely related genera (i.e. *Corynebacterium, Gordona, Nocardia, Rhodococcus* and *Dietzia*) have similar cell wall compounds and structure, and hence show some phenotypic resemblance. The mycobacterial lipid-rich cell wall can be stained with basic dyes such as carbol fuchsin and cannot be decolourised with acid-alcohol. This unique property is termed "acid-fastness" and is the basis of the Ziehl-Neelsen staining technique for the identification of mycobacteria.

1.1.3. Unique cell envelope of mycobacteria

The cell envelope is composed of three major constituents; the plasma membrane, the cell-wall core, and the extractable, non-covalently linked glycans, lipids and proteins. The structure of the cell envelope is illustrated in Fig.1 (Brennan & Crick, 2007). External to the membrane is peptidoglycan in covalent attachment to arabinogalactan, which in turn is attached to the mycolic acids with their long meromycolate and shorter a-chains. This portion is termed the cell-wall core (the mycolyl arabinogalactan-peptidoglycan complex (MAPc). Very long-chain fatty

acids, the mycolic acids, are covalently bound to the arabinogalactan-peptidoglycan co-polymer and were proposed to form the inner layer of an asymmetric outer membrane while other lipids constitute the outer leaflet (Brennan & Crick, 2007, Brennan & Nikaido, 1995) (Fig. 1). The mycolic acids extend perpendicular to the arabinogalactan / peptidoglycan, and other cell wall-associated glycolipids intercalate into the mycolic acid layer to form a 'pseudo' lipid bilayer. The cell wall of mycobacteria consists of large amount of lipids. The free lipids comprise the extractable material, which include the phthiocerol-containing lipids, the phosphatidylinositol mannosides, lipomannan, lipoarabinomannan, trehalose dimycolate (cord factor), trehalose monomycolate, and the diacyl- and polyacyl-trehaloses presumably intercalating with the a-and meromycolate chains of the mycolic acids (Russell, 2007). When the cell wall is subjected to treatment with various solvents, the free lipids and proteins are solubilised and the MAPc remains as an insoluble residue. Hence it was considered that these lipids, proteins, and lipoglycans are the signaling, effector molecules in the disease process, whereas the insoluble core is essential for the viability of the cell (Deres *et al*., 1989).

1.1.4. Proteome of the cell membrane of *M. tuberculosis*

The cell membrane of *M. tuberculosis* is likely to harbor proteins functioning as enzymes, receptors, transporters or signal transducers that could be of vital importance to the microbe (Nigou *et al*., 2003; Asselineau *et al*., 2002). Bioinformatic analysis of the *M. tuberculosis* genome predicts >65 lipoproteins of 'cell envelope' origin, some of which were identified previously as 'secreted' proteins, or enzymes involved in cell wall biogenesis. There are about 600 'putative' membrane proteins. They harbor a number of transmembrane hydrophobic segments, and include proteins belonging to the major facilitator and ATP-binding cassette (ABC) superfamilies (Tekaia *et al*., 1999). These proteins play a role in the uptake and effects of various metabolites, peptides, drugs and antibiotics. Proteome analysis of the plasma membrane of *M. tuberculosis* by Sinha *et al.* (2002) demonstrates the presence of important proteins such as heat shock proteins four HSPs (Rv2031c, α-crystallin; Rv0251c, HSP20; Rv3417c, groEL1; and Rv0440c, groEL2), the iron storage protein the bacterioferritin (bfrA, Rv1876) and ATP synthase (Rv1308, Rv1309, Rv1310). ESAT-6, a well characterised culture filtrate protein, which belongs to the family of early secretory antigenic target, was found to be expressed in the membrane of *M.*

tuberculosis (Sinha *et al.,* 2005). Along with ESAT-6, some of the components of its secretory machinery such as the products of the genes Rv3870, and Rv3877 (possible conserved transmembrane proteins) are present in the inner membrane of the cell (Abdallah *et al.,* 2007).

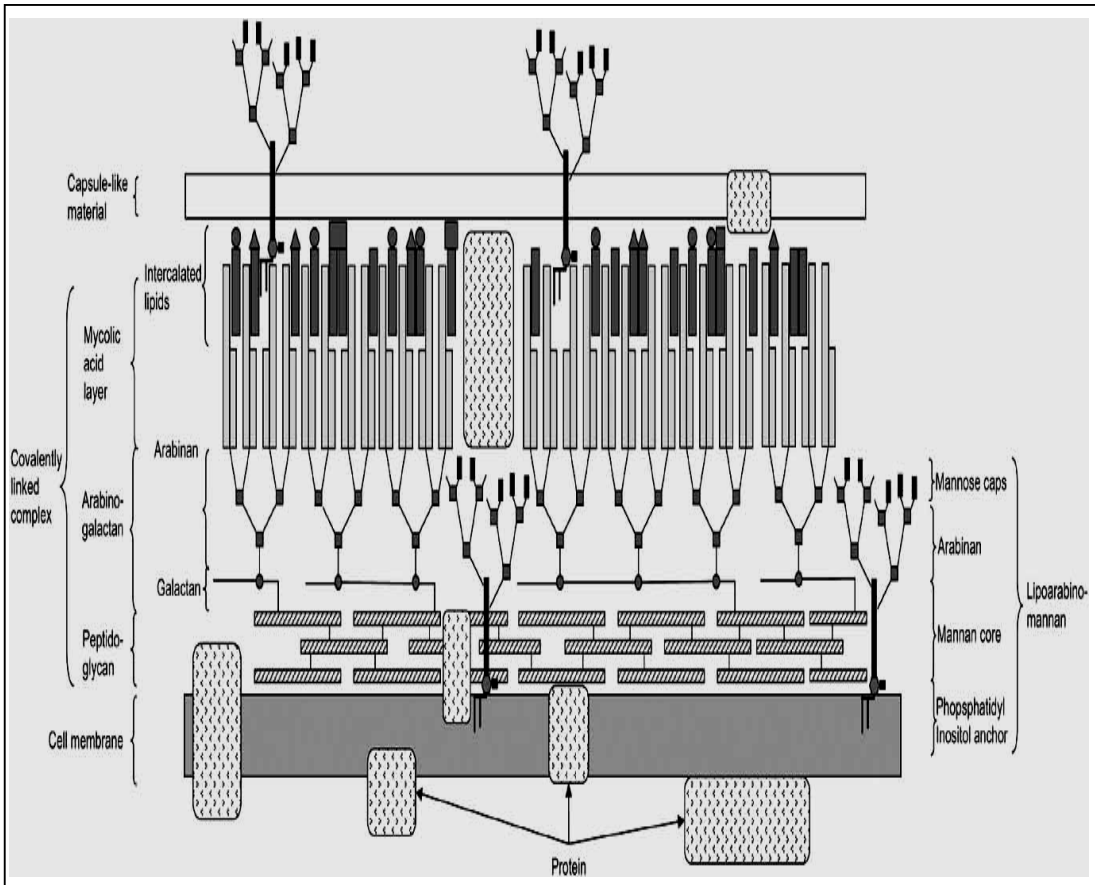


Fig 1. Schematic representation of the cell envelope of *Mycobacterium tuberculosis* (Brennan & Crick, 2007). Peptidoglycan (PG) of *Mycobacterium* spp. consists of a backbone of alternating units of *N*-acetylglucosamine (GlcNAc) and a modified muramic acid (Mur).

1.2. *Mycobacterium tuberculosis* and tuberculosis

1.2.1. Epidemiology of tuberculosis

Tuberculosis is a disease of great public concern as it is a leading cause of death among the infectious diseases. Despite extensive efforts to control the causative organism *M. tuberculosis* since its discovery by Robert Koch in 1882, it still remains an enigma and the disease remains a major health problem worldwide.

Each year, 55 million people become newly infected with the pathogen worldwide (WHO report, 2007 & 2006). Fig. 2 illustrates the estimated tuberculosis incidence rate worldwide. It has been estimated that one-third of the world's population is infected with *M. tuberculosis* and roughly 10% of these individuals will develop active tuberculosis within their lifetime. With the rise in HIV infections, tuberculosis has been on the rise and death due to tuberculosis in HIV infected people increase at least twice that of the person infected only with HIV. This alarming rise led the WHO to declare tuberculosis 'a global emergency' in 1993 (WHO report, 1993). In addition, about one third of human population is estimated to suffer from latent tuberculosis, which can be reactivated even after several decades (Glassroth, 2005). It is estimated that, between 2000 and 2020 nearly one billion people will be newly infected, the active disease will affect 200 million, and 35 million will die of TB, if control measures are not significantly improved (WHO report, 2007).

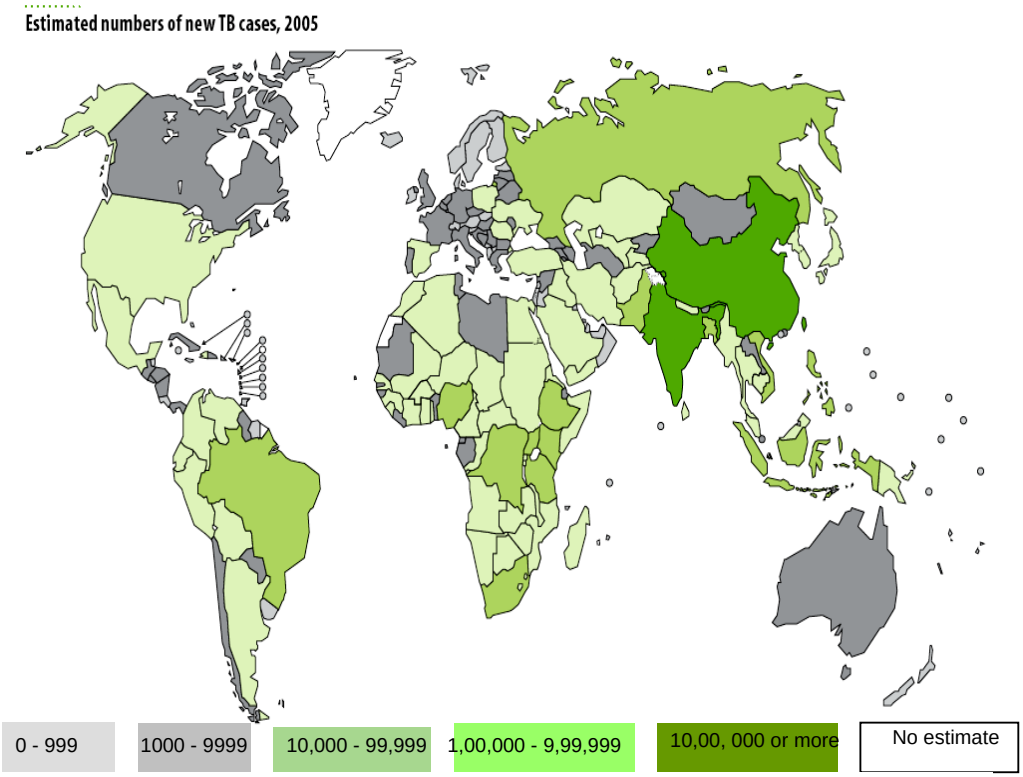


Fig. 2 - Estimated tuberculosis incidence rate. (WHO report, 2007)

1.2.2. Pathogenesis

1.2.2.1. Infection and granuloma formation

The first stage of mycobacterial infection begins with the inhalation of tubercle bacilli as droplets, released into the atmosphere from an infected individual /animal. Alveolar resident macrophages are the primary cells involved in the initial uptake of *M. tuberculosis*. Dendritic cells and monocyte-derived macrophages also take part in the phagocytic process (Henderson *et al.,* 1997). The bacilli are taken up by receptor-mediated phagocytosis using a variety of macrophage receptors including CR3, CR4 and mannose receptors.

The outcome of an infection in the new host depends on the balance between (i) host immune response and effective killing of the invading pathogen (ii) the extent of tissue necrosis, fibrosis, and regeneration (Van Crevel *et al.,* 2002). In the lungs, the bacteria are phagocytosed by alveolar macrophages and induce a localized proinflammatory response that leads to the recruitment of mononuclear cells from neighbouring blood vessels. These cells are the building blocks for the granuloma, or tubercle, which is the signature of tuberculosis. The granuloma consists of a kernel of infected macrophages, surrounded by foamy macrophages and other mononuclear phagocytes, with a mantle of lymphocytes in association with a fibrous cuff of collagen and other extracellular matrix components that delineates the periphery of the structure (Russell, 2007) (Fig. 3). The interactions of the pathogen with the macrophage are dominated by the ability of the pathogen to prevent phago-lysosome biogenesis (Vergne *et al*., 2004, Armstrong & Hart, 1971), by modulating the phagosomal compartment and preventing its fusion with acidic lysosomal compartments and actively excluding vesicular proton ATP-ases, resulting in an elevated pH of 6.3–6.5 (compared to the normal lysosomal pH of 4.5).

The granuloma formation typifies the ‘containment’ phase of the infection in which there are no overt signs of disease and the host does not transmit the infection to others. In the later stages, the granuloma develops a marked fibrous sheath and the number of blood vessels penetrating the structure diminishes markedly. Containment usually fails when the immune status of the host changes, which could be associated with old age, malnutrition or co-infection with HIV; essentially any condition that reduces the number, or impairs the function, of $CD4^+$ T cells. Following such a change in the immune status, the granuloma caseates (decays into a structure-less mass of cellular debris), ruptures and spills thousands of viable, infectious bacilli into

the airways (Fig. 3). This results in the development of a productive cough that facilitates aerosol spread of infectious bacilli. (Russell, 2007).

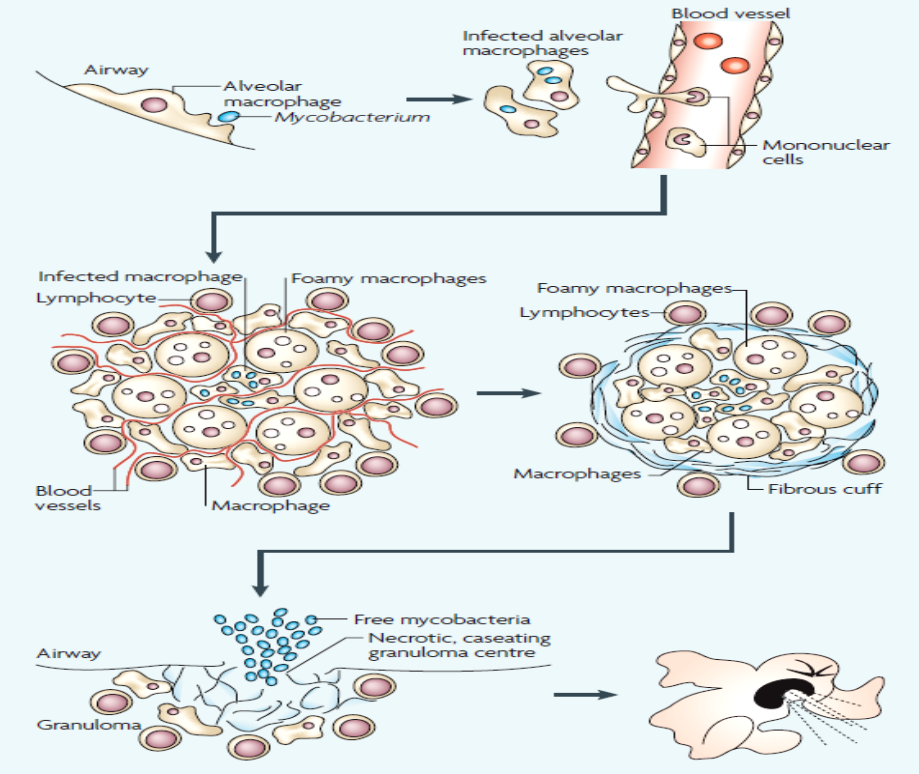


Fig. 3 - Infection with *Mycobacterium tuberculosis* follows a relatively well-defined sequence of events. (Russell, 2007)

1.2.2.2. Immune response and the outcome of infection

As *M. tuberculosis* is an intracellular pathogen, T-cell effector mechanisms play a role in the elimination of the pathogen. The proteins from the pathogen mediate the interactions between the pathogen and the host immune system; they are degraded in the phago-lysosomal compartment and the resulting peptides are complexed with the MHC class II and presented to CD4 cells (Andersen, 1997). To survive in macrophages, *M. tuberculosis* has evolved mechanisms to block immune responses. These include modulation of phagosomes, neutralization of macrophage effector molecules, stimulating the secretion of inhibitory cytokines, and interfering with processing of antigens for T-cells (Cooper & Flynn, 1995). The balance of the host–pathogen interaction in *M. tuberculosis* infection is determined by the interaction of

T-cells and infected macrophages. The outcome of this interaction results either in control of the infection or development of active disease.

The CD4 subset comprises the regulatory Th1 and Th2 cells each producing the regulatory and characteristic panel of cytokines. The Th1 cells are important players in the control of TB infection due to the production of IFN-γ and TNF-α, with both the cytokines playing an important role in macrophage activation. In addition to CD4+ T-cells, other T-cell subsets such as, γβ, CD8+ and CD1-restricted T-cells have roles in the immune response to *M. tuberculosis* (Schaible & Kaufmann, 2000). A diverse T-cell response allows the host to recognize a wider range of mycobacterial antigens presented by different families of antigen-presenting molecules, and thus greater ability to detect the pathogen. The slow growth and chronic nature of *M. tuberculosis* infection results in prolonged exposure to a large diversity of antigens (Boom *et al.,* 2003). This is likely an important factor in generating this T-cell diversity. CD8+ cells are also primed in response to TB infection but the pathway leading to MHC class I restricted antigen presentation is still not clear.

$CD4^+$ T cells recognize mycobacterial peptide fragments presented to them by MHC class II molecules on antigen-presenting cells such as macrophages. The loss of CD4+ T cell number and function results in progressive primary infection, reactivation of endogenous *M. tuberculosis* and enhanced susceptibility to re-infection (Branes *et al.,* 1991). Mice with deleted genes for CD4+ or MHC class II molecules are susceptible to *M. tuberculosis*, firmly establishing a central role for $CD4^+$ T cells in protection (Caruso *et al.,* 1999). A number of proteins, recognized by a majority of healthy tuberculin skin-test positive persons have been identified; they include the three 30–32 kDa 85 complex proteins, ESAT-6 and CFP-10, the 19 and 38 kDa lipoproteins. Recognition of the mycobacterial antigens by phagocytic cells results in a complex process of regulation and cross-regulation by the different cytokines. This cytokine network plays a crucial role in the inflammatory and the outcome of mycobacterial infections (Fig. 4) (Fenton & Vermeulen, 1996). Some of the pro-inflammatory cytokines are TNF-α, IL-1β, IL-6, IL-12, IFN-γ, IL-18 and IL-15. The anti-inflammatory cytokines are IL-10, TGF-β and IL-4 (Flynn & Chan, 2001).

IFN-γ is a key cytokine in control of *M. tuberculosis* infection (Cooper *et al.,* 1993). Mycobacterial antigen specific IFN-γ production *in vitro* could be used as marker of infection with *M. tuberculosis.* IFN-γ is produced by healthy subjects as well as those with active tuberculosis (Sahiratmadja *et al.,* 2007). Though IFN-γ

production varies among subjects, some studies suggest that IFN-γ levels are depressed in patients with active tuberculosis (Zhang *et al.,* 1995 & Lin *et al.,* 1996). IFN-γ has also been shown to influence the cellular iron status of the macrophage (Boelaert *et al.,* 2007). IFN-γ activation of human monocytes down-regulates transferrin receptors on the cell surface (Byrd & Horwitz, 1989) and the rate of macrophage iron acquisition from holo transferrin (Olakanmi *et al.,* 2002), thereby decreasing the availability of iron to the intracellular pathogen.

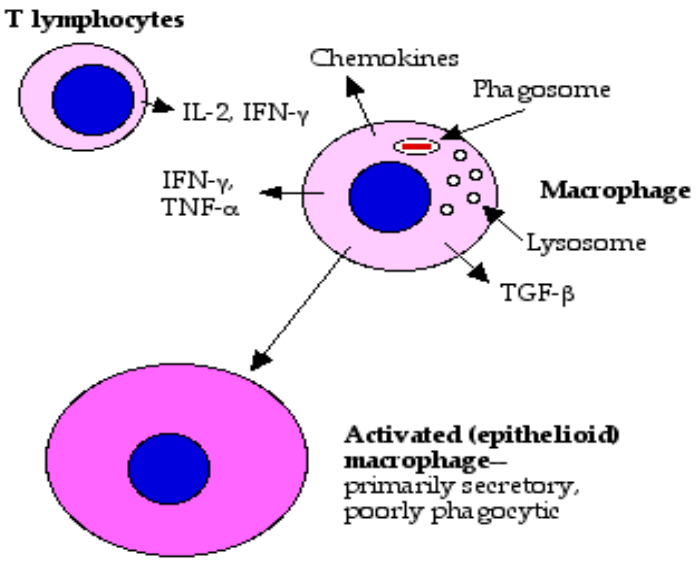


Fig. 4 - Activation of macrophages by cytokines. (Fenton & Vermeulen, 1996). After phagocytosis of tubercle bacilli, macrophages secrete chemokines that attract leukocytes. Interaction with lymphocytes stimulates macrophages to produce IFN-γ and TNF-α, thereby activating $CD4^+$ T lymphocytes. These lymphocytes produce cytokines, IL-2 and IFN-γ, which activate macrophages and other lymphocytes. TGF-β tends to dampen the immune responses.

1.2.3. Control measures

Control measures for tuberculosis include timely diagnosis and chemotherapy. Tuberculosis can be cured in 95% of patients with active, drug sensitive pulmonary TB (Spigelman & Gillepsie, 2006). Multi-drug therapy with a combination of three frontline drugs, isoniazid, rifampin and pyrazinamide and one or more of the second-tier antibiotics including ethambutol, ethionamide, *p*-aminosalicylic acid, D-cycloserine, streptomycin, capreomycin, kanamycin and thiacetazone considerably

reduced the incidence of TB, especially in developed countries. Nevertheless, this decline has been reversed in the past two decades due to several factors including poverty, overcrowding, travel and the synergy between HIV and TB (Corbett *et al.*, 2003). WHO initiated directly observed therapy short-course (DOTS), which is currently being adopted by 119 countries including all 22 high burden countries that contain 80% of all estimated cases (Collins & Kaufmann, 2001). Despite the efforts like the DOTS, numbers of drug resistant cases are on the rise, thereby necessitating newer and better drugs (Reece & Kaufmann, 2008).

Existing diagnostic methods can detect up to 60% of tuberculosis cases but tuberculosis management in developing countries is difficult as the existing diagnostic methods are non-specific and time-consuming. The present day diagnosis for human tuberculosis include clinical examination followed by radiological and laboratory testing comprising of chest X-ray, skin testing by Mantoux test, AFB testing of sputum smears and biopsies followed by culture confirmation. The latter is the gold standard but it is time consuming. Many patients in endemic areas are never diagnosed and in several cases, death due to disease progression results (Brodie & Schulger, 2005). Modern diagnostic methods like PCR, ELISA, and more rapid ways to detect the positive cultures are being developed.

Preventive measures like vaccination and early diagnosis will help in the control of this disease. Vaccination with *M. bovis* BCG (see section 1.2.4 below), however has not been promising. The variable efficacy of the BCG vaccine and the genetic heterogeneity between strains demands that a better vaccine be developed for the prevention of this disease (Agger & Andersen, 2001).

1.2.4. Vaccines: BCG as a vaccine

The BCG vaccine, derived from the virulent *M. bovis* is the only vaccine available for tuberculosis. The efficacy of this vaccine is however controversial. It is given to infants soon after birth in countries where tuberculosis is endemic. The efficacy of this vaccine in preventing adult pulmonary tuberculosis is low, as concluded from the extensive 10-year follow-up trial by Tuberculosis Research Centre (ICMR), Chennai of BCG vaccination in Chingleput (Tamil Nadu, India) involving 360,000 individuals (Tuberculosis Research Centre, 1999). Several researchers are making efforts to increasing the efficacy of BCG, by the cloning of specific genes into BCG (Olsen & Andersen, 2003).

1.2.5. Diagnosis of tuberculosis

1.2.5.1 AFB staining

AFB smear microscopy plays an important role in the early diagnosis of mycobacterial infections because culture results become available only after 4-6 weeks. Over 90% of the tuberculosis cases occur in the developing countries, where clinical diagnosis of tuberculosis is based primarily on microscopic examination of smears for acid-fast bacilli and radiological testing. Table 1 shows the recommended grading of the smear testing.

Table 1: Quantitation scale recommended by the WHO and the International Union against Tuberculosis and Lung Disease

Count	Report
0	No AFB observed
1-9/100 fields	Exact count
10-99/100 fields	1+
1-10/field	2+
> 10/field	3+

A high bacterial load is needed in the specimen to render an AFB microscopy result positive. Smear-positive cases constitute only about 50% of pulmonary tuberculosis cases and the sensitivity of the test ranges from 22 to 78% of culture-proven cases in different studies (Kim *et al.,* 2008). In addition, it is non-specific and does not confirm the disease diagnosis because of the detection of environmental mycobacteria other than *M. tuberculosis.*

1.2.5.2. Culture

Culture confirmation is the "gold standard" for tuberculosis diagnosis as it is specific and sensitive. However, this is time-consuming as *M. tuberculosis* has a long generation time; growth and subsequent biochemical analysis for species identification requires up to 6–8 weeks. Culture techniques, using conventional egg-

based Lowenstein Jensen media have been estimated to detect as many as 10–1,000 viable mycobacteria per mL of specimen. Middlebrook media are also used extensively for culture in several labs.

The BACTEC TB-460 system was the first semi-automated system used. This is based on the liquid media for mycobacteria culturing and allows for culture confirmation within 7-10 days. It is however expensive and is not economical for screening all samples. Other commercial systems include the Mycobacteria Growth Indicator Tube (MGIT), Bact / Alert, ESP Mice, MB Redox and KRD "Niche B", biphasic Septic-Check AFB and Mice-Acid, and BACTEC MGIT960 systems.

1.2.5.3. Mantoux test-tuberculin skin testing

Tuberculin skin testing (TST), first introduced in 1890 is the oldest diagnostic test in use (Lee *et al.,* 2002, Huebner *et al.,* 1993). 0.1 mL of 5 TU (tuberculin unit) purified protein derivative (PPD) is injected intra-dermally on the forearm and the induration is measured after 48-72 hours. A positive reaction is indicated by erythema and induration of > 10 mm in size.

The TST attempts to measure cell-mediated immunity in the form of a delayed-type hypersensitivity response to PPD, which is an unfractionated mixture of the culture filtrate proteins from the spent growth medium of *M. tuberculosis*. Infection with *M. tuberculosis* results in a cell mediated immune response giving rise to sensitized T lymphocytes (both CD4+ and CD8+) targeted to *M. tuberculosis* antigens. Stimulation by *M. tuberculosis* antigens causes these T-cells to release IFN-γ. The TST functions by eliciting this response in previously sensitized individuals. In such individuals, an intra-dermal injection of PPD evokes a delayed-type hypersensitivity response mediated by sensitized T-cells and results in cutaneous induration. The amount of PPD administered and the erythema produced varies from endemic region to a non-endemic region.

The antigens in PPD are shared among *M. tuberculosis*, *M. bovis* BCG, and several non-tuberculosis mycobacteria (NTM). As a result, the TST has lower specificity in populations with high BCG coverage and NTM exposure. The sensitivity may be low in individuals with depressed immunity (eg, AIDS and other immunosuppressive conditions, advanced tuberculosis, malnutrition). Despite these limitations, the test is still widely used because of its ability to predict active disease

in latently infected individuals. A major advantage of this test is its low material cost and it does not require any laboratory infrastructure.

1.2.5.4. Molecular methods

Using polymerase chain reaction, amplification of the insertion element IS*6110* was first developed as an important molecular tool for the diagnosis of tuberculosis (Eisanech, 1994). This was fairly successful in developed countries, where the IS*6110* element was present in multiple copies (from four to 20) in more than 95% of *M. tuberculosis* strains (Coros *et al.,* 2008). However in India, this has not been successful as many of the clinical isolates either harbor single copy of *IS6110* gene or it is absent (Krishnan *et al.,* 2007). Other successfully used DNA regions include the 65 kDa *hsp* gene, the gene encoding the 126 kDa fusion protein, and the gene encoding the β-subunit of ribonucleic acid (RNA) polymerase; all of them are present in single copies in *M. tuberculosis* complex genomes (Baba *et al.,* 2008, Negi *et al.,* 2007).

The two commercially available molecular techniques approved by U.S. FDA are the Amplified *Mycobacterium tuberculosis* direct test (MTD test; Gen-Probe, San Diego, CA, USA) and the Cobas Amplicor *M. tuberculosis* assay (Roche Diagnostics, Mannheim, Germany). They show a sensitivity >95% and 100% specificity when sputum positive samples were used. The sensitivity was lower (83–85%) when the test was used for testing smear-negative specimens, though the specificity was high (99%) (Reischl *et al.,* 1998). The use of radiometric systems in conjunction with nucleic acid probes has considerably reduced the detection time, as these procedures require a minimum of 1 week before a definitive laboratory diagnosis can be made. However, these techniques are too expensive and technologically complex for widespread application in laboratories in developing countries.

1.2.5.5. Tests based on secretory proteins

Antibody based tests

Assays based on the detection of antibody response to tuberculosis are alternatives to current methods for diagnosing active tuberculosis because they are simple, rapid, inexpensive and relatively non-invasive (Gennaro, 2000). Conventional immunoassays such as ELISA and Western blot employing CFPs have limits for

widespread diagnostic use. Immuno-chromatographic assays, also called lateral-flow tests (or strip tests), which are extension of latex agglutination tests use secretory proteins of *M. tuberculosis* as antigens to detect antibodies in tuberculosis infected individuals. The benefits of immuno-chromatographic tests include, they are less time consuming, have long-term stability over a wide range of climates and are relatively inexpensive to make, so that they can be used in developing countries (Houghton *et al.,* 2002). A rapid immunochromatographic assay, the VetTB STAT-PAK test, was recently developed by Chembio Diagnostic Systems, Inc., Medford, New York, to detect antibodies of three isotypes (IgM, IgG, and IgA) against mycobacterial antigens in various host species. The test employs a unique cocktail of *M. tuberculosis* or *M. bovis* antigens and a blue latex bead-based signal detection system. The ready-to-use disposable device consists of a plastic cassette containing a strip of nitrocellulose membrane impregnated with test antigen (Waters *et al.,* 2006). The important antigens employed in these assays include the 38 kDa protein (Bartoloni *et al.,* 2003), ESAT-6/CFP-10 complex and MPB 83 antigens (Lyashchenko *et al.,* 2007).

Cell mediated immune response assays

Different types of blood tests based on cell-mediated immune response have been suggested for diagnosis of tuberculosis. Most *M. tuberculosis* infected individuals mount a relatively strong $CD4^+$ T-cell response to a broad spectrum of *M. tuberculosis* antigens. Assays were developed based on $CD4^+$ T-cell response for use in diagnosis. Such assays were performed using the whole blood, purified PBMCs and stimulated *in vitro* with PPD, culture filtrate protein or defined *M. tuberculosis* antigens (Andersen *et al.,* 2000). The responses are monitored by measurement of the stimulation indices and / or production of IFN-γ. The important CFPs explored in T-cell responses include the Mtb 8.4, Mtb 9.8 (coller *et al.,* 1998), ESAT-6, CFP-10, (Arend *et al.,* 2000) Ag85 B (Nagai *et al.,* 1991) and MPT-64 (Johnson *et al.,* 1999). The ELISPOT assay for diagnosis of *M. tuberculosis* infection is based on the rapid detection of T cells specific for *M. tuberculosis* antigens. IFN-γ released *ex vivo* from these cells can be detected by ELISPOT (Lalvani *et al.*, 1998) (see section 1.3.6.1).

1.3. Culture filtrate proteins (CFPs)

1.3.1. Excretory - secretory antigens

Culture filtrate proteins (CFPs) are proteins released into the immediate environment by growing mycobacteria. Andersen and his group have done extensive work and divided CFPs of mycobacteria into three groups based on the antigens that are released (Andersen *et al.,* 1991). They are the secretory proteins, the excretory proteins and the cytoplasmic antigens. The excreted proteins are produced in large quantities into the medium by *M. tuberculosis* during the first few days of the culture. They accumulate in large quantities in the media and are present in minor quantities in intact bacilli. The secreted proteins are from the outer cell wall and they are gradually released during the growth of the bacilli. Short-term culture filtrate (ST-CF) is enriched in secreted antigens from *M. tuberculosis* (Andersen *et al.,* 1991). The concentration of the antigens increases steadily during the culture period. Cytoplasmic antigens are released from dead bacteria and they appear in high concentrations during the late logarithmic phase of the bacterial growth. The appearance of these proteins is related to the release of iso-citrate dehydrogenase (ICD), which is the indicator of autolysis.

1.3.2. Secretory proteome of *M. tuberculosis*

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) of the CFPs of *M. tuberculosis* followed by the identification of the separated proteins by protein sequencing has led to the creation of 2D-PAGE mycobacterial databases such as the Berlin database and Copenhagen database both displaying 2D images of both cellular and culture filtrate proteins. Table 2 lists the important CFPs identified in 2D-PAGE and their role.

Table 2 – Secretory proteome of *M. tuberculosis* **(**modified from Okkels *et al.,* 2003)

Antigen	Theoretical Mass (kDa)	Role
ESAT-6 *	9.9	DTH
Ag 85A	35.7	Fibronectin binding surface protein
Ag 85B	34.6	Mycolyl-transferase surface protein
MPT 51	27	Diagnosis
45-47 complex	45-47	DTH

MPT 32	32	Diagnosis
MPT 64	24.8	Vaccine
CFP 10*	10.8	Dimer with ESAT-6
TB 10.4*	10.4	DTH
Lipoprotein	38	Diagnosis
TB 9.8*	9.8	Diagnosis
Mtb 8.4*	10.9	Unknown
CFP 6*	12.2	Unknown
PstS-1	38.2	Phosphate uptake Surface protein
SOD	23	Removal of free radicals
MPT 63	16.5	Unknown
Mtb 41	41.4	Unknown

* indicates proteins unique to *Mtb* complex, while others are also seen in other mycobacteria.

1.3.3. CFPs as vaccine candidates

CFPs prepared from *M. tuberculosis* have been shown to be highly stimulatory to T cells of human tuberculosis patients (Demisse *et al*., 1999), mice (Olsen & Andersen, 2003) and cattle (Wedlock *et al.,* 2005). Immunization of mice and guinea pigs with CFP from *M. tuberculosis* gave high levels of protection against aerogenic challenge with *M. tuberculosis* (McMurry *et al*., 2005). The potential of the CFPs led to their separation and characterisation, especially the short term culture filtrate (ST-CF) proteins (Okkels *et al*., 2003, Skjot *et al.,* 2000). For eg., two narrow molecular mass fractions of ST-CF, containing molecules of low mass (<14 kDa) and medium mass (26–34 kDa) were particularly strongly recognized by cells from infected mice and patients with minimal tuberculosis (Boesen *et al*., 1995, Andersen *et al*., 1992). The immuno-dominant antigens in these pools were identified as ESAT-6 and the Antigen 85 complex respectively. The vaccine potential of these secretory antigens is studied either singly or as antigen pools (Dietrich *et al.,* 2006).

Horwitz and coworkers (Horwitz *et al.,* 1995) had proposed the following three hypotheses regarding the role of extracellular proteins of intracellular pathogens in protective immunity and the potential for their use in a subunit vaccine.

(i) Extracellular proteins play a key role in inducing cell-mediated immune responses that provide immuno-protection against these pathogens during natural infection.

Such proteins, by virtue of their release by live organisms into their intracellular compartment in the host cell, are available for proteolytic processing and subsequent presentation on the surface of the infected host cell as MHC-bound peptide fragments. These surface-exposed fragments would allow the host immune system to recognize live pathogens sequestered within a host cell and to exert an antimicrobial effect against them. In particular, T-cells may activate the host macrophage, allowing it to inhibit multiplication of intracellular organisms, or they may lyse the host cell, thereby denying the pathogens an intracellular milieu in which to multiply.

(ii) Immunization of a naive host with extracellular proteins of intracellular pathogens-particularly in the case of pathogens such as *L. pneumophila* and *M. tuberculosis*, which reside within a phagosome rather than free in the cytoplasm in host cells would induce a population of lymphocytes capable of later recognizing and exerting an immune response against infected host cells. These lymphocytes would recognize infected host cells by identifying MHC-bound fragments of extracellular proteins displayed on the host cell surface consequent to the release of the proteins by the intracellular pathogen.

(iii) Among the extracellular proteins of intracellular pathogens, the ones released in greatest abundance will be among the most effective in inducing immuno protection. Such proteins, due to their abundance in the phagosome, would be processed and presented most frequently and therefore induce a particularly strong cell-mediated immune response.

1.3.4. Mechanism of secretion of mycobacterial CFPs

Bacterial protein secretion involves either of the two types of signal peptide-dependent translocations: the essential Sec-dependent pathway (Pugsley, 1993) and the Sec-independent pathway (Berks *et al.,* 2000). The Sec Pathway is evolutionarily conserved and is thus a primary pathway for protein transport in most bacteria, including mycobacteria. Secreted proteins are produced as precursors containing signal sequences. Proteins secreted via the Sec pathway are accompanied to the membrane by SecB, a chaperone molecule. SecA, an ATPase required for translocation at the multisubunit translocase SecY-SecE-SecG, is found associated with both the signal sequence of secretable proteins, and also with the membrane at SecE-SecY-SecG. During translocation ATP is hydrolyzed, the protein is inserted across the membrane, the signal sequence is cleaved, and the protein is released.

Mycobacterium tuberculosis has a functional Sec pathway. The genome sequence includes homologues of all Sec pathway components with the exception of SecB a protein not found in all Sec systems (Braunstein & Belisle, 2000). There are exported or secreted proteins synthesized with consensus Sec signal sequences (Lim *et al.,* 1995). *M. tuberculosis* contains two homologues of SecA, namely the SecA1 and the secA2 (Fig. 5) (Braunstein & Belisle, 2000). *SecA1* is a house-keeping gene and *SecA2* is a non- essential gene. SodA of *M. tuberculosis* is secreted by the SecA2 mechanism (Braunstein *et al.,* 2003).

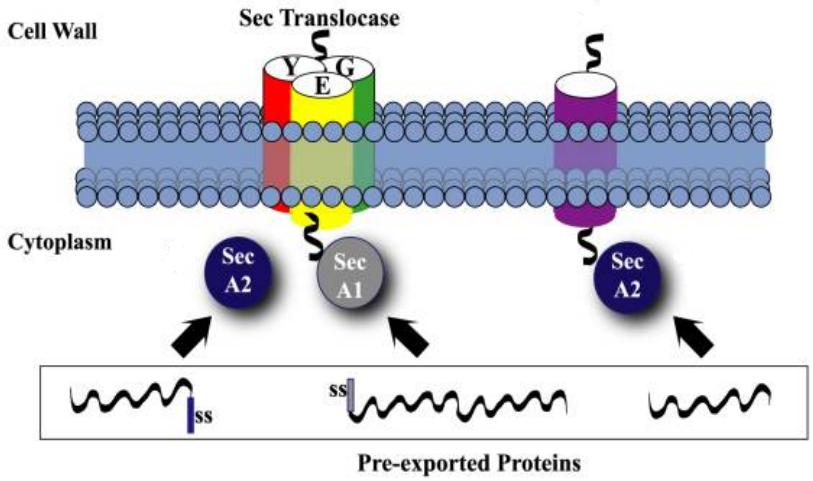


Fig. 5. SecA2-dependent export pathway in mycobacteria (Braunstein & Belisle, 2000). The SecA protein plays a central role in this export pathway.

Sec-independent system was named TAT for twin arginine translocation because the precursor proteins engaged in this mechanism of export contain a conserved motif, S/T-R-R-X-F-L-K, with two contiguous arginine residues near the N terminus of the leader peptide. It was shown that in certain organisms, the TAT system was responsible for the export of a wide variety of substrates and, in particular, virulence factors (Dilks *et al.,* 2003). The Tat pathway of *E. coli* includes at least four components: TatA, TatB, and TatE, predicted to be anchored to the cytoplasmic membrane via an N-terminal hydrophobic alpha-helix, and TatC, with six predicted transmembrane helices. In some prokaryotes such as *Bacillus subtilis*, only

tatA and *tatC* are present, suggesting that the minimal translocation system comprises TatA and TatC. The genome sequences of *M. tuberculosis* and *Mycobacterium leprae* revealed that both mycobacteria contained clearly identifiable *tatA*, *tatB*, *tatC*, and *tatD* genes which form functional Tat system. However, the *tatABCD* gene cluster identified in *E. coli* was not found in *M. tuberculosis*. It resembles *B. subtilis* by having a *tatAC* operon, with *tatB* and *tatD* located elsewhere (Saint-Joanis *et al.,* 2006). *Mycobacterium tuberculosis* genes encoding homologues of TatA (Rv 2094c), TatC (Rv 2093c) and TatB (Rv 1224) are present in the genome. Substrates for the TAT pathway are synthesized as precursor proteins containing N-terminal signal sequences (Braunstein & Belisle, 2000) (Fig. 6). Phospholipase C (Rv2439c), localized in the cell wall is among the several substrates for TAT system (Raynaud *et al*., 2002).

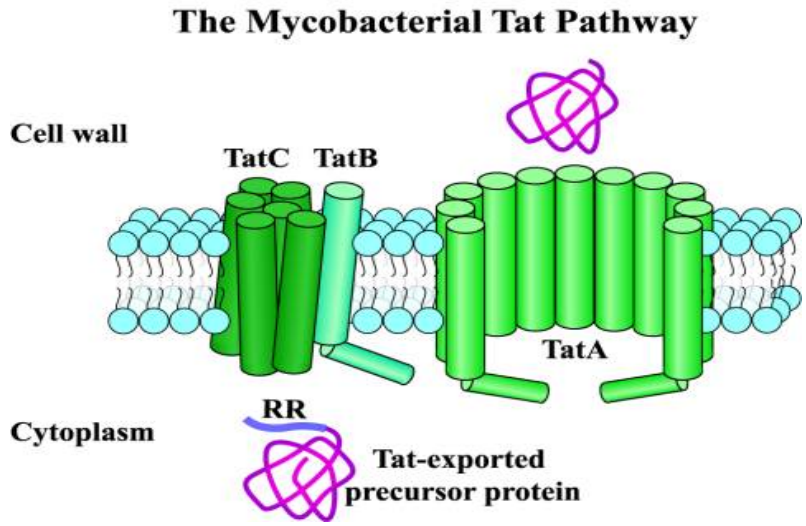


Fig. 6 - The mycobacterial Tat pathway (Braunstein & Belisle, 2000). Tat pathway operates independently of the Sec pathway and is distinguished by the ability to translocate proteins in a folded state. Substrates of the Tat pathway are synthesized as precursor proteins containing N-terminal signal sequences.

1.3.5. CFPs of vaccine/diagnostic potential

1.3.5.1. Heat shock proteins and Ag85 complex

The heat shock proteins, Hsp10, Hsp60 and Hsp70 are strongly immunogenic and serve as important T cell antigens (Young & Garbe, 1991). The proteins of the antigen 85 complex (85A, 85B, and 85C) represent a major part of the secreted

proteins of *M. tuberculosis* and are most well studied (Wiker & Harboe, 1992). The three proteins in this complex are encoded by three genes located at different sites in the mycobacterial genome and show extensive cross-reactivity as well as homology at amino acid and gene levels (Wiker *et al.,* 1990). The antigen 85 complex is the major secreted protein constituent of mycobacterial culture fluids, but it is also found in association with the bacterial surface. The antigens in this complex have been demonstrated to be associated with different functions like fibronectin binding and mycolyl transferase activity (Abou-Zeid *et al*., 1991, Ronning *et al.,* 2000).

1.3.5.2. ESAT family

ESAT-6 and CFP-10 are two major targets of the immune response in infected individuals (McLaughlin *et al*., 2007). Now, it is known that several proteins sequence similarities are present with the identification of 23 ESAT-6 family members in *M. tuberculosis* strain H37Rv, which are located in 11 genomic loci and 25 in strain CDC1551, and their genes have been given genetic names *esxA-W*, with *esxA* encoding ESAT-6 and *esxB* encoding CFP-10.

The ESAT-6 gene is seen in the members of the *M. tuberculosis* complex (Harboe *et al.,* 1996) and is absent in *M. bovis* BCG strains. The latter, by virtue of the loss of RD1 region encompassing the *esat-6 gene* does not elaborate the protein (Mahairas *et al.,* 1996). The ESAT-6 and CFP-10 have been shown to form a 1:1 hetero-dimeric complex and adopt a stable fully folded structure, which is biologically active (Renshaw *et al.* 2002).

As ESAT-6 expression is restricted to the *M. tuberculosis* complex and not the vaccine strain *M. bovis* BCG and the majority of environmental mycobacteria, it is used as a diagnostic marker to differentiate between the BCG vaccinated healthy individuals and individuals with tuberculosis (Andersen *et al*., 2000). ESAT-6 stimulates T-cells from tuberculosis patients to proliferate and produce IFN-γ. The antigenicity of ESAT-6-CFP-10 has been utilized to develop diagnostic T-cell assays such as the skin test (Weldingh & Andersen, 2008). The combination of ESAT-6 and CFP-10 was found to be highly sensitive and specific for both *in vivo* and *in vitro* diagnosis. In humans, the combination had a high sensitivity (73%) and a much higher specificity (93%) than PPD (7%) (Van Pinxteren *et al.,* 2000).

Mori *et al.* (2004) have demonstrated the use of two of these antigens, CFP-10 and ESAT-6, in a whole blood IFN-γ assay as a diagnostic test for tuberculosis in

BCG-vaccinated individuals. The results demonstrate that the whole blood IFN-γ assay using CFP-10 and ESAT-6 was highly specific and sensitive for *M. tuberculosis* infection and was unaffected by BCG vaccination status.

Humoral response to ESAT-6 has been demonstrated to have a diagnostic potential in tuberculosis patients, cattle and non-human primates. The data by Kanaujia *et al*. (2004) have demonstrated that use of synthetic peptides in lieu of the full-length ESAT-6 protein in diagnostic antibody detection assays.

1.3.6. Commercial tests based on CFPs

1.3.6.1. Interferon-gamma (IFN-γ) tests

One of the current tests includes the assay of interferon-gamma (IFN-γ) released by blood cells in response to mycobacterial antigens. The first generation assay utilises PPD as antigen to stimulate the lymphocytes for IFN- γ production. The second generation assay uses *M. tuberculosis* specific antigens ESAT-6 and CFP-10. The third generation is also similar to second one with the inclusion of TB7.7 (Rv2654) to the earlier antigens. IFN-γ assays are used as an alternative to the TST in the form of a new type of *in-vitro* T-cell-based assay (Ewer *et al.,* 2003). They are based on the principle that T-cells of individuals sensitised with tuberculosis antigens produce IFN-γ when they re-encounter mycobacterial antigens. IFN-γ assays are useful in various applications, such as diagnosis of active tuberculosis, distinguishing between non-tuberculous mycobacterial and *M. tuberculosis* infection, differentiating between *M. tuberculosis* infection and previous BCG vaccination (Pai *et al.,* 2004). IFN- γ assay serves to assess vaccine efficacy, prediction of reactivation of the disease and monitoring treatment response (Barnes *et al.,* 2004).

The commercial IFN-γ assay kits include the QuantiFERON-TB assay (Cellestis Limited, Carnegie, Victoria, Australia) and the T SPOT-TB assay (Oxford Immunotec, Oxford, UK). Both tests measure cell-mediated immunity by measuring IFN-γ released from T cells in response to tuberculosis antigens, using methods such as ELISA and enzyme-linked immunospot (ELISPOT) assay (Pai, 2005). The first generation QuantiFERON-TB is a whole-blood assay that measures IFN-γ response to PPD with ELISA. This test is approved by the US Food and Drug Administration (FDA), and is commercially available in many countries. The enhanced QuantiFERON-TB Gold assay utilises ESAT-6 and CFP-10. The ELISPOT assay for diagnosis of *M. tuberculosis* infection is based on the rapid detection of T cells

specific for *M. tuberculosis* antigens. IFN-γ released *ex vivo* from these cells can be detected by the sensitive ELISPOT (Lalvani *et al.,* 1998). Each such T cell gives rise to a dark spot and the read-out is the number of spots. The T cells enumerated by the ELISPOT assay are effector cells that have recently encountered antigen *in vivo* and can rapidly release IFN-γ when re exposed to the antigen (Kaech *et al.,* 2002). The T-SPOT-TB assay employs ESAT-6 and CFP-10 as antigens and detects (by use of ELISPOT) the number of T-cells producing IFN-γ. Studies that used antigen cocktails with ESAT-6 and CFP-10 yielded sensitivity estimates higher than PPD-based assays (Brock *et al.,* 2004). The best combination of sensitivity and specificity was seen in studies with cocktails of RD1 antigens (QuantiFERON TB Gold and T SPOT-TB assays). Studies among individuals with suspected latent tuberculosis show that IFN-γ assays detect about 80% of this population (Adetifa *et al*., 2007, Brock *et al.,* 2004,). IFN-γ assays were less affected by BCG vaccination than is the TST. Lalvani *et al.* (2001) studied the correlation between ELISPOT (using PPD and ESAT6) and BCG status in a contact investigation study and showed that the ESAT-6 based ELISPOT showed no correlation with BCG vaccination status whereas TST results were positive in BCG-vaccinated contacts.

1.3.6.2. Tests based on the detection of antibodies against CFPs

A 38 kDa protein antigen was demonstrated in the majority of the sera of the pulmonary tuberculosis individuals but absent in the normal controls (Espitia *et al.* 1989). This antigen is also included as a component in three different commercial immunochromatographic test kits (ICT Tuberculosis AMRAD-ICT (Amrad, Sydney, Australia), RAPID test TB, and PATHOZYME-MYCO), which detect TB in from 25 to 64% of patients with smear-negative and smear-positive TB (Perkins *et al*., 2003). However the 38 kDa antigen was poorly recognized in by HIV- infected Tb patients. Weldingh *et al*., (2005) identified immuno-dominant serological antigens using 35 recombinant antigens. TB9.7, TB15.3, TB16.3, and TB51 are four potential candidate antigens, which were recognized with recognition frequencies ranging from 31 to 93% with a specificity of 97%.

1.4. Iron and mycobacteria

1.4.1. Role of iron in bacteria

Iron is essential as a co-factor for many enzymes involved in vital cellular functions ranging from respiration to DNA replication (Sritharan, 2000). It exists in two oxidation states, ferric Fe^{3+} and ferrous Fe^{2+} forms, with the oxidation reduction potential for the Fe^{2+} / Fe^{3+}couple varying between +300 mV to –500 mV, which enables it to serve as a carrier molecule in the electron transport chain. Iron is biologically unavailable due to the inherent insolubility of the ferric iron. Though it was earlier thought that iron existed as insoluble $Fe(OH)_3$, it has been shown that it exists as $Fe(OH)_2^+$ at pH 7, with a solubility of approximately $1x10^{-09}$ M (Chipperfield & Ratledge, 2000). At physiological pH free (Fe^{3+}) is limited to 10^{-18} M, whereas virtually all-living microorganisms require a minimum effective concentration of 10^{-8} M for growth. In mammals, 99.9% of iron is held as protein bound iron. Free iron is not readily available in the host as it is bound to high affinity iron binding proteins like the lactoferrin, transferrin and ferritin. The host limits the availability of iron as part of its innate defense mechanism, which is called nutritional immunity (Kohan, 1976).

1.4.2. Adaptation of bacteria to conditions of iron limitation

Bacteria have adapted to conditions of iron limitation by the elaboration of novel iron acquisition machinery (Ratledge, 2004; Sritharan, 2000). They elaborate two types of iron acquisition machinery, namely

1. Direct removal of the protein-bound iron by specific transferrin and lactoferrin receptors as seen in *Neisseria* (Genco & Desai, 1996).
2. Siderophore-mediated uptake: Siderophores are low molecular weight ligands with a high specificity for ferric iron. The ferric siderophores are then taken up by a receptor-mediated membrane bound protein.

1.4.3. Iron acquisition in mycobacteria

Iron is an obligate cofactor for at least 40 different enzymes encoded in the *Mycobacterium tuberculosis* genome (Cole *et al.,* 1998). Mycobacteria are unique in that they produce two kinds of siderophores namely the intracellular mycobactins and the extracellular carboxymycobactins / exochelins. Two forms of mycobactin are produced, which differ in the length of an alkyl substitution and in polarity and

solubility. The more polar form (carboxymycobactin) is released into the medium, whereas the less polar form (mycobactin) remains cell-associated (Ratledge, 1999). Carboxymycobactin has the capacity to remove iron that is bound to iron-binding proteins of the host (Gobin & Horwitz, 1996). Non-virulent mycobacteria such as *M. smegmatis* produce mycobactin as the intracellular siderophore and a peptidic siderophore known as exochelin (Ratledge & Ewing, 1996). The essential nature of siderophores for iron acquisition was demonstrated by the generation of an *mbtB* mutant *M. tuberculosis* strain, which is unable to produce either carboxymycobactin or mycobactin. This mutation impairs the ability of *M. tuberculosis* to replicate in low-iron media and in infected macrophages (De Voss *et al.,* 2000). Based upon the type of siderophore(s) expressed, mycobacteria can be classified into four groups, namely

1) Those producing mycobactin and both the types of extracellular siderophores such as *M. smegmatis*.
2) The strains producing mycobactin and carboxymycobactin, such as *M. tuberculosis.*
3) Those producing only the exochelins and no mycobactin, e.g. *M. vaccae.*
4) Those that not produce any siderophores e.g. *M. paratuberculosis*.

1.4.3.1. Mycobactins

These are found in the cell wall of mycobacteria and are extracted using organic solvents. Ferric ion in the mycobactin is reduced to ferrous ion and thus release of iron by mycobactin reductase in the presence of NAD(P)H. Ferrous ion has little affinity towards the mycobactin and then is inserted in to the carrier molecule. Mycobactin belong to the mixed ligand type wherein they have two hydroxamate groups and the third pair being provided by an oxygen atom on the aromatic residue and nitrogen in the oxazoline ring. Snow (1970) elucidated the structures of several mycobactins isolated from different mycobacterial species. The structure of mycobactin is represented in Fig. 7.

Mycobactin from different mycobacterial species have the core nucleus that consists of a 2-hydroxyphenyloxazoline moiety linked by an amide bond to an acylated ε-N-hydroxylysine residue. The second ε-N-hydroxylysine is cyclised to form the seven membered lactam and is attached to β-hydroxyacid via an amide bond.

This, in turn is connected to the α-carboxyl of the first lysine residue. Within this core, a methyl group may or may not be present at the 6th position of phenolic ring and the 5′ position of the oxazoline (Dover & Ratledge, 2000). Mycobactin chelates iron forming very stable complexes, the coordinating ligands being the two hydroxamate groups, the third pair being provided by the nitrogen atom of the oxazoline group and the hydroxyl group of salicylic acid. The long alkyl / alkenyl chain at R1 accounts for its hydrophobicity.

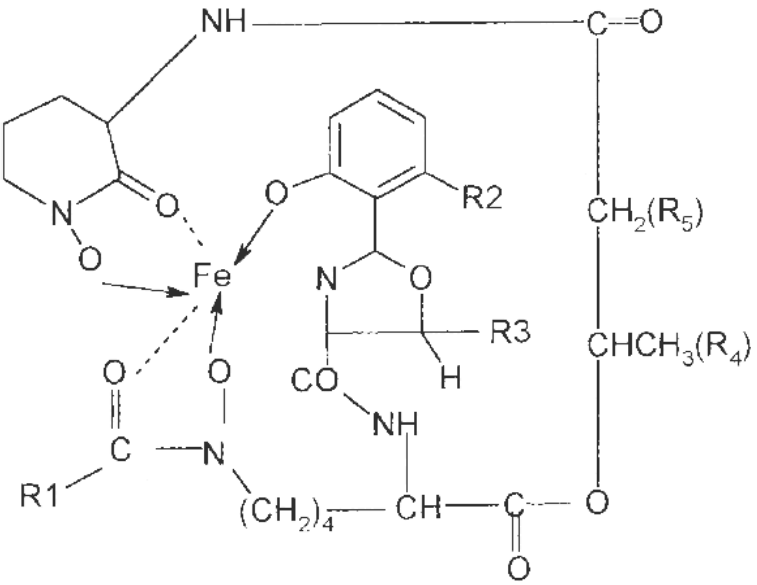


Fig. 7. General structure of mycobactin. (Snow, 1970). The side chains at R1 are usually long chain alkyl or alkenyl groups with a cis double bond at C2. Substituents at R2, R3 and R5 are usually H or CH_3 and at R4 could be simple CH_3, C_2H_5 or long chain alkyl groups.

Mycobacterium tuberculosis genome codes for a cluster of 10 genes (*mbt A-J*) called the *mbt* operon which is involved in the biosynthesis of mycobactin. They encode two polyketide synthetases (MbtC and MbtD), three peptide synthetases (MbtB, MbtE and MbtF), and isochorismate synthase (MbtI) (Quadri *et al.,* 1998).

1.4.3.2. Carboxymycobactins

Carboxymycobactins were previously referred to as chloroform-soluble exochelins (due to their extractability into chloroform in their ferric form). Their structure has been elucidated in *M. avium* (Fig. 8) (Lane *et al*., 1995), *M. tuberculosis*

(Gobin *et al*., 1995; Wong *et al*., 1996), *M. bovis* and *M. bovis* BCG (Gobin *et al*., 1999). They are expressed as a family of related molecules. They differ from the mycobactin in the side chain R1, terminating in either -COOH or –CO.CH3 that renders them more polar and thus water-soluble. They can solublise iron from transferrin, lactoferrin and ferritin. The synthesis of carboxymycobactin is similar to mycobactin and differs from mycobactin after the formation of the common precursor. The final step in the synthesis of carboxymycobactin differs as the alkyl chain terminates in a carboxylic acid (Ratledge, 2004).

1.4.3.3. Exochelins

Exochelins are water-soluble, ornithine derived siderophores produced by non-pathogenic mycobacteria and well characterised in *M. smegmatis* and *M. neoaurum* (Sharman *et al.,* 1995a and 1995b) (Fig. 9 a, b). The exochelins are peptidyl in nature but involve D-amino acids and thus there are no conventional peptide bonds present (Ratledge & Dover, 2000). The coordination center with Fe^{3+} is hexadendate in an octahedral structure involving the three-hydroxamic acid groups, which are from ornithine. The exochelin MS from *M. smegmatis* is a formylated pentapeptide derived from three molecules of δ-N-hydroxyornithine, β-alanine and a threonine (Fig. 9 a). Exochelin MN from *M. neoaurum* is a hexapeptide with two δ-N-hydroxyhistidines, providing the coordination center for iron chelation and two β-alanine and an ornithine (Fig. 9 b) (Sharman *et al.,* 1995 a and 1995 b).

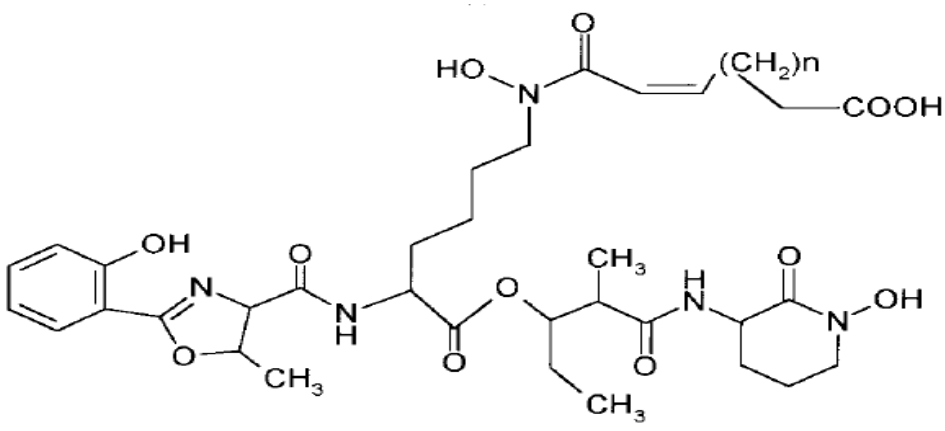


Fig. 8. Structure of the extracellular carboxymycobactin (n = 2 – 9). The structure has been elucidated in *M. avium* (Lane *et al*., 1995), *M. tuberculosis* (Gobin *et al*., 1995; Wong *et al*. 1996), *M. bovis* and *M. bovis* BCG (Gobin *et al*. 1999). They differ from the mycobactin in the side chain R1, terminating in either a COOH or $COCH_3$ that renders them more polar and thus water-soluble.

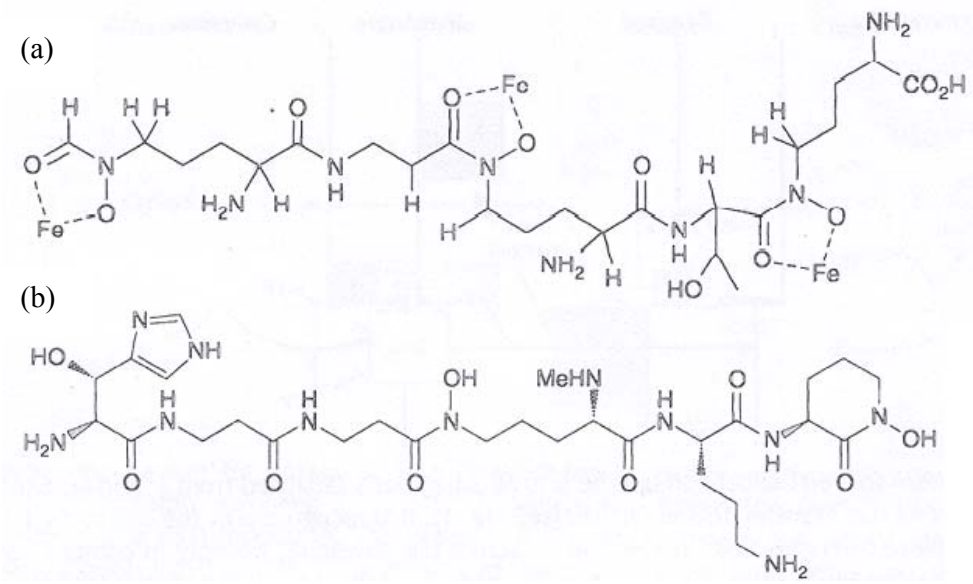


Fig. 9 - Structure of exochelins (a) MS and (b) MN from *M. smegmatis* and *M. neoaurum* respectively (Sharman *et al.,* 1995 a & b) (a) represents exochelin MS from *M. smegmatis* (Formyl-D-ornithinel-β-alanine-D-ornithine2-D-allo threonine-l-ornithine3) and (b) represents exochelin MN from *M. neoaurum* (L-threo-β-hydroxy histidine- β alanine-L-α methyl ornithine-l-ornithine-L- (cyclo)ornithine).

1.4.3.4. Identification of IREPs in mycobacteria

Several bacteria express iron regulated envelope proteins (IREPs) / Iron regulated membrane proteins (IRMPs) that function as receptors for ferric siderophores. Mycobacteria also express IREPs as part of the iron acquisition machinery (table 3) (Sritharan, 2000). Hall *et al*. (1987) first demonstrated the expression of IREPs in *M. smegmatis*. The 29 kDa IREP was found to be a potential receptor for the siderophore exochelin MS by specifically blocking its uptake with monospecific antibodies. This specificity was subsequently used to purify the 29 kDa protein using affinity chromatography (Dover & Ratledge 1996). Subsequently, IREPs were identified in other mycobacterial species not only under defined lab conditions of established iron status but also under *in vivo* conditions. *Mycobacterium avium* isolated from infected tissues of C57 black mice showed the expression of IREPs of 180, 29, 21, 14 kDa. *Mycobacterium leprae*, isolated from infected armadillo liver tissue showed the 21 kDa protein in the cell wall fraction. The expression of the IREPs in *in vivo* grown mycobacteria strongly suggests that an iron-

deficient environment exists under *in vivo* condition (Sritharan, 2000). Table 3 lists the IREPs identified in mycobacteria (Sritharan 2000).

Table 3. Iron-regulated envelope proteins in mycobacteria grown *in vitro* and *in vivo*. (Sritharan, 2000)

Organism	**Expression of IREPs of size (kDa) with reference to iron status**									
	Low iron							**High iron**		
	180	84	29	25	21	14	11	250	240	12
Defined iron status *(in vitro*-derived mycobacteria)										
M. smegmatis	+	+	+	+	-	+	-	+	+	-
M. neoaurum	+	-	+	-	+	+	-	+	+	+
ADM 8563	-	-	+	-	-	+	+	?	?	-
M. avium	+	-	+	-	+	+	-	+	+	-
Undefined iron status (*in vivo*-derived mycobacteria)										
M. avium	+	-	+	-	+	+	-	+	+	-
M. leprae	-	-	+	-	+	+	-	+	+	-
+ and - denote the presence or absence of the protein										
? denotes a very faint band.										

1.4.4. Global response of *M. tuberculosis* to iron availability

In addition to expressing iron-uptake systems during iron deficiency, *M. tuberculosis* displays several changes in response to iron availability detected at the level of protein and mRNA. Differences in mRNA levels in bacteria subjected to iron deprivation versus those replicating in iron-rich medium were examined by Rodriguez *et al*. (2002) revealed 155 iron regulated genes. Iron deficiency induced about two-thirds of those genes, whereas the remainders were induced in iron-rich medium. Half of the genes induced under low iron conditions are of unknown function. The other half includes iron acquisition genes such as *mbtA–J*, the mbt-2 cluster and the irtAB operon in addition to genes encoding membrane proteins, members of the glycine-rich PE/PPE protein family, putative transporters and several genes encoding proteins involved in basic metabolism (Rodriguez *et al.,* 2002). High iron levels in the culture medium results in induction genes including *bfrA* and *bfrB*,

which encode putative iron-storage proteins (*i.e*. bacterioferritin and ferritin, respectively) and *katG*, which encodes a catalase–peroxidase.

1.4.5. Iron-dependent regulation at molecular level

The adaptive changes that occur in response to iron availability are controlled in bacteria by regulating the expression of genes, whose regulation is controlled at the level of transcription. The control is mediated by iron-dependent transcriptional regulators (Bagg & Neilands, 1987). The ferric uptake regulator (Fur) and the diphtheria toxin repressor (DtxR) are two families of transcriptional regulators that control iron homeostasis in bacteria. Proteins of these two families are widely distributed in many bacteria and they are functional homologs. *Mycobacterium tuberculosis* contains two Fur-like proteins (FurA and FurB) and two members of the DtxR family (IdeR and SirR) (Cole *et al*., 1998). FurA negatively regulates the expression of katG (the gene immediately downstream of furA), thereby modulating the response against oxidative stress (Pym *et al*., 2001). The furB promoter is induced by Zn^{2+}.

Mycobacterium tuberculosis relies on the iron-dependent regulator (IdeR) to maintain iron homeostasis. IdeR is a 230-amino acid protein that is closely related to the DtxR protein (Schmitt *et al*., 1995). It functions as a homodimer and each monomer has three functional domains with two metal-binding sites. The N-terminal region contains the DNA binding domain, which has a helix-turn-helix motif, and the dimerization domain, which includes most of the metal-binding residues. The third domain, located in the C-terminal region, folds like a SH3 (Src homology domain 3) domain. This domain interacts with the two first domains and contributes two ligands for metal binding, which are required for stable dimer formation and full DNA binding activity. IdeR binds to the DNA as a double dimer with each dimer on opposite sides of the DNA (Rodriguez, 2006). IdeR can be activated *in vitro* by several metals but iron (the natural cofactor) is the optimal metal for IdeR function. The direct effect of IdeR on the transcription of iron sensitive genes correlates with its ability to bind to a 19 bp inverted-repeat consensus sequence (TTAGGTTAGGCTAACCTAA) which is present in the promoters of IdeR regulated genes (Gold *et al*., 2001). In the presence of $Fe^{2+,}$ IdeR binds to the promoters of siderophore synthesis and iron-storage genes but it affects their expression in opposite ways. It represses transcription of the *mbt* genes, whereas it activates that of *bfrA* and

bfrB (Fig. 10). IdeR combines with Fe^{2+} and binds to specific sequences (iron boxes) in the promoter region of iron-regulated genes modulating their transcription (Fig. 10) (Rodriguez & Smith, 2003). In these conditions, IdeR–Fe^{2+} down regulates iron uptake by repressing siderophore production and increases iron storage by activating transcription of *bfrA* and *bfrB* encoding bacterioferritin and ferritin.

In an indirect manner, IdeR also positively modulates protection against oxidative stress. In low-iron conditions, the IdeR–Fe^{2+} complex is not formed, and IdeR-repressed genes are transcribed while iron storage genes are not expressed (Rodriguez & Smith, 2003).

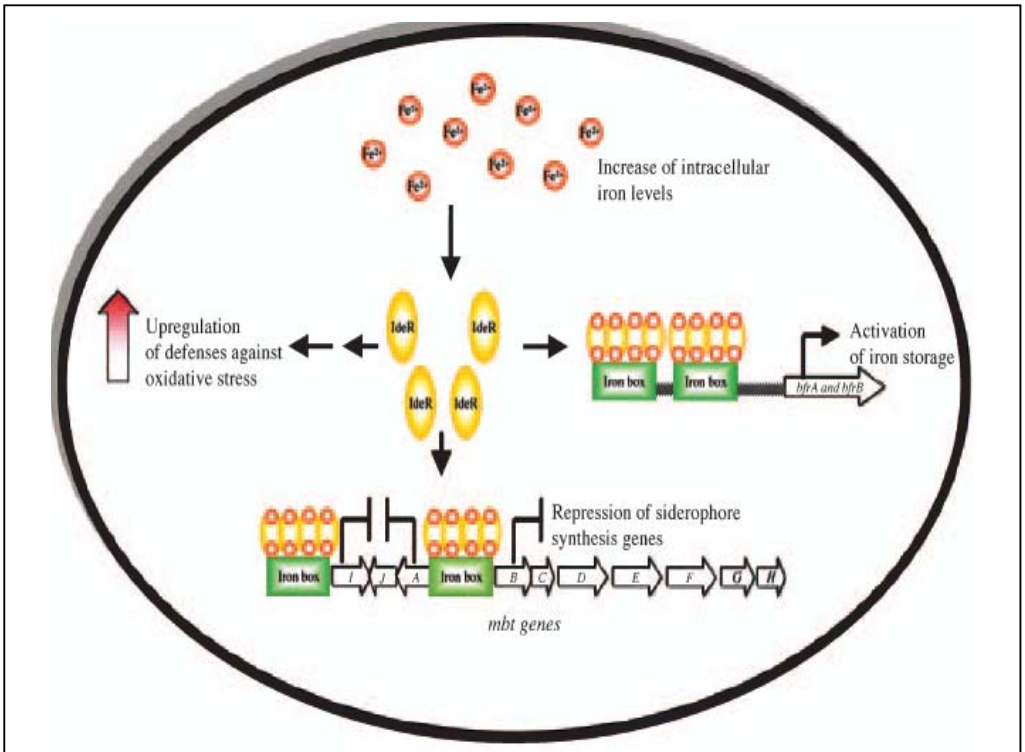


Fig. 10. Iron-dependent regulatory function of IdeR. (Rodriguez & Smith, 2003). When intracellular iron levels increase, IdeR combines with Fe^{2+} and binds to specific sequences (iron boxes) in the promoter region of iron-regulated genes modulating their transcription.

1.4.6. Iron and virulence factors

Iron levels and expression of virulence factors have been demonstrated in many bacterial systems like *E. coli, C. diphtheriae, P. aeruginosa, V. cholerae*. (Salyers & Whitt 1994). Diphtheria toxin is encoded by the *tox* gene, carried only in those strains of *C. diphtheriae* lysogenized by beta and omega phages. The DtxR

repressor, as the DtxR-Fe complex binds to the ± 10 region of the promoter and the transcription start site of the tox gene and blocks its transcription. Under low iron conditions, the repressor no longer binds and toxin production is increased. The toxin lyses the host cells and releases the iron from within these cells. It is thus clear that toxin production is part of the iron acquisition machinery of this pathogen. In *V. cholerae*, virulence is associated with the expression of two genes, the irgA and the hemolysin gene, whose expression is controlled by iron (Sritharan, 2000).

In *M. tuberculosis*, mycobactin is recognized as a virulence determinant (Quadri *et al*., 1998; De Voss *et al*., 1999, 2000). De Voss *et al*. (1999) by generation of mutants defective in the biosynthesis of mycobactin T, highlighted the importance of this siderophore not only for normal growth *in vitro* but also within the macrophages. Dussurget *et al*. (1996) showed an increase in sensitivity to hydrogen peroxide in IdeR mutants due to the decreased activity of catalase-peroxidase (KatG) and superoxide dismutase (SodA) activity, implying that IdeR is most likely to play a central role in oxidative stress. Manabe *et al.* (1999) by the introduction of a dominant Dtx (E175K) iron-dependent repressor into *M. tuberculosis* proved that the IdeR repressor controlled events influenced the virulence in a murine model of infection. Iron and *ideR* were reported to play a role in the expression of ESAT-6 proteins (Rodriguez *et al.,* 2002).

Objectives of the study

In this study, the major objectives are to

I. Study the influence of iron levels on the protein profile of the secretory / excretory proteins of *Mycobacterium tuberculosis*: identification of iron-regulated culture filtrate proteins.

II. Assess the cell-mediated immune response of peripheral blood mononuclear cells from tuberculosis patients to defined culture filtrate antigen (CF-Ag) pools.

III. Screen serum samples of tuberculosis patients for antibodies against CF-Ags.

CHAPTER 2

MATERIALS AND METHODS

2.1.1. Sources of chemicals

Culture media Middlebrook 7H9 / 7H10 / 7H11, supplements OADC / ADC, Lowenstein Jensen base, iron free chemicals were purchased from Becton & Dickinson, Hi-Media and Alfa AESAR respectively. Organic solvents and acids were purchased from Qualigens. All fine chemicals, including hexadecyltrimethyl ammonium bromide (HDTMA), Chrome azurol sulphonate (CAS) and anhydrous piperazine and cocktail protease inhibitor were purchased from Sigma-Aldrich. Cell culture reagents including RPMI media, fetal calf serum, Ficoll -Histopaque (Density 1.77), heparin sulfate, phyto-hemagglutinin were from Sigma - Aldrich. Penicillin and streptomycin used in lymphocyte proliferation studies were obtained from Hi-Media. Others included Amicon filters (Millipore), IPG strips and other items for 2D-PAGE (Biorad, USA), nitrocellulose membranes (Pall Scientifics, USA) and 0.22 μm cellulose acetate filters (Sartorius). IFN-γ Opt EIA kit was obtained from Becton and Dickson. All antibody-enzyme conjugates (Alkaline phosphatase conjugate, HRP-conjugates) and the substrate tetra-methyl benzidine (TMB) and hydrogen peroxide were obtained from Bangalore Genei, India.

2.1.2 Bacterial strains

Mycobacterium tuberculosis H37Rv (ATCC 27294) and strains of *M. bovis* BCG - Denmark, Birkhaug, Moreau, Phipps, Sweden, Russia, and Pasteur (a kind gift from Dr. Peter Small) were used in the study.

2.2.1. Growth of *M. tuberculosis* and BCG strains under high and low iron conditions (Hall *et al*., 1987)

Mycobacterium tuberculosis H37Rv and all the BCG strains, maintained as glycerol stocks at -80°C was revived in 7H9 middle brook medium supplemented with ADC. This was used to inoculate iron-free Proskauer and Beck medium supplemented with calculated amounts of iron.

All glassware used for preparation of medium was made iron free and glass double-distilled water was used for all experiments. Proskauer and Beck medium was prepared by dissolving 5 g of L-asparagine, 5 g of potassium dihydrogen orthophosphate

and 20 mL of glycerol in 900 mL of glass double distilled water. The pH was adjusted to 6.8 with 10 N NaOH and the medium was made up to 1 L. Alumina (5 g) was added and autoclaved at 121°C at 15 lbs / inch^2 for 15 min. The media was cooled and later filtered using Whatman grade 541 filters to remove the alumina. The media was aliquoted as 100 mL volume in 250 mL conical flasks and then re-autoclaved.

Before inoculation, added 1 mL of salt solution containing (16.8 mM Mg^{2+}, 5.01 µM Mn^{2+} and 17 µM Zn^{2+}) to 100 mL medium. Iron was added at 8 µg Fe / ml (144 µM of Fe) and 0.02 µg Fe / ml (0.36 µM of Fe) for high and low iron media respectively. A homogenous suspension of the organisms grown in 7H9 medium was prepared by vortexing the sample in the presence of glass beads, diluted to McFarland 4 and 1 mL of this was added to each of the flasks. The organisms were maintained at 37°C with shaking at 150 rpm for 14 days. The organisms were separated by centrifugation at 6000 rpm for 15 minutes and the spent growth medium was used for further studies. Growth was measured by determination of the cell dry weight.

2.2.2. Growth of *M. tuberculosis* with varying concentrations of iron

Mycobacterium tuberculosis was grown as above in medium supplemented with iron at 0.02, 0.05, 0.1, 0.2, 0.4, 1, 4, 8 and 12 µg Fe / mL respectively.

2.2.3. Time-dependant growth of *M. tuberculosis*

Mycobacterium tuberculosis was inoculated separately into 5 flasks each of high (8 µg of Fe/ml) and low iron (0.02 µg Fe / ml) media respectively and grown as mentioned above. The organisms were harvested on days 4, 8, 12, 16, 20 and 24 respectively.

2.3. Assay of mycobactin and carboxymycobactin

Mycobactin and carboxymycobactin were assayed in the cell pellet and the culture filtrate from an aliquot of 15 mL from each culture flask. Carboxymycobactin was assayed by Universal CAS assay and mycobactin was assayed by measuring the absorbance of the ferric mycobactin at 450 nm.

2.3.1. Assay of carboxymycobactin by Universal CAS assay (Schwyn & Neilands, 1987)

Preparation of reagents

Solution A

6 mL of hexadecyltrimethyl ammonium bromide (HDTMA) from 10 mM stock (3.645 g / L of DDW) was pipetted in a standard flask and diluted with 10 mL of DDW. To this, 1.5 mL of a freshly prepared 1 mM ferric chloride taken from 10 mM stock (0.1622 g / L) in 10 mM HCl and 7.5 mL of 2 mM Chrome azurol sulphonate (CAS) (1.211 g / L) were added slowly.

Solution B

Anhydrous piperazine (4.3 g) was dissolved in a minimum volume of water and 7.25 mL of concentrated HCl was added. All the above solutions were made in iron-free glassware. To prepare working CAS assay solution, solution B was added slowly to solution A and made up to 100 mL with iron free DDW.

Procedure

2 mL of CAS assay solution was added to 2 mL of water (blank) and 2 mL of culture filtrate (test), incubated for 30 min at RT and the absorbance read at 630 nm. The absorbance is converted to siderophore units, using the equation below:

$$\text{Siderophore Unit} = (A_b - A_t / A_b) \text{ X } 100$$

(A_b - $OD_{630\ nm}$ of blank and At - $OD_{630\ nm}$ of test).

Carboxymycobactin was expressed as siderophore units per 100 mg dry weight.

2.3.2. Assay of mycobactin (Yeruva *et al.,* 2006)

Mycobactin was extracted by adding 750 µL of ethanol chloroform mixture (1:1) to the cell pellet and leaving it at RT for o/n. The cells were centrifuged at 5000 rpm for 15 min and chloroform layer was transferred into new tube and saturated $FeCl_3$ in alcohol was added and left for 10 min at RT. The ferric mycobactin was extracted into chloroform, washed thrice with water and the concentration was determined by reading at

450 nm using chloroform as blank. Mycobactin was expressed as OD_{450} units per g dry weight.

2.4. Analysis of culture filtrate proteins (CFPs)

2.4.1 Concentration of CFPs by ammonium sulphate precipitation (Malen *et al.* 2007)

The spent growth medium was filtered through 0.20 µm filter to remove any viable bacteria. The protease cocktail inhibitor (AEBSF, E-64, bestatin, leupeptin, aprotinin, and sodium EDTA) was added to a final concentration of 1 mM. All subsequent handling was done by keeping on ice.

Solid ammonium sulphate was added slowly with continuous stirring to 80% saturation (516 g / L) over a period of 1 hour. It was then subjected to centrifugation at 10,000 rpm at 4°C for 30 min. The protein pellet was taken up in 10 mL of 50 mM Tris-HCl (pH 6.8), centrifuged at 5000 rpm at for 5 min at 4°C to remove any insoluble debris and then subjected to ultrafiltration using Amicon filtration cones with 5-kDa cut-off. This was done over a period of 3h at 4°C till the volume was reduced to 500 µL. Protein was estimated by BCA method and the samples stored as aliquots at -80°C.

CFPs from organisms grown under high and low iron conditions were separated by SDS-PAGE.

2.5. Separation of CFPs by sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970)

Stock solutions

1. Acrylamide and N, N'-bisacrylamide mix (30:0.8): 30 g of acrylamide and 0.8 g of bisacrylamide were dissolved in 60 mL of water and made upto 100 mL.
2. Resolving gel buffer: Tris-HCl (1.5 M, pH 8.8) with 0.4% SDS
3. Stacking gel buffer: Tris-HCl (0.5 M, pH 6.8) with 0.4% SDS
4. Sample buffer (2X): 0.125 M Tris-HCl (pH 6.8) containing 4% SDS, 20% glycerol and 0.002% bromophenol blue

5. Running buffer: Tris-glycine buffer (25 mM Tris, 250 mM glycine and 0.1 % SDS), pH 8.8

6. Staining solution: 0.25% Coomassie Brilliant Blue in 50% MeOH and 10% acetic acid.

7. Destaining solution: 10% methanol and 10% acetic acid

Table 4- Preparation of gel mix

Reagent	**Volume of the components (mL) for**		
	5-20% Gradient gel		**10%**
	5%	**20%**	
Acrylamide: Bisacrylamide	2.75	10.6	10.6
Resolving gel buffer	4.00	4.0	8.00
Double distilled water	9.30	1.4	13.4
10% ammonium persulphate	0.08	0.08	0.160
TEMED	0.008	0.008	0.008
Total volume	**15**	**15**	**30**

The resolving gel was prepared using the recipe as given above either for 5-20% gradient gel or 10% gel. The gradient was prepared using a gradient maker. The resolving gel was allowed to polymerize and then the stacking gel was poured over it, with the appropriate comb positioned.

5% Stacking gel mix

Components	Volume (mL)
Acrylamide: Bisacrylamide	1.5
Stacking gel buffer	2.5
Double distilled water	6.0
10% ammonium persulphate	0.03
TEMED	0.010

Equal volumes of the protein sample and 2X sample buffer were mixed, boiled for 10 min and centrifuged at 10,000 rpm for 15 min to remove any insoluble material. The clear supernatant was loaded on the gel and subjected to electrophoresis at 100 V till the bromophenol dye entered the resolving gel and then at 125 V. The tracking dye was allowed to run out of the gel, followed by electrophoresis for an additional 30 min. Gels were stained with Coomassie Blue for 2 h and then the excess dye was removed with the destaining solution.

2.5.2. Tris-Tricine gel electrophoresis for the separation of low molecular weight proteins (Schagger & Jagow, 1987)

Stock solutions

1. Acrylamide-bisacrylamide mix (49.5:3): 48 g of acrylamide and 1.5 g of bisacrylamide were dissolved in 50 mL of water and made up to 100 mL**.**
2. Gel buffer (for both stacking and resolving gel) - 3 M Tris and 0.3% SDS, pH 8.45.
3. Running buffer-
    a) Anode buffer - 0.2 M Tris-HCl, pH 8.9.
    b) Cathode buffer - 0.1 M Tris-HCl, 0.1 M Tricine and 0.1% SDS, pH 8.25.

Table 5- Preparation of 10% gel for Tris-Tricine gel electrophoresis

Composition	**Stacking gel (4% T, 3%C)**	**Resolving gel 10% T, 3%C**
Acrylamide: bisacrylamide	1 ml	6.1 ml
Gel buffer	3.1 ml	10 ml
Glycerol	-	3.17 ml
Double distilled water	8.4 ml	10.8ml
10% ammonium persulphate	100 ul	100 ul
TEMED	10 ul	10 ul

The resolving gel was first poured and layered with the stacker. Samples, boiled with sample buffer were loaded in the wells and electrophoresis was carried out at RT, initially at 30V till the bromophenol tracking dye entered the resolving gel and then at 150V till the dye reached the bottom of the glass plates.

2.5.3. Two-dimensional polyacrylamide gel electrophoresis: 2D - PAGE

2D-PAGE was done as described by Rosenkrands *et al.* (2000). Ready-to-use 11 cm length pre-cast IPG gel strips (BioRad) with a pH gradient of 4-7 were used.

2.5.3.1. Sample preparation

125 µg of total protein was precipitated with 5 volumes of chilled acetone and kept at -20°C o/n. The protein was pelleted by centrifugation at 10,000 rpm for 30 min and dried at RT for 10 min.

2.5.3.2 Rehydration

This was done as per manufacturer's instructions. To the protein pellet 185 µl of rehydration buffer (7M of urea, 2M thiourea, 2% CHAPS, 18 mM DTT, 0.5% IPG buffer pH 3-10) was added. The resultant mixture was centrifuged at 6000 rpm for 5 min to remove any insoluble debris. The protein sample was loaded on the IPG strip, let stand for 1 h, layered with mineral oil to prevent evaporation of the sample and left o/n at RT.

2.5.3.3. First dimension separation (IEF)

IEF was performed according to manufacture's instructions. Paper wicks were placed at both ends of the channels covering the wire electrodes of the IEF focusing tray. Nanopure water (8 µL) was pipetted onto each wick so as to wet them. The strips were transferred to the IEF focusing tray with the forceps after draining the mineral oil for 1 min on the filter paper. The gel side of the strips should be in contact with the electrodes and air bubbles should not be introduced. The strips were layered with 2 mL of mineral oil and the proteins were subjected to isoelectric focusing in PROTEAN IEF cell, Biorad, using the step-wise programme for 11 cm strip given below.

Step-wise programme for 11 cm strips

	Voltage (V)	Time (h)	Volt-hours	Ramp
Step 1	250	0.20 h	---------	Linear
Step 2	8000	2.5 h	---------	Linear
Step 3	8000	-------	30,000	Rapid
Total		5.3 h	~ 40,000	

2.5.3.4 Second dimension separation-using SDS-PAGE

Reduction and alkylation of the separated proteins was done prior to SDS-PAGE. The strips were first equilibrated in buffer I containing 6 M urea, 0.375 M Tris-HCl, pH 8.8, 2% SDS, 20% glycerol, 2% w/v DTT for 20 min followed by equilibration in buffer II with the same composition as buffer I except DTT was replaced by 2.5% iodoacetamide for 20 min.

Second dimension electrophoresis was done using 10% Tris-Glycine or 10% Tris-Tricine SDS-PAGE. The strips were washed in 1 x SDS buffer, and then layered on the resolving gel, with the positive side of the strip towards the marker. The strips were then over laid with the stacking gel (containing the tracking dye) mix. The gel was run constantly at 100 V. The gel was removed and the separated proteins detected using Coomassie blue or silver staining.

2.5.3.5 Silver Staining

The gel was initially fixed in a solution containing of methanol: water: glacial acetic acid (48:40:12) with 0.01% formaldehyde for 2 h. The gel was washed with DDW and then fixed in 50% methanol solution for 1 h. The gel was washed thrice with DDW and soaked in 0.001% solution of sodium thiosulphate for about 1 min. The gel was thoroughly washed with DDW three times and soaked in 0.01% solution of silver nitrate containing 0.01% of formaldehyde for 20 min. The gel was washed with water and developed with solution containing 5% sodium carbonate, 0.001% of sodium thiosulphate, and 0.001% of formaldehyde.

If the background was high, the background stain was removed using the protocol by Biorad. Solution A was prepared by dissolving 37 g of sodium chloride and 37 g of anhydrous copper sulphate in 80 mL of DDW. Concentrated solution of ammonia was added until the precipitate formed dissolves. Solution B was prepared by dissolving 436 g of sodium thiosulphate in 1 L of DDW. The working solution was prepared by mixing equal volumes of solutions A and B. The destaining was done for a period that was sufficient to remove the background without destaining the protein spots. The gel was washed in water to prevent fogging. The reaction was stopped by soaking the gel in 10% glacial acetic acid.

2.5.3.6 Fast Coomassie Brilliant Blue staining

The staining involved three stain solutions with decreasing concentration of Coomassie Brilliant Blue (Table 6).

Table 6- solutions used for fast coomassie brilliant blue staining

	Coomassie blue (g)	**Acetic acid** (mL)	**Isopropanol** (mL)	**Water** (mL)
Solution A	0.5	100	250	650
Solution B	0.05	100	100	800
Solution C	0.02	100	--	900
Solution D	--	100	--	900

Procedure

The gel was kept in solution A for 2- 3 h and then heated until the solution boiled for 5 min in a microwave oven, cooled to RT for 5 min with gentle shaking. The solution A was discarded and gel was rinsed in DDW for 5 min. The gel was treated with solutions B and C similarly with boiling for 7 and 10 min respectively. The gel was destained in solution D for 20 min followed by washing with water.

2.5.3.7 Scanning and image analysis of the 2D-gel proteins

The gel was scanned and the image was analysed using Image Analysis, Melanine 2D platinum software (GE Healthcare). The gel spots were picked manually after wearing gloves, taking care not to contaminate the samples with keratin.

2.6. Identification of CFPs by MALDI-TOF MS analysis

2.6.1. Processing of 2D PAGE spots for MALDI-TOF MS analysis

Solutions used

1. Freshly prepared 25 mM ammonium bicarbonate (NH_4HCO_3).
2. Dehydrating solution: acetonitrile (ACN) and NH_4HCO_3 in 2:1 ratio (100 mL of acetonitrile and 50 mL of 25 mM NH_4HCO_3).
3. Promega Trypsin Gold: Stock solution 1 µg / µL solution.
4. Acetonitrile with 2% formic acid.

Washing and In-gel digestion of gel pieces

Dehydrating solution (100 µL) was added to dehydrate the gel slices and left for 15 min. The supernatant was removed and the spots were re-hydrated for 10 min by adding 50-100 µl of 25 mM NH_4HCO_3. The dehydration and rehydration steps were repeated twice. Finally enough acetonitrile was added to shrink the gel pieces and they were dried in Speed Vac. The gel slices were pre-incubated in a minimal volume (10-20 µl) of the Trypsin solution at RT for 1 h. After the gel spots were fully hydrated the gels were covered with a minimal volume of 25 mM NH_4HCO_3 and the digestion was continued o/n at RT.

Extraction of peptides

The peptides were extracted by adding 2 volumes of ACN containing 2% formic acid. The extract was vortexed and incubated for 10-30 min. The supernatant was transferred into a new tube and dried in Speed Vac for 15-30 min to remove the ACN. The extracted peptides were subjected for mass spectrometric analysis.

MALDI-TOF MS

One micro-litre of the sample eluted from the matrix was applied to the steel target plate and analysed by MALDI-TOF MS (Autoflex & Ultraflex, Bruker Daltonics). The obtained mass spectra were searched against the *M. tuberculosis* complex database, using MASCOT (http:// www.matrixscience.com). A protein was regarded as identified if the matched peptide mass fingerprint covered 20% of the complete protein sequence (Malen *et al.,* 2007).

2.6.2. Western blotting analysis of CFPs

Polyclonal antibodies against the whole culture filtrate proteins of *M. tuberculosis* H37Rv, monoclonal antibody HYB 76.8 against ESAT-6 and monoclonal antibody L24B5 against MPT64 (kind gift of Dr. Ida Rosenkrands) were used to probe CFPs of high and low iron grown *M. tuberculosis* in this study.

Western blotting

The CFPs separated by SDS-PAGE analysis was transferred on to nitro-cellulose membrane (Towbin *et al.,* 1979) using the electroblotting buffer (48 mM Tris pH 8.3, 39 mM glycine and 20% MeOH) using the transblot apparatus (Balaji Scientific). Transfer was done at 30 mA for 15 h at 4ºC o/n. After the transfer, the proteins were visualized by staining with 10% Ponceau S. The nitro-cellulose membrane was blocked for 1 h at RT with 5% non-fat milk in Tris buffered saline (50 mM Tris, pH 7.5 plus 150 mM NaCl). Monoclonal antibodies (1:50 dilution) / polyclonal serum (1:1000 dilution) was added and incubated o/n. The blots were washed and the antibody-conjugate (1: 500 dilution) was added and incubated for 2 h. The blots were then developed with BCIP and NBT.

2.7. CF-Ag pools: preparative gel electrophoresis

CFPs of *M. tuberculosis* grown under high iron and low iron conditions were subjected to preparative SDS-PAGE using 5-20% gradient gel. The gel was stained for about 30 min to visualize the bands. Gel slices containing the separated proteins between defined molecular marker bands were cut out (Table 7) and eluted as described below.

The fractions corresponding to high and low iron grown organisms were labeled as H and L respectively

Table -7. Molecular range of the proteins in the CF-Ag pools of *M. tuberculosis*

Fractions	Molecular weight (kDa)
H1 / L1	116 – 66
H2 / L2	66 – 45
H3 / L3	45 – 25
H4 / L4	25 – 12
H5 / L5	Below 12

The gel slices was crushed and the proteins eluted in Tris-HCl buffer (25 mM, pH 8.8) with 5% glycerol and 2% SDS. The proteins in the eluate were subjected to acetone precipitation; five volumes of chilled acetone was added, left o/n at - 20ºC and centrifuged at 10,000 rpm for 30 min. The pellets were air-dried and solubilised either in 5 mM Tris -HCl pH 6.8 (for electrophoretic separation) or in 50 mM phosphate buffer (for immune proliferation studies). The CF-Ag pools were re-run on 10% SDS-PAGE using either Tris-glycine or Tris-Tricine system, the latter for the separation of low molecular weight proteins.

2.7.1. Immune proliferation studies with CF-Ag pools

2.7.1.1. Preparation of RPMI media

RPMI medium was prepared by dissolving the contents of one bottle of RPMI 1640 (with glutamine and without $NaHCO_3$) in 800 mL of Milli-Q water. Added 2 gm of $NaHCO_3$, pH was adjusted to 7.0 with 1N HCl and the volume made up to 1 L. The medium was filter sterilized using 0.2µm filter. Fetal calf serum (5% final concentration), penicillin (100 units / mL) and streptomycin (100 µg / mL) were added just before use. This is referred to as complete RPMI medium.

2.7.1.2. Study group: selection of tuberculosis patients and normal control individuals

The study group consisted of tuberculosis patients, attending the clinic in Andhra Pradesh Government General Chest Hospital, Hyderabad. Three categories of patients, namely the smear positive, smear negative, extrapulmonary were chosen and the fourth group consisted of normal healthy individuals as controls (Table 8).

Table 8- Categories of tuberculosis patients and normal healthy individuals included in the study

Category[a]	No. of cases		Diagnostic procedures		
	Male	Female	AFB[b]	Chest X-Ray	Clinical history
Smear (+) (SP) (n = 25)	19	6	(+)	(+), with characteristic lesions in two or more segments of the lungs	Cough with expectoration, loss of appetite, fever, and loss of weight (symptoms for 2 months)
Smear (-) (SN) (n = 20)	16	4	**(-)**		
Extra-pulmonary[c] (EP) (n = 15)	11	4	NA	NA	The diagnosis was by histological examination of the specimens.
Normal controls[d] (NH) (n =20)	15	5	ND	ND	Student donors

[a]Tuberculosis patients were categorised as per RNTCP (Revised National Tuberculosis control program) guidelines.

[b]Smear test of sputum samples was done to check for AFB positivity and was classified as smear (+) if positive for AFB on three consecutive days.

[c]Extra pulmonary cases included patients with pleural effusions, cervical lymphadenitis, spine TB and miliary tuberculosis

[d]Normal healthy control individuals were from University of Hyderabad with no previous history of tuberculosis or serious illness and no contact with patients with active tuberculosis.

NA- not applicable; ND- not determined.

Mantoux test was not performed for any of the individuals due to its low specificity in this population and the unwillingness of the patients to remain in the clinic for measuring the induration after 48 –72 hours. Clearance from the Ethical committee of Government General Chest Hospital and consent from the patients were obtained before collection of samples. Individuals who had history of HIV infection, diabetes, earlier history of tuberculosis, defaulters of ATT and individuals with other serious illness were excluded from the study.

2.7.1.3. Isolation of lymphocytes from blood

Peripheral venous blood (10 mL) was collected in to 60 units of heparin sulphate (Stock of 3000 units / mL). The blood was diluted with equal volume of normal saline (0.9% NaCl) and layered onto 4 mL of Ficoll - Histopaque (in a 15 mL Falcon tube) and centrifuged at 1500 rpm for 30 min. The buffy coat layer containing the lymphocytes, resuspended in 6 mL of RPMI was centrifuged for 8 min at 1000 rpm. The supernatant was discarded, the cells gently re-suspended in 1 mL of RPMI and the viability was checked using 0.4% Trypan blue. 10 µL of the cell suspension was diluted with trypan blue solution (0.2% w / v in 0.9% NaCl), loaded on a hemocytometer and the cells in the four large squares were counted. The cells that did not take up the colour were treated as viable cells and the total of viable cells was calculated using the formula

Cells / mL = (Average number per large square) x 10^4 / mL x 1 / dilution factor.

2.7.1.4. Lymphocyte proliferation assay

Antigen induced proliferation of lymphocytes was performed according to standard procedures (Surekha *et al.,* 2005). 200 µl of the cell suspension containing 2 x 10^5 cells were seeded into the each of 96-well flat bottom tissue culture plates. The lymphocytes were stimulated with 3 µg of each CF-Ag pool in triplicates. Phyto-hemagglutinin (PHA) (2.5 µg) was used as the positive control. The plates were incubated at 37ºC in an atmosphere of 5% CO_2. After 72 h the plates were centrifuged at 2000 rpm for 10 min to separate the supernatants. 100 µl of the supernatant obtained from each reaction was stored in - 80ºC for IFN-γ assay.

10 µL of MTT (3, (4, 5 dimethyl thiazol-2-yl) 2,5 diphenyl tetrazolium bromide) from a stock solution of 5 mg / mL (prepared in PBS) was added to each well. The plates were incubated for 4 h at 37ºC in atmosphere of 5% CO_2. The purple formazan crystals were dissolved by the addition of 100 µl of acidified isopropanol. The absorbance was read at 570 nm with 630 nm as the reference. Proliferation was expressed as stimulation index (SI), which is the ratio of OD_{test} (stimulated with antigen) with $OD_{control}$ (without antigen). SI value > 2 were used as cut-off and values greater than 2 were considered as positive (Sable *et al.,* 2007).

Data analysis was done with SPSS ver. 15.00 software and Minitab. Non-parametric test Mann-Whitney-U test and One-way ANOVA was performed to check the variance among groups. P value of < 0.05 was considered as significant.

2.7.2 Assay of interferon – γ in the culture supernatants of stimulated T cells

The IFN-γ level in the culture supernatant of stimulated T cells (from above) was done using the commercial OptEIA kit, with a detection limit of 2.35 $pg.mL^{-1}$.

Reagents

a) Capture antibody. Stock was diluted 1: 250 in 0.1 M carbonate buffer, pH 9.5 (8.4 g of $NaHCO_3$ and 3.56 g of Na_2CO_3 in 1 L of DDW).

b) Phosphate buffered saline (PBS), pH 7.0. This was prepared by dissolving 8 g of NaCl, 1.6 g of Na_2HPO_4, 0.2 g of KH_2PO_4 and 0.2 g of KCl in 1 L of water.

c) Assay diluent: PBS with 10% Fetal Bovine Serum.

d) PBS/T as wash buffer: PBS with 0.05% Tween-20 (freshly prepared).

e) Primary antibody: The standards for IFN-γ were prepared by dissolving the lyophilized standard in water to a final concentration of 100 µg / mL. It was aliquotted and stored at -80ºC. Standards were prepared by serial dilution of the stock to yield 300, 150, 75, 37.5 and 18.25 pg / mL respectively.

f) Working detector solution: This consists of biotinylated anti-human IFN-γ monoclonal antibody and streptavidin - horseradish peroxidase conjugate, which were prepared fresh and used at 1: 250 dilution.

g) Substrate: Tetramethyl benzidine-hydrogen peroxide (TMB-H_2O_2) was used.

h) Stopper solution: 2N H_2SO_4

Procedure

The assayed was performed according to the manufacturer's instructions. The plates were coated with 100 µL / well of capture antibody and incubated o/n at 4ºC. After three washes, the plates were blocked with 200 µL / well of assay diluent and incubated for 1 h at RT. The plates were washed three times in PBS-T. The standards and the samples were added to their respective wells (100 µL / well) and incubated for 2 h at RT. The assay was performed in duplicates. After washing five times with PBS-T, 100 µL of the working detector was added to each well and incubated for 1 h at RT. The plates were washed 7 times, with 30 sec soaking between each wash. 100 µL of substrate solution (TMB - H_2O_2 at 1: 20 dilution) was added to each well and incubated for 30 min at RT. The reaction was stopped by the addition of 50 µL of stop solution into each well. The absorbance was read at 450 nm. Each sample was tested in duplicate. A standard curve was drawn and the level of IFN-γ in the unknown sample was read from the graph.

2.8.1. Detection of antibodies against the CF proteins in the serum of patients with tuberculosis

2.8.1.1. Enzyme linked immunosorbent assay (ELISA)

Reagents

a) Carbonate / bicarbonate buffer (pH 9.8): 2.9 g of $NaHCO_3$ and 1.5 g of Na_2CO_3 was dissolved in 1 L of double distilled water.

b) 10 X PBS: 80 g of NaCl, 2 g of KCl, 2 g of KH_2PO_4 and 29 g of Na_2HPO_4 was dissolved in 1 L of double distilled water.

c) Blocking solution: 2 % BSA in PBS.

d) Wash solution (PBS-T): PBS with 0.05% Tween 20.

e) The secondary antibody anti-human IgG antibody conjugated to HRP (ready to use solution) was used.

f) TMB / H_2O_2 diluted in 1:20 were used as the substrate.

g) Stop solution- 1N H_2SO_4 was used as the stop solution.

Procedure

500 ng of the different pools of CF-Ags, diluted in carbonate buffer was used to coat the wells of 96-well micro-titre plate and incubated at 37^{o}C for 1 h. The plate was then kept at 4^{o}C o/n. The plate was washed thoroughly in PBS-T 4 times. Blocking was done with 200 µl of 2% BSA and incubated at 37^{o}C for 1 h. After washing, 100 µl of serum (1: 40 dilution) was added to each well and incubated at 37^{o}C for 2 h. After three washes, 100 µl of secondary antibody was added to each well and incubated at 37^{o}C for 1 h. After 3 washes with PBS-T, 100 µl of substrate (TMB / H_20_2) was added to each well and incubated for 10 min at RT. The reaction was stopped with 100 µl 1N H_2SO_4. The colour developed was read at 450 nm. The cut off OD for each fraction was determined by taking the mean OD of the healthy population plus two times the standard deviation of the OD obtained from the healthy individuals (Weldingh *et al*., 2005).

2.8.1.2 Mycotest, a commercial lateral flow device

All serum samples were tested by using the commercial Mycotest (GVK Biosciences, India), which is a lateral flow device coated with recombinant antigens for secretory proteins specific for *M. tuberculosis* (with a sensitivity and specificity of 87 and 90%, respectively, as per manufacturer's instructions). 80 - 100 µl of serum was added, and the detection of a pink band in addition to the control band within 15 min after addition of serum was considered as positive for tuberculosis.

CHAPTER 3

RESULTS

3.1 Iron levels in *Mycobacterium tuberculosis*

3.1.1 Effect of iron levels on mycobactin and carboxymycobactin

3.1.1.1 Establishment of growth of *M. tuberculosis* under high and low iron conditions: assay of the mycobactin and carboxymycobactin

Mycobacterium tuberculosis was grown under low (0.02 µg Fe / mL) and high iron (8µg Fe / mL) conditions after inoculation with 1 ml of culture re-suspended to a density corresponding to McFarland 3. The organisms were grown with shaking and harvested after 14 days of growth.

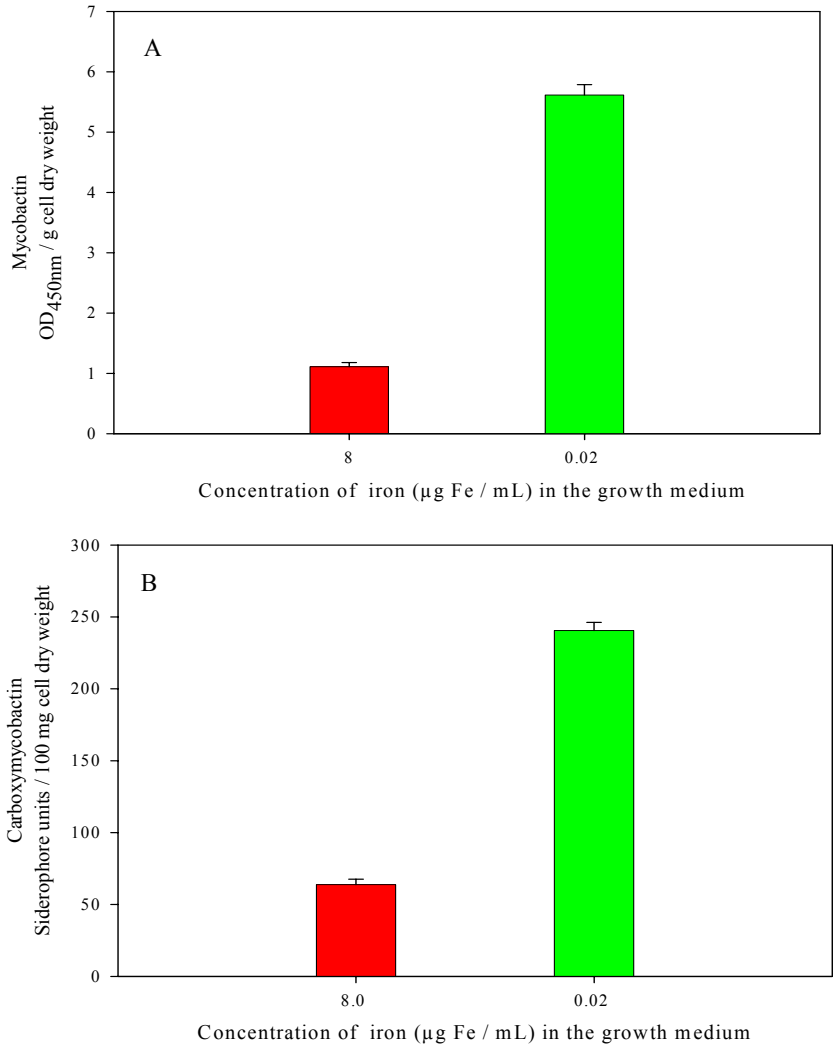


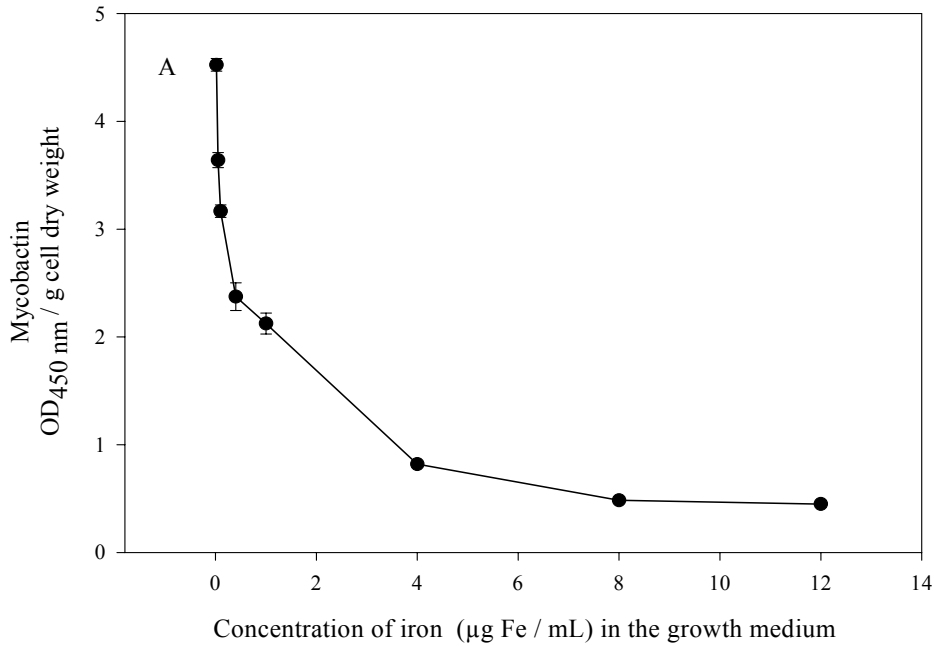
Fig. 3.1 – Iron levels and expression of mycobactin (A) and carboxymycobactin (B) in *M. tuberculosis*. Cells were grown under high (8 µg Fe / mL) and low iron (0.02 µg Fe / mL) conditions. Mycobactin was expressed as OD_{450} units / g cell dry weight and carboxymycobactin is expressed as siderophore units / 100 mg cell dry weight. . The vertical bars represent the standard deviation of the mean from three independent experiments.

Mycobactin was extracted from the cells as ferric mycobactin and assayed spectrophotometrically and carboxymycobactin was assayed by the CAS assay using the spent growth medium directly. Low level of expression of the siderophores were seen in cells subjected to high iron growth while organisms subjected to low iron growth showed about 5 - 6 fold increase in both mycobactin and carboxymycobactin respectively (Fig. 3.1 A & Fig. 3.1 B respectively).

3.1.1.2 Iron-dependant expression of mycobactin and carboxymycobactin

Mycobacterium tuberculosis was subjected to iron levels added to a final concentration of 0.02, 0.05, 0.1, 0.2, 0.4, 1, 4, 8 and 12 µg Fe / mL respectively and grown under the same conditions as above.

14-day old cultures showed that maximal expression of both mycobactin and carboxymycobactin were seen at 0.02 µg Fe / ml (Fig. 3.2 A & B respectively). There was a steady decrease in the levels of both these siderophores with increasing iron concentration in the growth medium, with appreciable levels of both the siderophores upto 0.4 µg Fe / mL. Further increase in the iron levels results in a marked decrease in their expression, with no further changes as can be observed by the plateau seen from 4 – 12 µg Fe / mL.



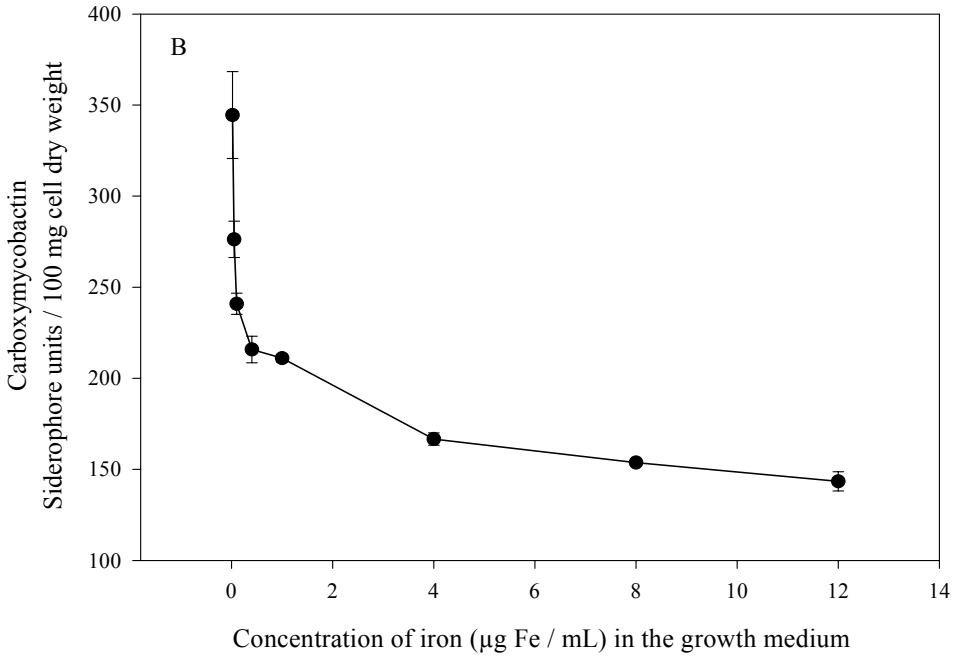


Fig. 3.2 – Iron-dependent expression of mycobactin (A) and carboxymycobactin (B). *Mycobacterium tuberculosis* was grown with iron added at 0.02, 0.05, 0.1, 0.2, 0.4, 1, 4, 8, and 12 µg of Fe / ml respectively and the siderophores were assayed as described in methods. The vertical bars represent the standard deviation of the mean from three independent experiments.

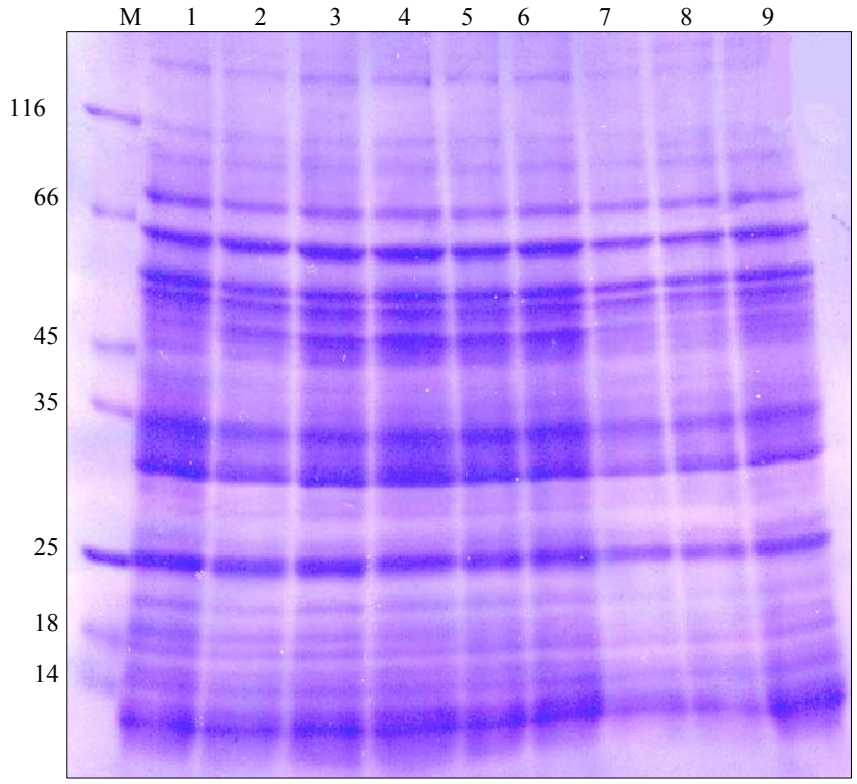


Fig. 3.3 – SDS-PAGE analysis of CFPs of *M. tuberculosis*. Cells were grown with varying levels of iron. The CFPs were analysed on a 5-20% gradient by SDS-PAGE. Lanes 1-9 represent the profile of cells grown with 0.02, 0.05, 0.1, 0.2, 0.4, 1, 4, 8 and 12 µg of Fe / mL respectively; lane M represents molecular weight marker.

3.1.2. Effect of iron levels on the expression of culture filtrate proteins (CFPs): analysis by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

After the removal of cells by centrifugation, the culture filtrate proteins (CFPs) in the spent growth medium were analysed by SDS-PAGE on a 5-20% gradient gel. Due to the low levels of the proteins, they were concentrated by precipitation with varying levels of saturation with ammonium sulphate. As most of the proteins precipitated with 80% saturation, this was followed for subsequent experiments. No significant up-regulation of the CFPs could be observed using single dimension SDS-PAGE (Fig. 3.3).

3.1.3. Time-course studies

3.1.3.1 Time-course expression of mycobactin and carboxymycobactin

The organisms were grown under high (8 μg of Fe / mL) and low iron (0.02 μg of Fe / mL) conditions, as described earlier and the cells were harvested on days 4, 8, 12, 16, 20 and 24 respectively. Both the siderophores were assayed and the CFPs were analysed on a 5-20% gradient gel by SDS-PAGE using the traditional Tris-glycine buffer system. In addition, as low molecular weight proteins of *M. tuberculosis* are of importance in host-pathogen interactions, the CFPs were additionally subjected to Tris-Tricine buffer system using 10% gel.

Fig. 3.4 A & B show the alterations in the levels of mycobactin and carboxymycobactin with increasing period of growth. Both mycobactin and carboxymycobactin levels were unaffected in organisms grown under high iron conditions. The levels are relatively higher than that seen in Fig. 3.1 as the inoculum used for this experiment was higher, with 2 ml of McFarland 3 used to obtain appreciable cell dry weight.

When grown under low iron conditions, there was a steady increase in the level of expression of both the siderophores. A steady increase in the expression of both of them was observed, with maximal expression of carboxymycobactin on day 16 with the mycobactin level reaching steady state on day 20. There is a slight decrease apparently on day 24 in both the siderophores, the reason for which is unclear.

3.1.3.2 Time-course expression of CFPs

As observed earlier, SDS-PAGE using Tris-glycine buffer system showed no obvious differences in the protein profile (Fig. 3.5 A). Tris-Tricine buffer system, on the other hand resulted in better protein resolution (Fig. 3.5 B). Several proteins were up regulated under low iron conditions. Notable among them were four proteins of approximate molecular mass 60, 34, 27, 19 and 8 kDa respectively.

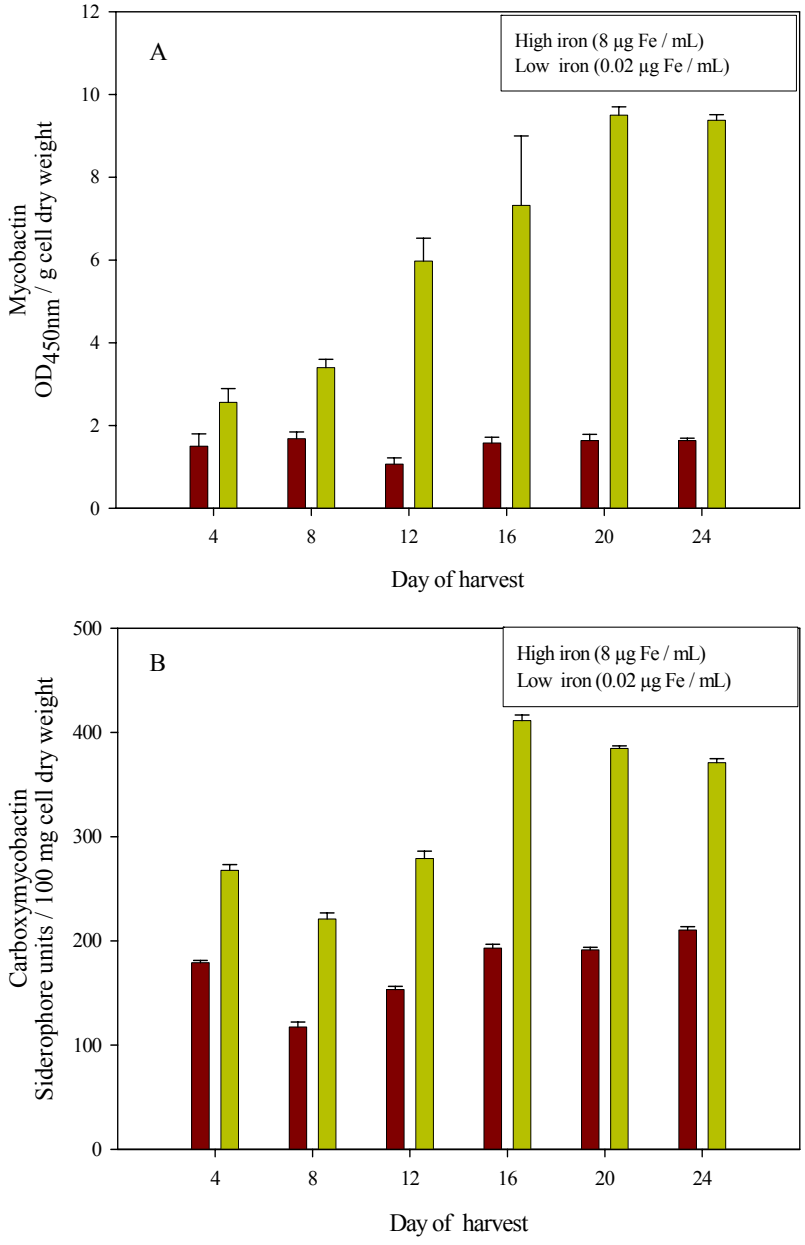


Fig. 3.4 – Time-course expression of mycobactin (A) and carboxymycobactin (B). *Mycobacterium tuberculosis*, grown under high iron (8 µg Fe / mL) and low iron (0.02 µg Fe / mL) were harvested on days 4, 8, 12, 16, 20 and 24 respectively and mycobactin and carboxymycobactin levels assayed**.** The vertical bars represent the standard deviation of the mean from three independent experiments.

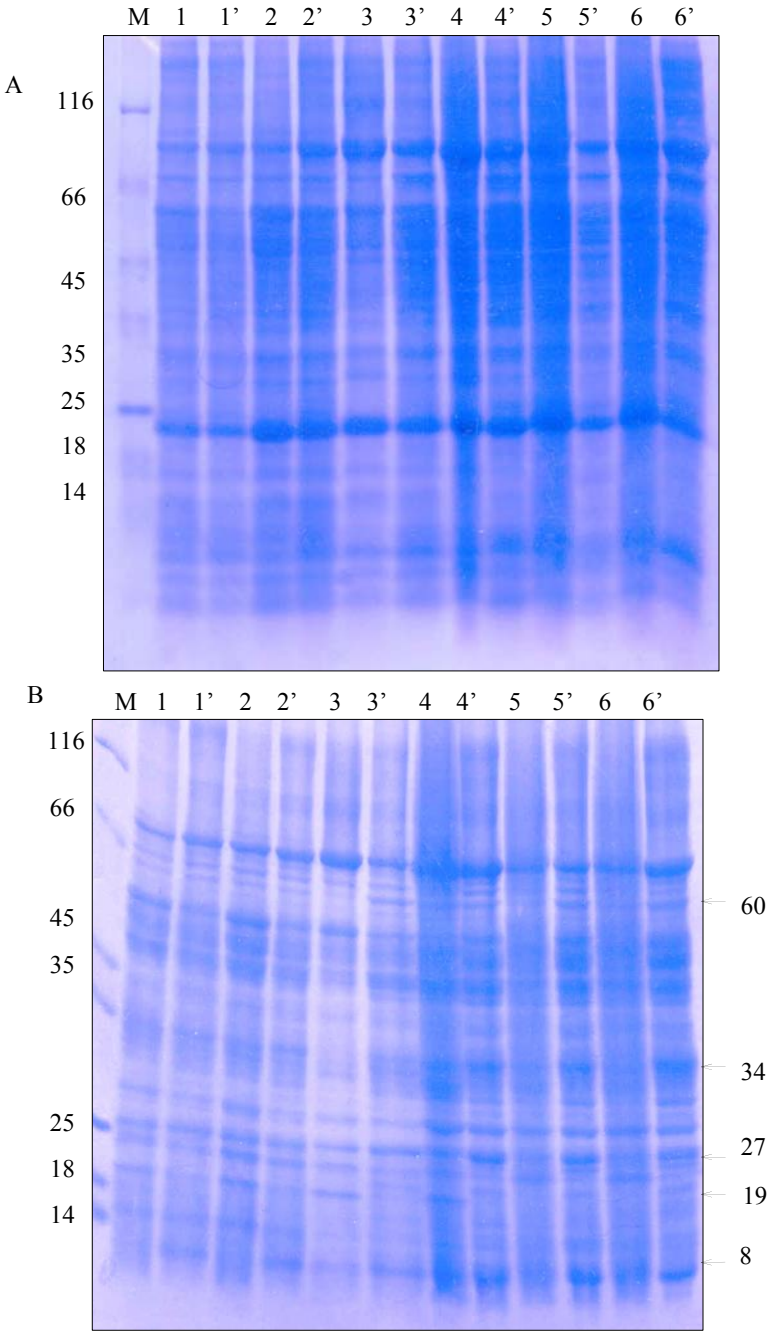


Fig. 3.5 – CFP profile of *M. tuberculosis* - time course study. Panels A and B represent identical sets of CFPs separated by Tris-glycine and Tris – Tricine buffer system respectively. Lanes 1 to 6 represent CFPs harvested on days 4, 8, 12, 16, 20 and 24, with each pair of lanes (for eg. 1 and 1') representing high (8 µg Fe / mL) and low iron (0.02 µg Fe / mL) cells respectively.

3.1.4 Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) of CFPs

Though no apparent differences were observed in the protein profiles of CFPs separated by single dimension SDS-PAGE, 2D-PAGE resolved the proteins into discrete spots and several proteins influenced by iron levels were identified. Proteins up regulated in organisms grown under high (8 µg Fe / mL) and low (0.02 µg Fe / mL) iron conditions are represented in Fig. 3.6. The iron-regulated proteins are represented as CFP with their respective molecular mass written as superscript, with Panel A showing those proteins up-regulated under high iron conditions and Panel B showing all the proteins induced upon iron limitation. Identification of all these proteins was not possible and Table 10 lists some of the proteins identified. The spots were randomly selected and subjected to MALDI-TOF analysis (shown in red in Fig. 3.6), while some were identified by co-migrational analysis by comparison with the profile from Statens Serum Institute (Fig. 3.7). Additionally, antiserum against specific CFPs, obtained from Statens Serum Institute were used to identify some of the spots (shown in green in Fig. 3.6).

3.1.4.1 MALDI-TOF analysis of CFPs

Several proteins were up regulated in the presence of 8 µg Fe / mL in the medium. Two of these proteins, namely CFP^{32} and CFP^{23} were subjected to MALDI-TOF analysis and identified as proteins encoded in the genome of *M. tuberculosis*. The former was annotated as MCE1B (Rv0170), a member of the MCE family of proteins, with a putative role in host cell invasion. The latter was identified as Rv0660, a conserved hypothetical protein, possibly an antitoxin that is part of toxin-antitoxin (TA) operon along with Rv0659c, encoding the toxin.

Among the several proteins induced upon iron limitation, 7 proteins were identified by MALDI-TOF analysis. CFP^{70} was shown to be DnaK (Rv0350), a probable chaperone protein induced under conditions of stress, implicated mainly as a heat shock protein. It possibly has an ATPase activity. CFP^{31} (Rv2096c) was identified as a hypothetical protein whose function is unknown.

Three proteins spots of about 22 kDa with slightly differing pI were labeled as CFP^{22a}, CFP^{22b} and CFP^{22c} respectively; the former and latter were up regulated upon iron limitation, while CFP^{22b} was secreted in organisms grown under high iron conditions. CFP^{22a} was shown to be Rv0881 with possible rRNA methyl transferase

activity, belonging to the rRNA methylase family of proteins. The function of CFP^{22c} remains unknown as it is annotated as a hypothetical protein.

Among the low molecular weight proteins, CFP^{10} protein was found to be the pre-protein translocase SecE1 subunit and CFP^{8} (Rv0651) protein was identified as probable 50 S ribosomal proteins L10RP.

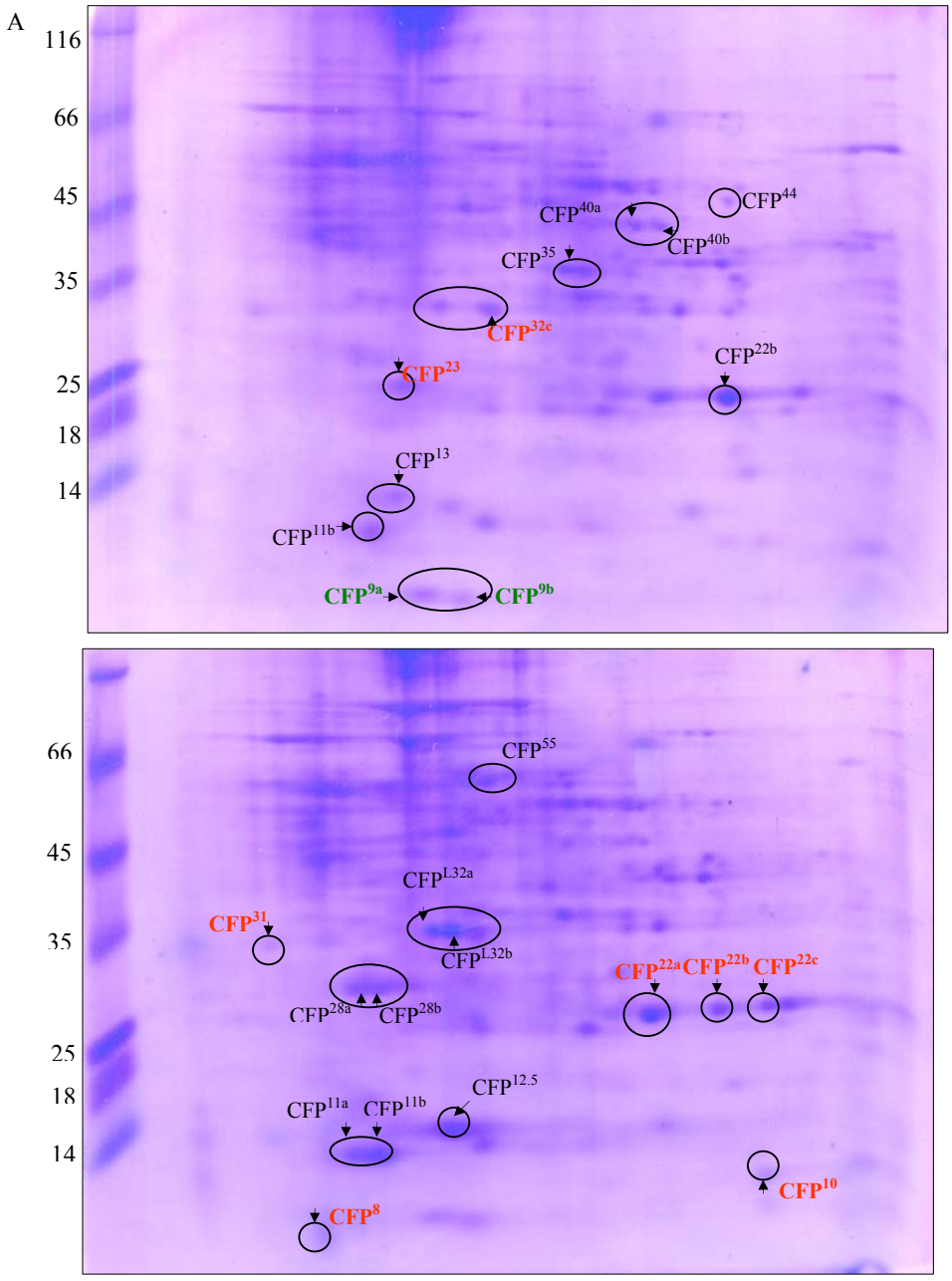


Fig. 3.6 – 2D PAGE analysis of CFPs of *M. tuberculosis*. CFPs of cells grown under high (8 µg Fe / mL; Panel A) and low iron (0.02 µg Fe / mL; Panel B) were subjected to 2D – PAGE. The second dimension was done using the Tris-Tricine system. The up-regulated CFPs were labeled in the respective high iron or the low iron condition.

Table 9- Identification of iron-regulated proteins in *Mycobacterium tuberculosis* grown under high (8 ug Fe / mL) and low (0.02 ug Fe / mL) iron conditions respectively.

Iron-regulated CFP	Up regulation in medium with		Identification of the iron-regulated CFP
	8 µg Fe /mL (High Iron)	0.02 µg Fe / mL (Low Iron)	
CFP^{70}		+	Rv0350, DnaK, a probable chaperone protein-DnaK (MALDI-TOF MS)
CFP^{65}	+		ND
CFP^{55}		+	Rv2220, Glutamine synthetase (co-migration studies)
CFP^{44}	+		ND
CFP^{40a}	+		ND
CFP^{40b}	+		ND
CFP^{35}	+		ND
CFP^{30a}		+	ND
CFP^{30b}		+	ND
CFP^{30c}	+		Rv0170, Mce1B protein (MALDI-TOF MS)
CFP^{31}		+	Rv2096c, hypothetical protein (MALDI-TOF MS)
CFP^{28a}		+	ND
CFP^{28b}		+	ND
CFP^{23}	+		Rv0660c, Conserved hypothetical protein (MALDI-TOF MS)
CFP^{22a}		+	Rv0881, possible rRNA methyl transferase (MALDI-TOF MS)
CFP^{22b}	+		Rv2602, conserved protein (MALDI-TOF MS)
CFP^{22c}		+	Rv0543c, hypothetical protein (MALDI-TOF MS)
CFP^{13}	+		ND
CFP$^{12.5}$	+		ND
CFP^{11a}		+	Rv3418, GroES protein (co-migration studies)
CFP^{11b}		+	ND
CFP^{10}		+	Rv0638, Probable pre-protein translocase-SecE1 (MALDI-TOF MS)
CFP^{9a} & CFP^{9b}	+		Rv3875, ESAT-6 group of proteins (immuno-blotting with specific monoclonals)
CFP^{8}		+	Rv0651, Probable 50S ribosomal protein (L10 RP) (MALDI-TOF MS)

CFPs from low iron organisms are indicated in blue and CFPs from high iron organisms is represented in red.

3.1.4.2. Identification of CFPs by co-migrational analysis and immunoblotting with anti-CFP antibodies.

Figure 3.7 shows the 2D-PAGE profile of CFPs of *M. tuberculosis*, published by Statens Serum Institute. Comparison of the spots in Fig. 3.6 with the above figure and immunoblotting analysis (Fig. 3.8) with polyclonal antibodies against whole CFPs (gift from Dr. Ida Rosenkrandts (Statens Serum Institute) helped to identify several of the CFPs. Among them, the two iron-regulated proteins CFP^{55} and CFP^{11a} were identified as Rv2220, glutamine synthetase glnA belonging to the glutamine synthetase family of proteins involved in glutamine biosynthesis and Rv3418, the chaperonin GroES protein, also called as 10 kDa protein.

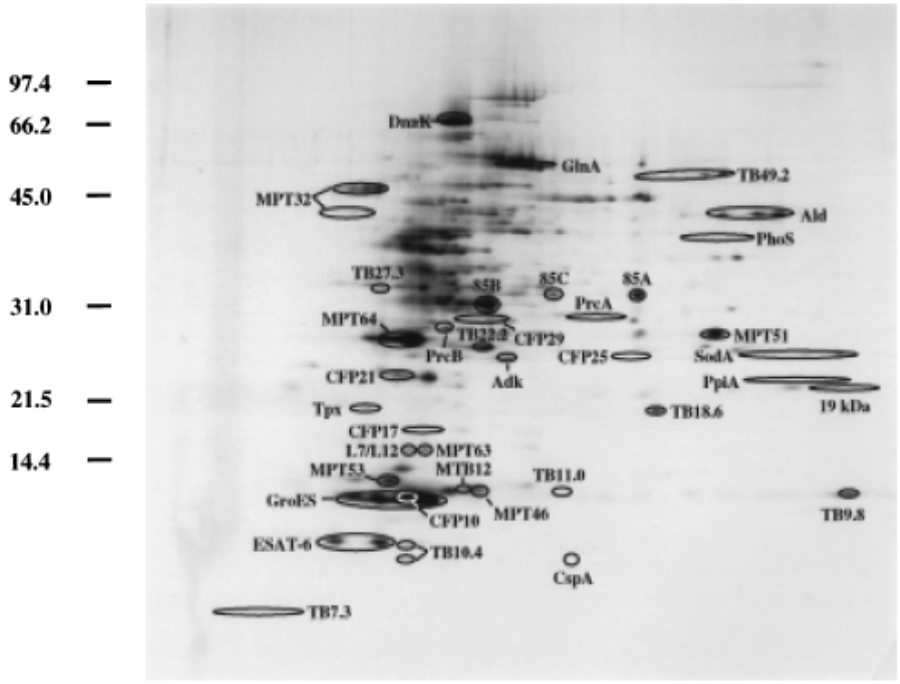


Fig. 3.7 - 2D map of the CFPs published by Statens Serum Institute. Proteins were identified by 2D-PAGE analysis followed by N-terminal sequencing and Immunoblotting with monoclonal antibodies. (Rosenkrands *et al.*, 2000).

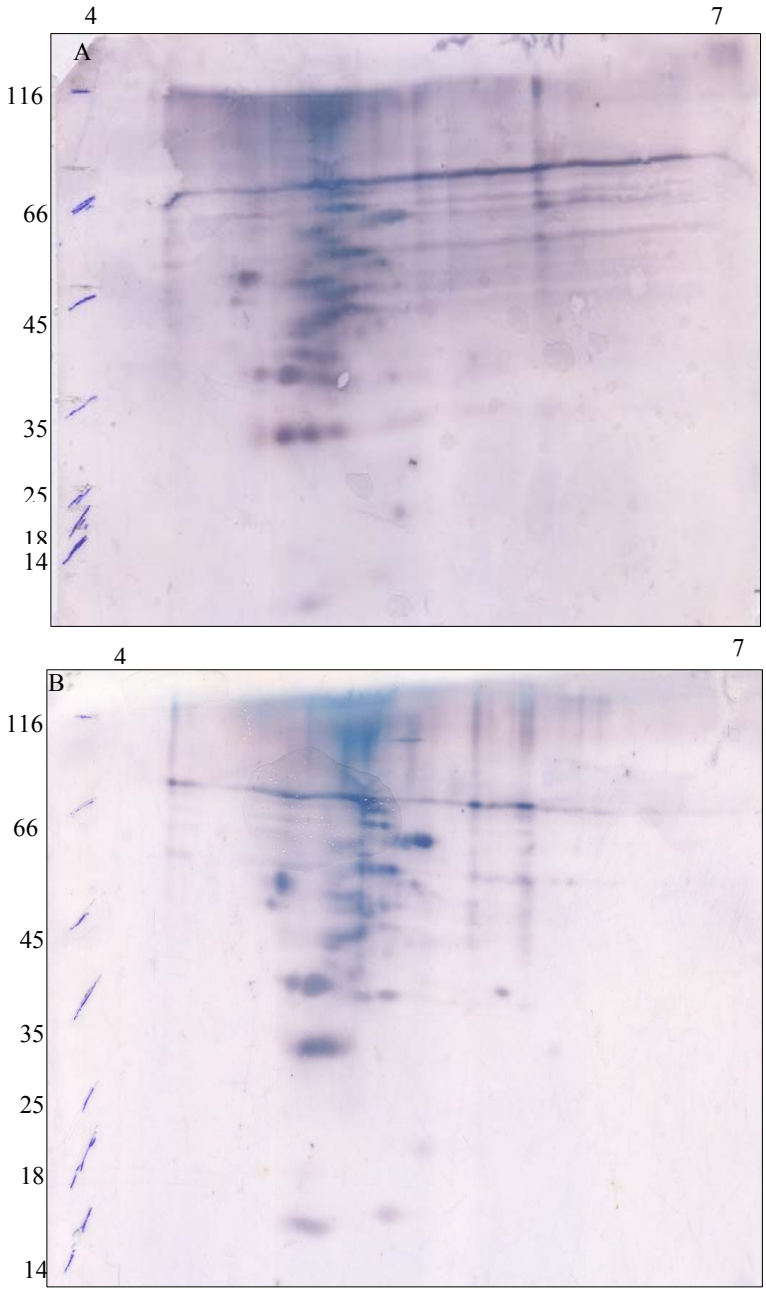


Fig. 3.8 – Immunoblot analysis of *M. tuberculosis* CFPs with polyclonal anti-H37Rv CFP antibodies. Panels A and B represent CFPs of *M. tuberculosis* grown under high iron and low iron condition that were subjected to 2D-PAGE and Western blotting with anti-H37Rv CFP antibodies.

Monoclonal antibody against MPT-64 protein identified the 24 kDa protein in both high and low iron preparations (Fig. 3.9).

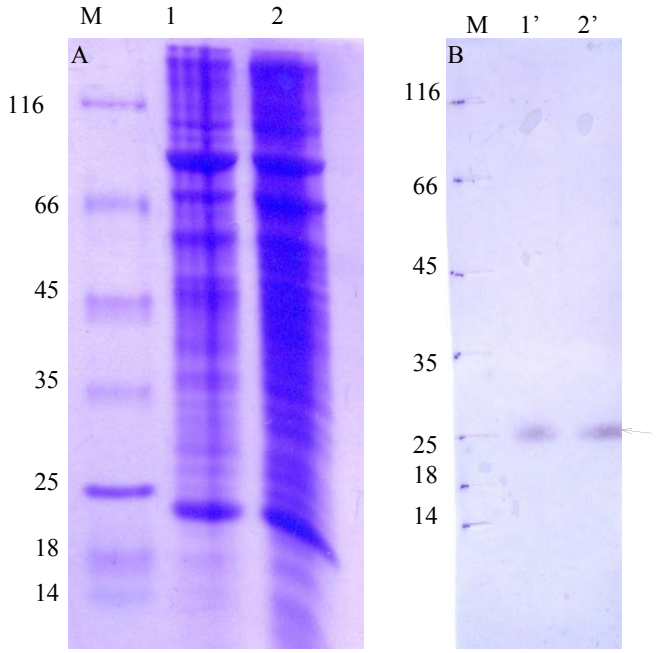


Fig. 3.9 – Immunoblot analysis of *M. tuberculosis* CFPs with monoclonal antibodies against MPT64. Panels A and B represent 10% Tris-Tricine SDS-PAGE and immunoblot probed with L24B5 (anti-MPT64) antibodies. Lanes 1 and 2 represent high and low iron CFPs respectively

3.1.4.3. Identification of ESAT-6 proteins with specific monoclonal antibodies

ESAT-6 specific monoclonal antibody (HYB 76.8) was used in independent experiments to identify the ESAT-6 group of proteins. The CFPs of high and low iron *M. tuberculosis*, separated by SDS-PAGE using Tris-Tricine buffer system, were probed with HYB 76.8 (Fig. 3.10). Two clear bands of activity at about 9 and 8 kDa was seen, with a relatively higher signal with the upper band. Correspondingly, the low iron sample showed lower level of immunoreactivity with the 9 kDa band with no visible 8 kDa band.

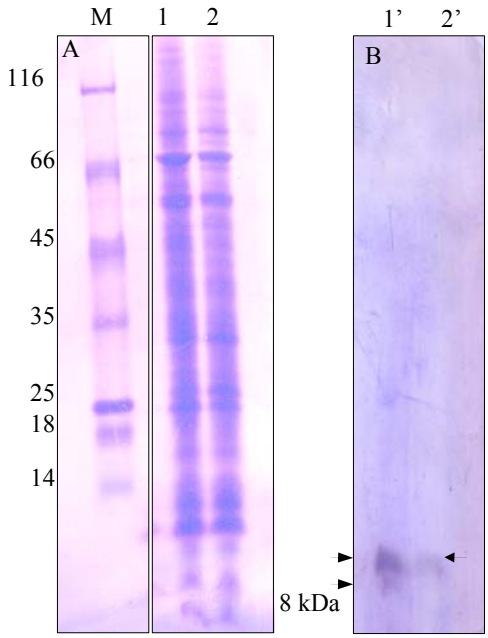


Fig. 3.10 – Immunoblot analysis of ESAT-6 proteins of *M. tuberculosis.* CFPs of cells grown under high 8 µg Fe / mL and low iron 0.02 µg Fe / mL were subjected to SDS-PAGE on 10% Tris –Tricine system. Immuno-blotting was done with HYB 76.8 (ESAT- 6 specific) monoclonal antibodies. Lanes1 and 2 represent whole CFPs of cells grown under high (8 µg Fe / mL) and low iron condition (0.02 µg Fe / mL) respectively.

2D-PAGE immunoblotting further confirmed this observation. CFPs from high (Panel A) and low (Panel B) iron cultures, subjected to 2D-PAGE and immunoblotting with HYB 76.8 is represented in Figure 3.11. At a pI of about 4.8, two distinct protein spots of approximate molecular mass of 9 kDa, with a slight difference in the pI were seen in high iron grown organisms. An additional ESAT-6 protein of about 8 kDa was seen in Panel A, which was notably absent in the low iron sample in which the two 9 kDa spots were clearly evident.

ESAT-6 expression was monitored in the culture filtrate by harvesting the organisms on days 4 – 20 after inoculation (Fig. 3.12). The monoclonal antibody HYB 76.8 did not detect any ESAT-6 in the early samples. This protein was detected in the culture filtrate of *M. tuberculosis* harvested on days 12, 16 and 20, with high level of expression under high iron conditions.

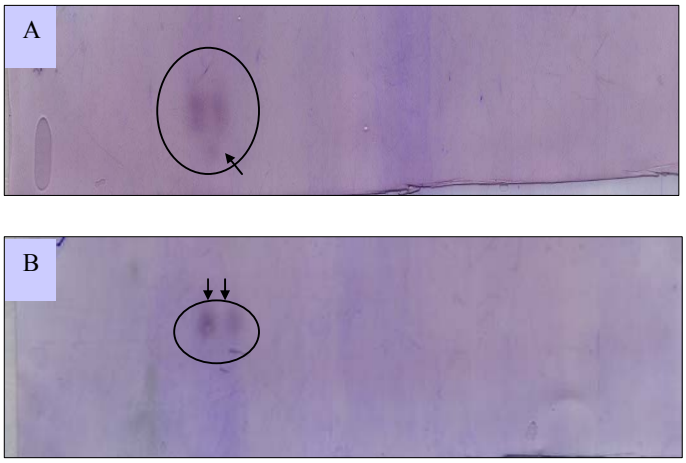


Fig. 3.11. Identification of ESAT-6 antigens with monoclonal antibody HYB 76.8. Panels A & B represent the CFPs of high and low iron organisms of *M. tuberculosis*, subjected to 2D-PAGE and Western blotting with HYB 76.8 (obtained from Statens Serum Institute). The arrows show the two protein spots in Panel A and the additional spot in Panel B.

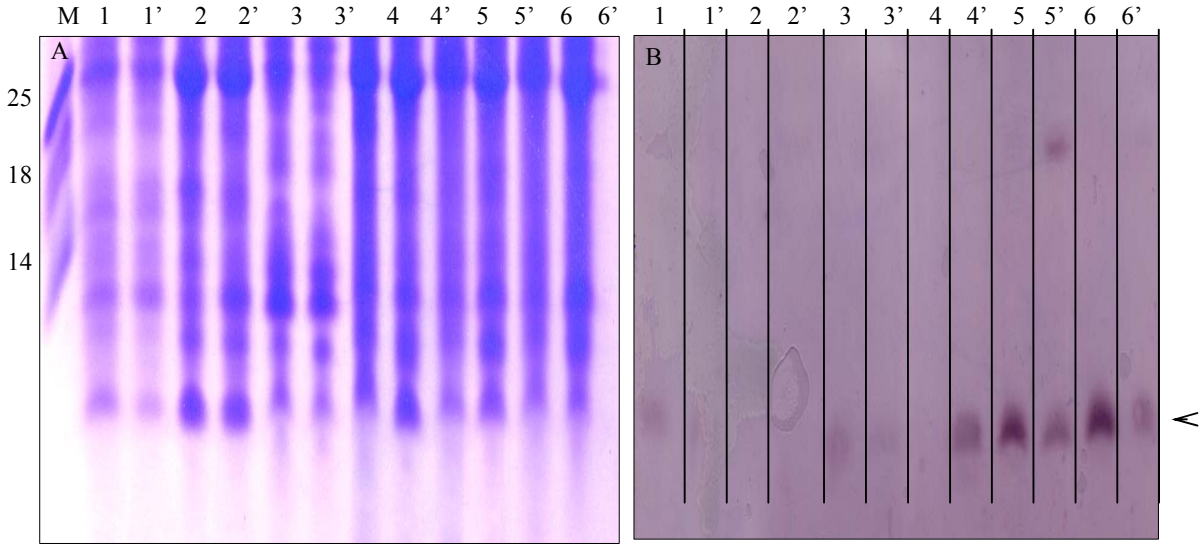


Fig. 3.12 – Time course expression of ESAT 6. Panel A and B represent identical sets of CFPs separated on 10% Tris-Tricine system. Panel A represents the proteins stained with Coomassie Blue and Panel B is the Western blot developed with HYB.76.8. Lanes 1 to 6 represent CFPs harvested on days 4, 8, 12, 16, 20 and 24, with each pair of lanes (for eg. 1 and 1') representing high (8 µg Fe / mL) and low iron (0.02 µg Fe / mL) cells respectively.

3.2 Iron levels in *M. bovis* BCG strains

Mycobacterium bovis BCG comprises several strains and in this study, the response of the various strains to iron levels was analysed. The different strains included *M. bovis* BCG Denmark, Birkhaug, Phipps, Moreau, Sweden and Pasteur.

All the BCG strains responded to iron deprivation and expressed significant levels of both mycobactin and carboxymycobactin (Fig. 3.13 A & B). Maximal level of mycobactin was expressed by Danish strains, with the lowest expression in Russia. Significant expression of carboxymycobactin was observed with almost equal level of expression seen in the strains in this study.

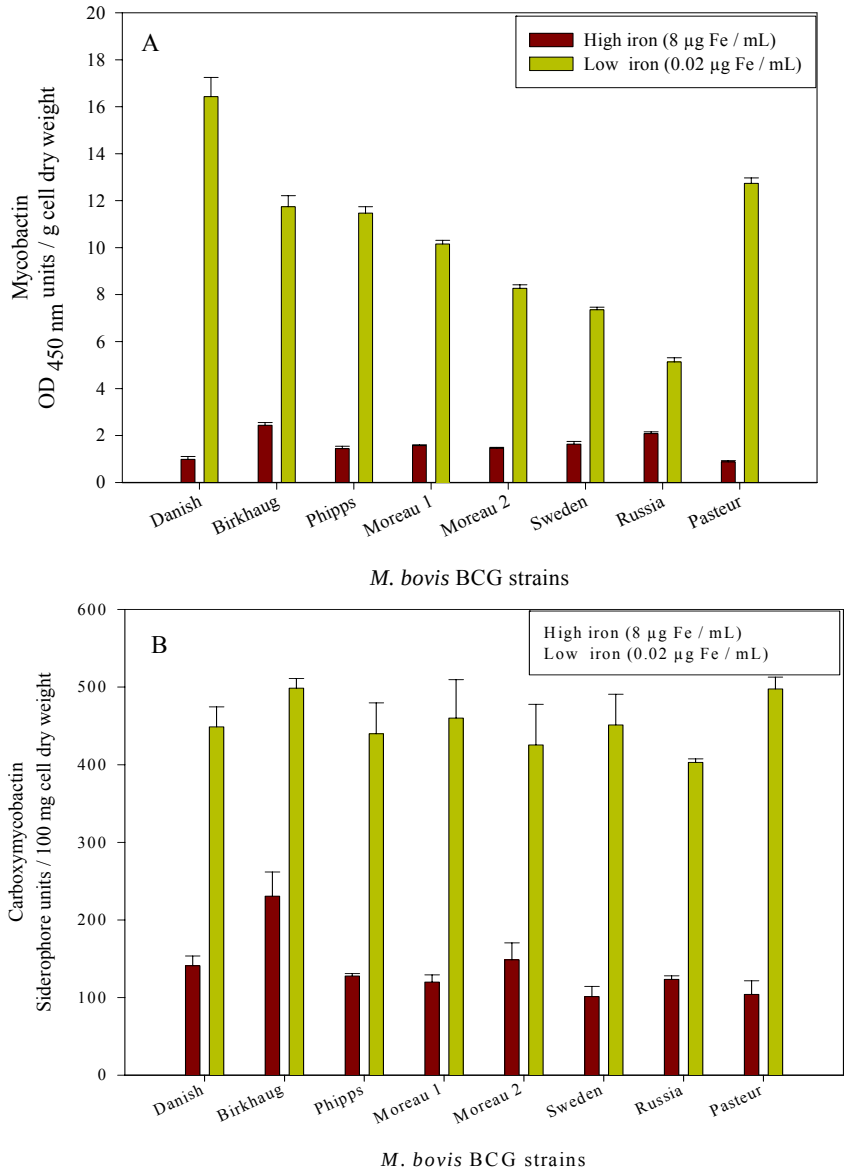


Fig. 3.13 – Iron-dependent expression of siderophores by different *M. bovis* BCG strains. Panel A and B represent expression of mycobactin and carboxymycobactin respectively by different BCG strains grown under high iron (8 µg Fe / mL) and low iron (0.02 µg Fe / mL) conditions. The vertical bars represent the standard deviation of the mean from three independent experiments.

The CFPs of all the strains were concentrated by 80% ammonium sulphate precipitation and subjected to SDS-PAGE on a 5-20% gradient gel using the Tris-glycine buffer system (Fig 3.14). Marked differences in the protein profile were observed, both between high and low iron strain of BCG as well as among the different BCG strains.

A constitutively expressed 60 kDa protein was seen in Danish, Birkhaug and Phipps and was absent from other strains. A 52 kDa protein was seen as a major band in CFPs from high iron grown Russia strain, with a prominent band of about 50 kDa seen in low iron sample. The protein profile of strain Russia was quite different from other strains. The 54 kDa protein was also seen in Phipps in both high and low iron grown cells, while in Moreau, it was evident in high iron grown cells with no detectable band in low iron grown organisms. Danish, Sweden, Pasteur and Birkhaug showed very faint bands corresponding to the 54 kDa band (Fig. 3.14).

Another notable protein expressed under low iron conditions is the 29 kDa protein, reaching about 7-8 times the levels in high iron grown organisms. This protein is seen in Danish, Birkhaug, Phipps, Moreau and Sweden and is not detectable in other strains. As this corresponded to the cell-wall associated iron-regulated HupB protein (Yeruva *et al*., 2006), western blot analysis was done with anti-HupB antibodies (Fig. 3.15) However, anti-HupB antibodies did not show any reactivity with the 29 kDa band nor with any other protein band.

Like the 29 kDa protein, a low molecular weight 8 kDa protein was iron-regulated, with high levels of the protein seen upon iron limitation seen in the strains Danish, Birkhaug, Phipps, Moreau, Sweden and Russia. A 16 kDa band was present only in strain Phipps and Sweden. The former strains, except BCG Russia also elaborated a 5 kDa protein under low iron conditions of growth, though the absolute amounts of the expressed protein was low.

Table 10. Summary of Proteins induced in different BCG strains.

Protein (kDa)	Russia	Moreau	Sweden	Birkhaug	Danish	Phipps	Pasteur
60	-	-	-	C	C	C	-
54	-	-	?	?	?	C	?
29	-	I	I	I	I	I	-
16	-	-	I	-	-	I	-
8	I	I	I	I	I	I	-
5	-	I	I	I	I	I	C
HupB (IREP)	-	I	C*	C*	I	C*	C*

I - induced under iron limitation and C – constitutively expressed proteins.

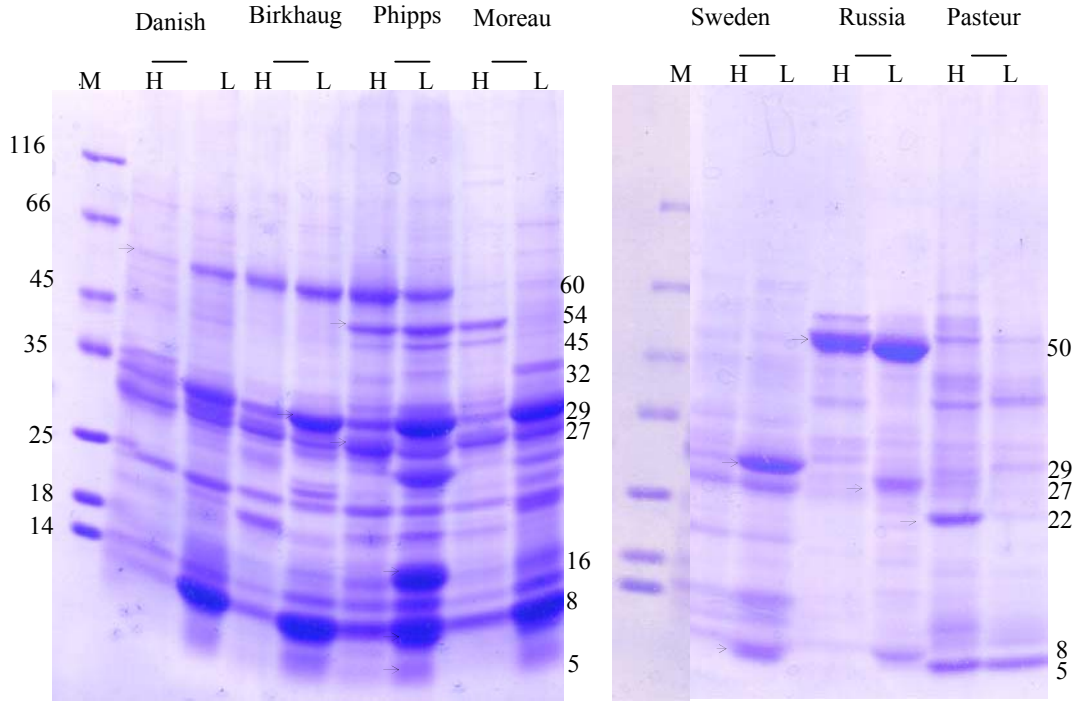


Fig. 3.14- Protein profile of the CFPs in *M. bovis* BCG strains. H and L represent high and low iron CFPs from the different *M. bovis* BCG strains as indicated. M represents molecular weight marker.

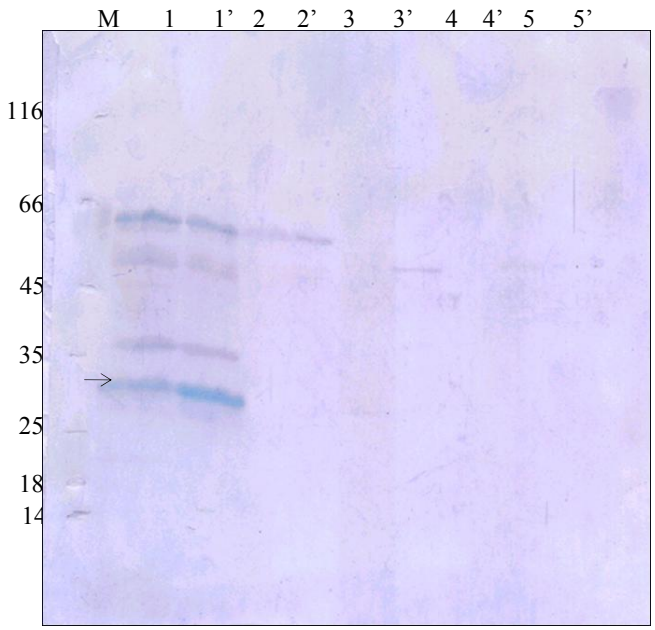


Fig. 3.15. Immunoreactivity of cell wall proteins and CFPs of *M. tuberculosis* and *M. bovis* BCG strains with anti-HupB antibodies. Lanes 1-5 represent proteins of cell wall detergent phase of *M. tuberculosis*, CFPs of *M. tuberculosis,* CFPs of *M. bovis* BCG strains Moreau 1,2 and Danish respectively with each pair of lanes (for eg. 1 and 1') representing high (8 µg Fe / mL) and low iron (0.02 µg Fe / mL) cells respectively. The HupB is expressed as a 28 kDa protein only in the cell wall detergent phase of *M. tuberculosis.*

3.3 Gel elution and preparation of culture filtrate antigen pools (CF-Ag pools) from *Mycobacterium tuberculosis*

3.3.1. Preparative SDS-PAGE and gel elution of CF-Ag pools

The CFPs were separated by SDS-PAGE using the Tris-glycine buffer system, Fig.3.16. 5 sets (each set consisting of the respective high and low iron sample) of antigen pools were prepared by cutting gel slices between defined molecular markers and eluting the protein into Tris-HCl buffer with overnight stirring. These samples were concentrated by ultrafiltration and subjected to single dimension SDS-PAGE to verify the different fractions. Fig. 3.17 A & B shows the 5 sets of CF-Ag pools; the Tris-Tricine buffer system was used for the low molecular weight proteins.

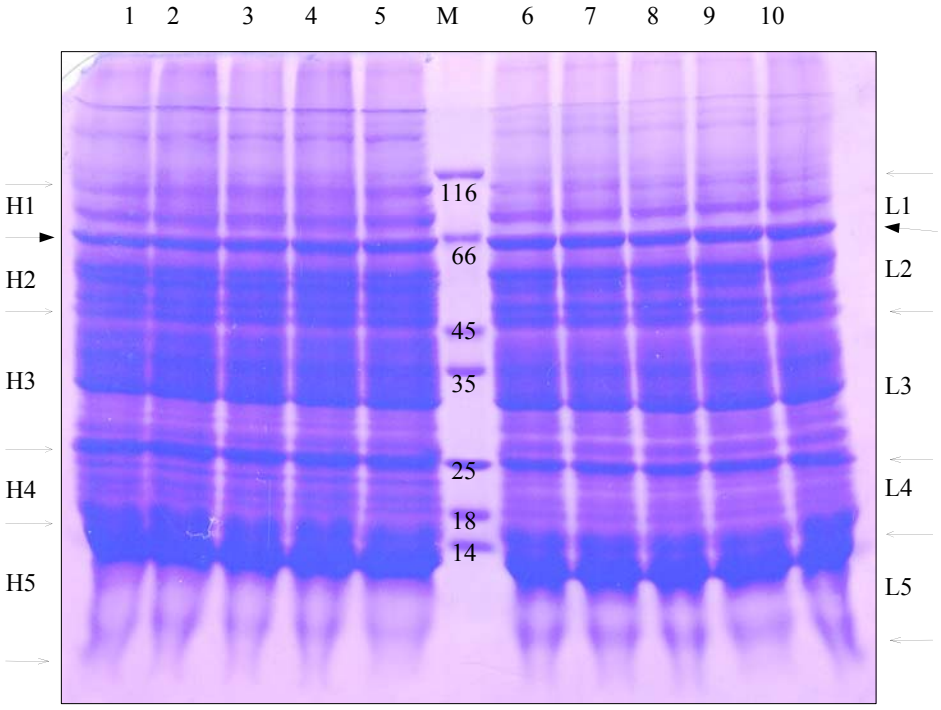


Fig. 3.16 - Preparative SDS –PAGE analysis of *M. tuberculosis* CFPs. CFPs were subjected to preparative 5-20% gradient SDS –PAGE. Lanes 1 - 5 represent CFPs of cells grown under high iron (8 µg Fe / mL) condition and lanes 6 -10 represent those of low iron (0.02 µg Fe / mL) condition.

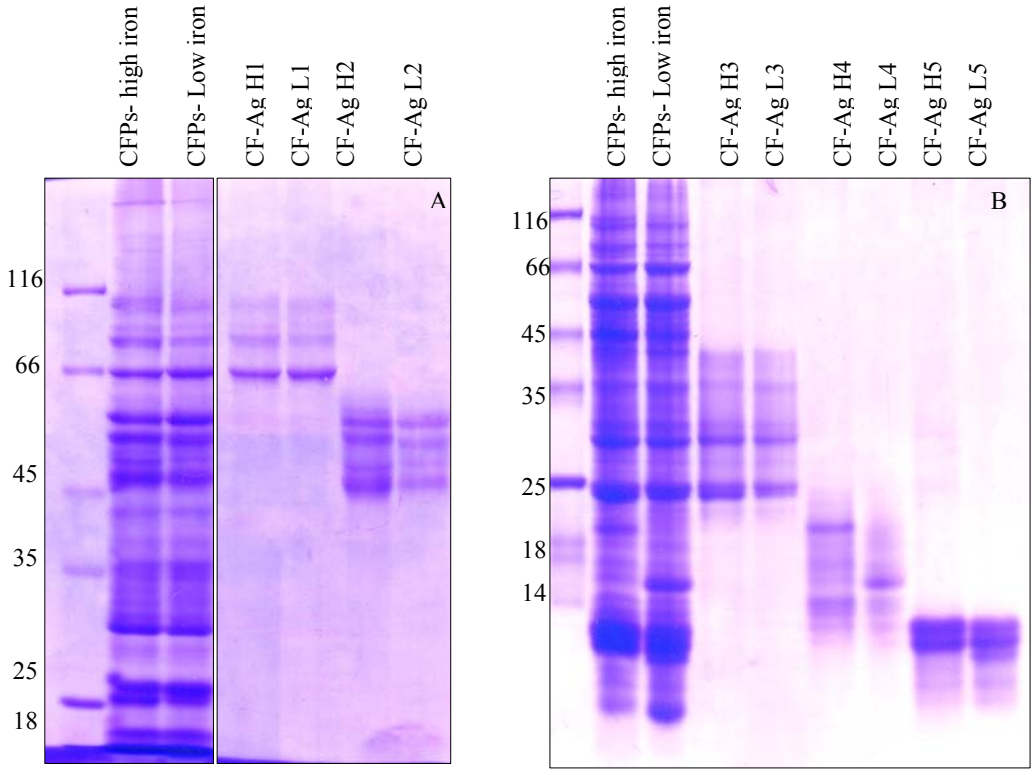


Fig. 3.17 – SDS-PAGE analysis of CF-Ag pools. Panels A and B represent the crude CFPs and CF-Ag pools subjected to electrophoresis using Tris-Glycine and Tris-Tricine buffer system.

3.3.2. 2D-PAGE of the CF-Ag pools

All the five sets of CF-Ag pools were analysed by 2D-PAGE analysis. Fig. 3.18 shows the 5 sets compiled together. The protein profile of these CF-Ag pools were compared with the protein profile of the whole CFP profile of high and low iron grown cells. All these antigen pools contained the iron-regulated proteins plus the proteins seen in both high and low iron grown samples. Based on comigrational analysis, some of the antigens could be identified. For eg. the CF-Ag H3 / L3 fraction was probably comprised of the probable iron-regulated elongation factor (EF-Tu, 43.59 kDa, pI 5.1), the periplasmic phosphate binding protein (pst SI) a (38.2 kDa pI 5.02), the secreted antigen 85 complex including the Antigen 85 A, 85 B, 85 C (33.6 kDa, pI 6.5, 32.5 pI 5.7, 33.7 pI 6.2) were present in this antigen pool. The secreted MPT 51 (31 kDa, pI 6.6) and MPT 64 (24.8 kDa, pI 4.5) were included in this fraction. The CF-Ag pool H4 / L4 was supposedly containing CFP 21 (21.78 kDa pI 5.8), TB 22.2 (24.4 pI 5.05),19 kDa lipo-protein (15.1 kDa, pI 7.18) and TB 18.6 (18.6 kDa, pI 5.52).

The proteins ranging within the molecular mass of 12 kDa and 5 kDa were included in the CF-Ag H5 / L5 pools. The prominent proteins in this antigen pool include those with approximate molecular mass 13, 11, 10 and 9 kDa. The ESAT-6 family of proteins were detected by immunoblotting with ESAT-6 monoclonal antibodies (HYB 76.8). CFP^{10} and CFP^{8} up regulated in the CF-Ag L5 were absent in the corresponding high iron sample CF-Ag H5. The reported proteins in this region are the ESAT – 6, (9.9 kDa), the 10 kDa chaperonin GroES protein (10 .8 kDa pI 4.34) and TB 10.4 (10.3 kDa and 4.3 pI).

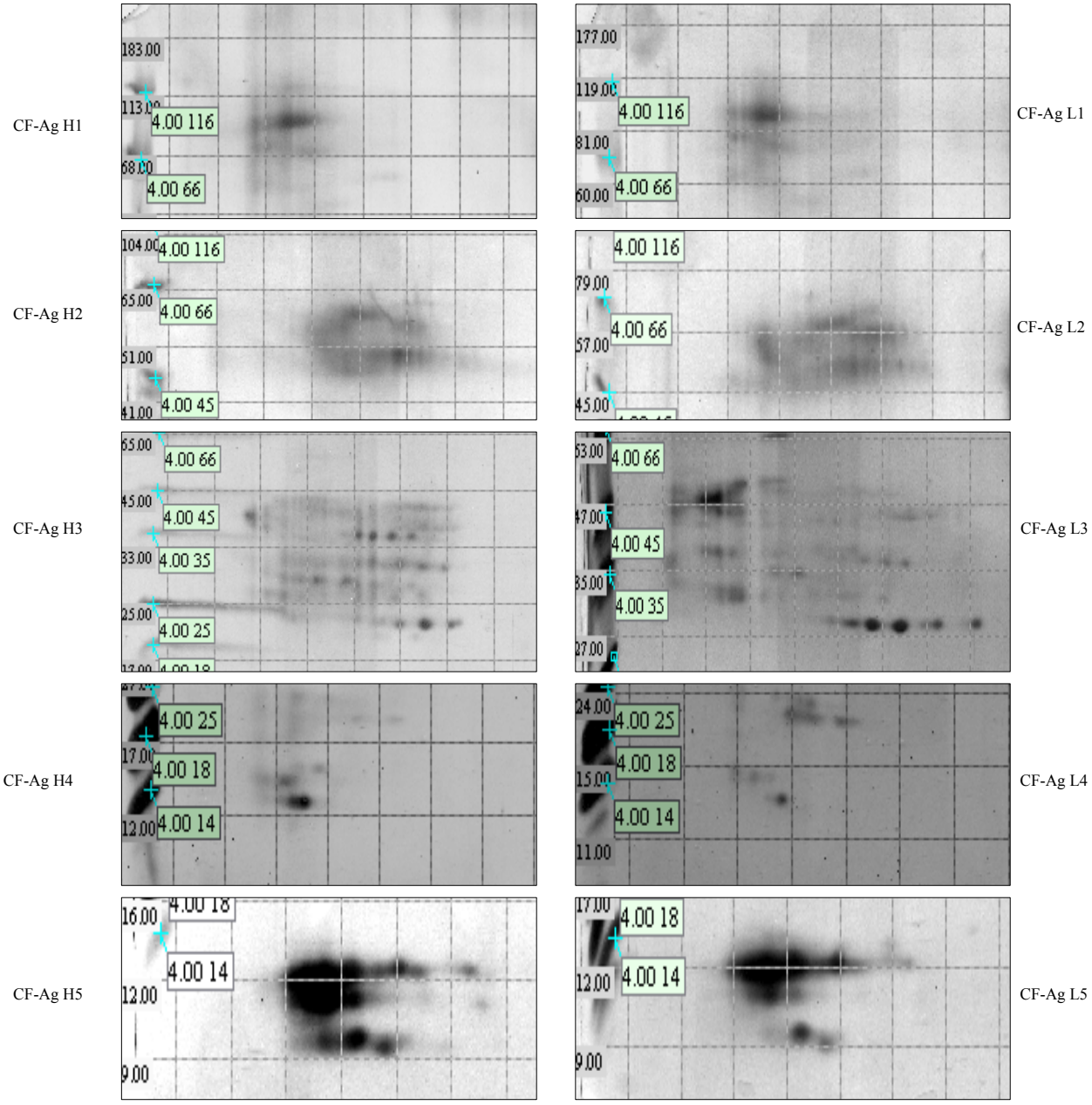


Fig. 3.18 – Analysis of CF-Ag pools by 2D-PAGE. CF-Ag pools. CF-Ag pools represented in Fig. 3.16 were separated on 2D-PAGE. CF-Ag pools H1-H5 represent fractions from CFPs of high iron grown cells and L1-L5 represent CF-Ag pools from low iron grown cells.

3.4. Immune response studies with iron-regulated culture filtrate antigens

T cell immune response of tuberculosis patients to iron-regulated culture filtrate antigens

The T cell immune response studies were done with PBMCs isolated from smear positive (n = 25), smear negative (n = 20), extra-pulmonary (n = 15) and control group consisting of normal healthy individuals (n = 20). The lymphocyte proliferation and interferon - gamma (IFN-γ) levels produced by the four groups of individuals on stimulation with the crude CFPs and CF-Ag pools (culture filtrate antigen pools) were measured and compared with the response produced by PPD. CF-Ag pools included proteins from high iron (H1-H5) and low iron (L1-L5) grown *M. tuberculosis* (prepared as discussed in Methods). Data was analysed using SPSS ver. 15 software.

3.4.1. Immune response of PBMCs to whole CFPs and PPD

Initially, the proliferation response of the PBMCs to unfractionated high and low iron CFPs was assayed in the four groups of subjects in the study and compared with the response evoked by PPD. All of them induced prominent lymphocyte proliferation (Fig.3.19) in smear positive pulmonary tuberculosis patients (median SI of about 2.5) with slightly lower values in smear negative cases. The proliferative response of extra pulmonary cases and normal individuals showed relatively lower levels of stimulation to both CFPs and PPD (median SI < 2.0). No major differences were observed in the proliferative response of the PBMCs to high and low iron CFPs.

3.4.2. Immune response studies with defined CF-Ag pools

3.4.2.1. Lymphocyte response of normal healthy individuals

Normal healthy individuals, serving as controls in this study did not react to any of the antigens including PPD (Fig. 3.20). The stimulation index was significantly lower than tuberculosis patients. About 40% recognition was seen with CF-Ag pool L1 and CF-Ag pool H3, with much lower levels of recognition (20% to 35%) by the other antigen fractions (Table 11).

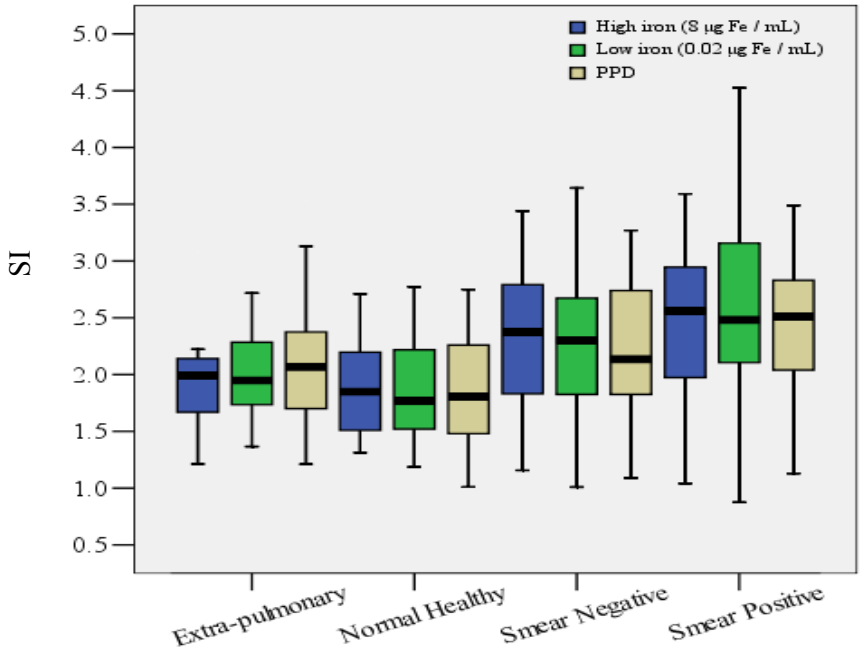


Fig. 3.19 - Lymphocyte proliferation of PBMCs to whole high iron and low iron CFPs and PPD. The study groups include smear positive (25), smear negative (20), extra-pulmonary (15) and normal healthy individuals (20). Stimulation index (SI) is represented as box plots; the median SI value is indicated with a line in the centre and the maximum and minimum values as whiskers.

Table 11- Lymphocyte proliferation of normal control subjects by CF-Ag pools

CF-Ag pool	% recognition[a]	Stimulation Index (SI)
H1	35	1.79
L1	40	1.67
H2	35	1.69
L2	35	1.67
H3	40	1.58
L3	35	1.64
H4	30	1.68
L4	30	1.65
H5	20	1.52
L5	20	1.72
PPD	30	1.80

[a] considered positive when SI > 2

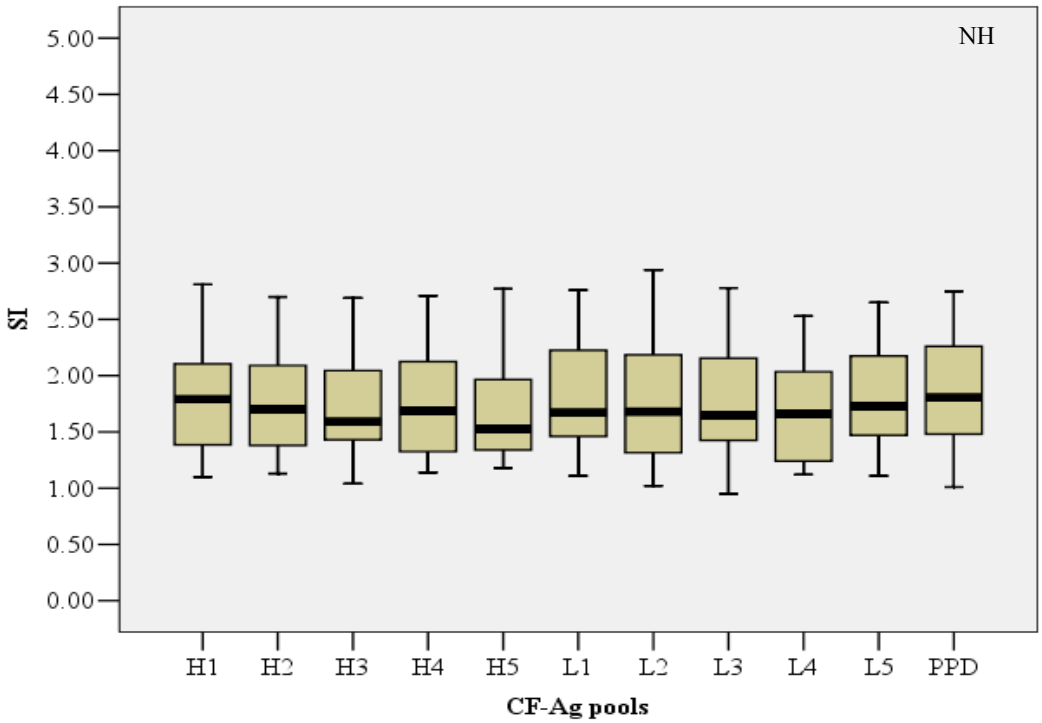


Fig. 3.20 - Lymphocyte proliferation response of normal healthy individuals to CF-Ag pools and PPD. The box plots represent the stimulation index (SI) of 20 normal healthy individuals to CF-Ag pools (H1 – H5) and (L1 – L5), prepared from high and low grown organisms respectively. The median value is represented as a line in the box plots.

3.4.2.2. Lymphocyte response of smear positive group of tuberculosis patients

PPD induced significant stimulation of the PBMCs of smear positive cases of tuberculosis with median SI of 2.5 and 76% recognition (Fig. 3.21, Table 12). In comparison, the CF-Ag pools H1, L1, L2 and L4 also showed good stimulation (median SI > 2.0) with appreciable recognition of 68 and 72% respectively by L1 and L2 fractions. It can be seen that the stimulation index and recognition of cases by low iron CF-Ag pools L1, L2 and L4 (median SI > 2.0; 68, 72 and 52% recognition respectively) was much higher than the respective high iron preparations (median SI < 2.0, 60, 44 and 28% recognition respectively). Compared to the low proliferation seen with H3 and L3 CF-Ag pools, CF-Ag pool H5 evoked an immune response equivalent to PPD but with a lower recognition of 50% (Table 12). The immune response of the smear positive group of subjects was compared with that of normal healthy individuals and was found to be statistically significant with P value < 0.05.

Table 12- Comparison of the lymphocyte proliferation of smear positive tuberculosis patients with normal healthy individuals

CF-Ag pool	% recognition[a]	Stimulation Index (SI)	P value
H1	60	2.30	0.0275*
L1	68	2.47	0.0027*
H2	44	1.94	0.0888
L2	72	2.25	0.0029*
H3	44	1.77	0.1601
L3	44	1.73	0.3549
H4	28	1.74	0.5004
L4	52	2.06	0.0275*
H5	52	2.01	0.0124*
L5	50	1.94	0.2779
PPD	76	2.50	0.0023*

*Statistically significant with $P < 0.05$, [a] considered positive when $SI > 2$

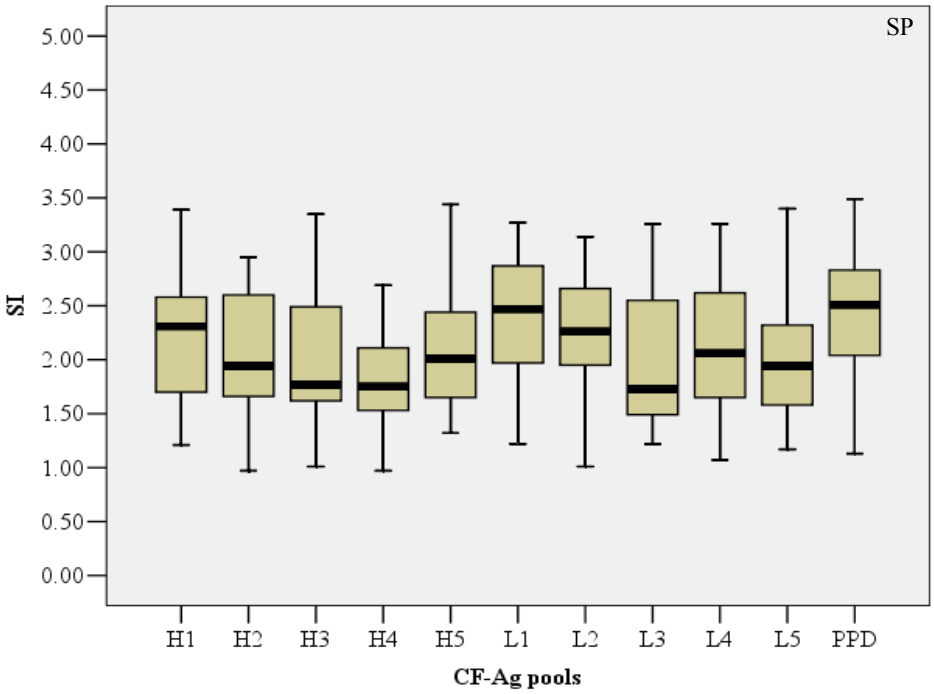


Fig. 3.21 -Lymphocyte proliferation of smear positive (SP) individuals to CF-Ag pools and PPD. The box plots represent the stimulation index (SI) of 25 SP cases in response to CF-Ag pools (H1 –H5) and (L1 –L5), prepared from high and low iron grown organisms respectively. The median value is represented as a line in the box plots.

3.4.2.3. Lymphocyte response of smear negative group of tuberculosis patients

The lymphocyte response of smear negative tuberculosis patients to PPD was lower than that of smear positive cases with a median SI of 2.13 and 55% recognition (Fig. 3.22, Table 13). The individual antigen pools also showed low response with SI < 2.0 for most of the fractions except for CF-Ag pools H1, L1 and L2 (median SI > 2.0). The latter two antigen pools showed higher recognition of positive cases (60 and 65% respectively) than PPD. L2, L4 and H5 showed a statistically significant response when compared to normal healthy individuals ($P < 0.05$).

Table 13 - Comparison of the lymphocyte proliferation of smear negative tuberculosis patients with normal healthy individuals

CF-Ag pool	% recognition[a]	Stimulation Index	P value
H1	55	2.12	0.0962
L1	60	2.08	0.0679
H2	40	1.82	0.1333
L2	65	2.17	0.0167*
H3	45	1.83	0.1017
L3	50	1.84	0.3648
H4	35	1.69	0.5375
L4	45	1.95	0.0385*
H5	45	1.92	0.0239*
L5	40	1.75	0.2977
PPD	55	2.13	0.0468*

*When compared to normal healthy controls, proliferation was statistically significant with $P < 0.05$, [a] considered positive when SI > 2

3.4.2.4. Lymphocyte response of extra pulmonary tuberculosis patients

The PBMCs of the extra-pulmonary group did not respond well to any of the antigens, all of them showing median SI < 2 (Fig.3.23) and failed to recognise tuberculosis cases (Table 14). PPD alone had given positive stimulation of the lymphocytes (median SI of 2.07 and 60% recognition). Among the CF-Ag pools L5 showed comparatively higher stimulation (median SI 1.93), though lower than PPD.

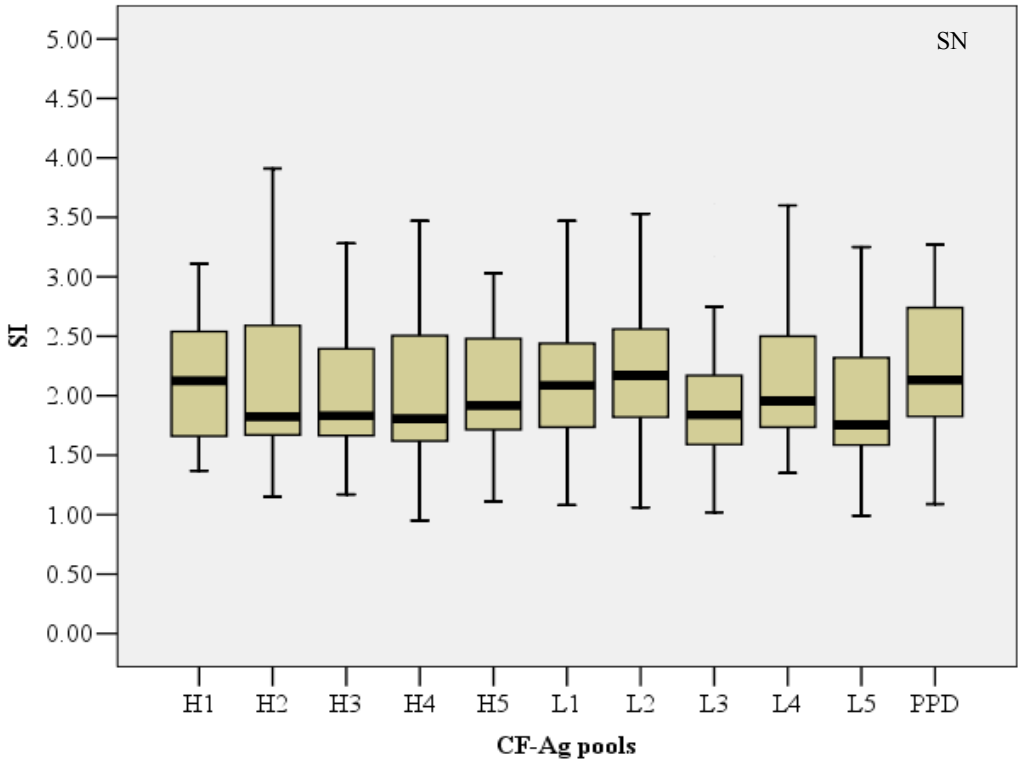


Fig. 3.22- Lymphocyte proliferation of smear negative (SN) individuals to CF-Ag pools and PPD. The box plots represent the stimulation index (SI) of 20 SN cases in response to CF-Ag pools (H1 –H5) and (L1 –L5), prepared from high and low iron grown organisms respectively. The median value is represented as a line in the box plots.

Table 14 - Comparison of the lymphocyte proliferation of extra-pulmonary tuberculosis patients with normal healthy individuals

CF-Ag pool	% recognition[a]	Stimulation Index	P value
H1	20	1.78	0.8545
L1	3	1.72	0.9336
H2	26	1.88	0.7015
L2	26	1.89	0.6054
H3	46	1.88	0.3093
L3	33	1.73	0.473
H4	33	1.80	0.3104
L4	20	1.84	0.5597
H5	26	1.83	0.1471
L5	46	1.93	0.2237
PPD	60	2.07	0.1770

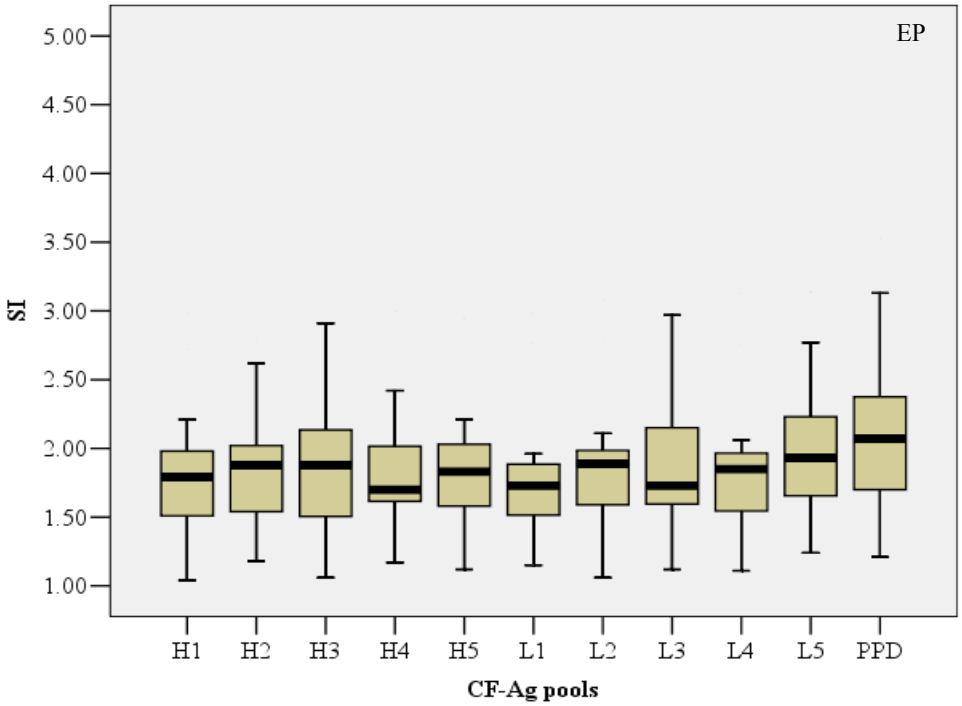


Fig. 3.23 - Lymphocyte proliferation response of the extra-pulmonary cases of tuberculosis to CF-Ag pools and PPD. The box plots represent the stimulation index (SI) of 15 EP cases to CF-Ag pools (H1 –H5) and (L1 –L5), prepared from high and low iron grown organisms respectively. The median value is represented as a line in the box plots.

3.4.3. Expression of IFN-γ: stimulation with whole CFPs and PPD

IFN-γ level was estimated in the culture supernatants of antigen-stimulated PBMCs of normal healthy individuals. Whole CFPs, like PPD produced a three-fold increase in IFN-γ levels (Fig. 3.24) that was statistically significant when compared to tuberculosis patients (Table 16.)

3.4.4. Expression of IFN-γ: stimulation with CF-Ag pools

3.4.4.1. Normal healthy individuals

Significantly high levels of IFN-γ was detected in the supernatants of lymphocytes from normal healthy control individuals (Fig. 3.25), with an approximate average value greater than 100 pg mL^{-1} with most of the antigens and a marginally lower level with L1 (Table 15). Markedly high level of the cytokine was induced with CFP antigen pool L5 (233.1 pg mL^{-1}), reaching levels greater than that observed with PPD (177.3 pg mL^{-1}, Table 15). Also, significant levels of IFN-γ (approximately 200

pg mL^{-1}) were expressed in the culture supernatants of lymphocytes stimulated with CF-Ag pools H3 and L3 (Table 15).

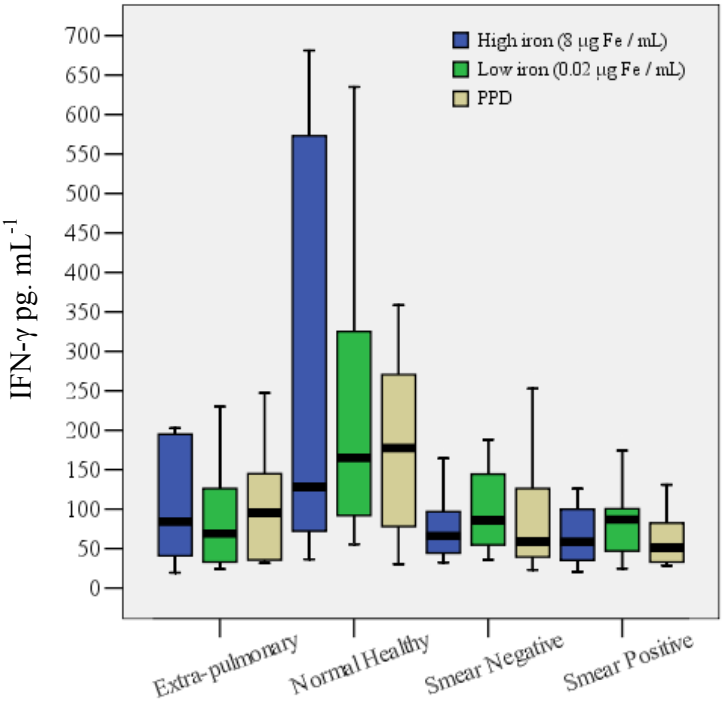


Fig. 3.24 - IFN-γ response of PBMCs to whole CFPs of high iron and low iron grown cells and PPD. The study groups include smear positive (22), smear negative (13), extra-pulmonary (12) and normal healthy individuals (20). Interferon **γ (**IFN-γ**)** levels are expressed as pg .mL^{-1} and are represented as box plots; The median IFN-**γ** value is indicated with a line in the centre and the maximum and minimum values as whiskers.

3.4.4.2. Smear positive tuberculosis patients

IFN-γ levels in the supernatants of lymphocytes from smear positive individuals were much lower, irrespective of the antigen pool used for stimulation (Table 16). These values, compared to healthy individuals were statistically significant with P value of < 0.05. It is to be noted that PPD also failed to evoke a strong IFN- γ response (median IFN- γ 50.9 pg.mL-1) (Fig. 3.26). Among the different antigen pools, the maximal response was seen with CF-Ag pool H5 (median IFN- γ 52.7 pg.mL-1).

Table – 15. IFN- γ levels in the (CF-Ag) stimulated culture supernatants of lymphocytes of normal human subjects

CF-Ag Pool	IFN- γ levels (pg mL^{-1})
H1	179.65
L1	95.50
H2	151.51
L2	110.02
H3	205.5
L3	206.5
H4	102.51
L4	169.3
H5	140.43
L5	233.1
PPD	177.3

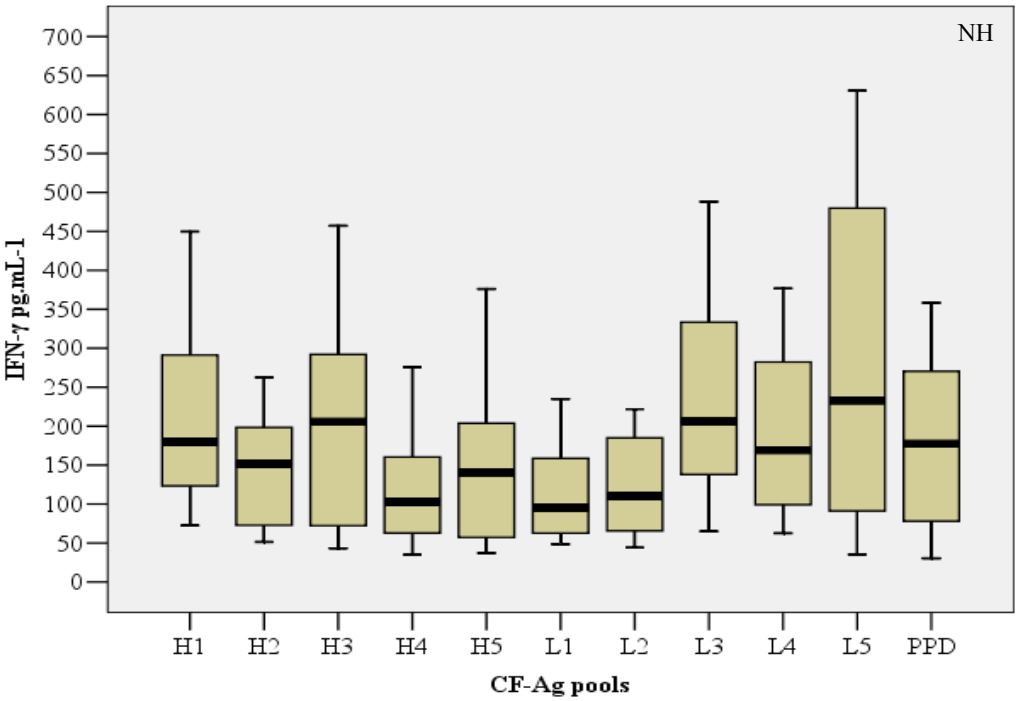


Fig. 3.25 - IFN-γ levels (pg. mL^{-1}) of the normal healthy individuals in response to CF-Ag pools and PPD. The box plots represent the IFN- γ levels expressed in pg.mL^{-1} of 20 normal healthy individuals to CF-Ag pools (H1 –H5) and (L1 –L5), prepared from high and low grown organisms respectively. The median value is represented as a line in the box plots.

Table 16 - IFN- γ levels in the (CF-Ag) stimulated culture supernatants of lymphocytes of SP cases of tuberculosis

CF-Ag pool	Median IFN- γ levels (pg mL^{-1})	P value
H1	41.26	0.0000
L1	40.41	0.0000
H2	39.45	0.0000
L2	43.06	0.0000
H3	37.9	0.0000
L3	43.3	0.0000
H4	52.70	0.0114
L4	46.2	0.0000
H5	38.72	0.0000
L5	43.0	0.0000
PPD	50.9	0.0008

3.4.4.3. Smear negative tuberculosis patients

When compared to the healthy individuals, this group of individuals showed low levels of IFN- γ with values ranging below 60 pg. mL^{-1} (Table 17). The level of expression was however relatively greater than the smear positive group. The CF-Ag pools H4, L4 and H5 showed a similar response as PPD (Fig 3.27, Table - 17).

Table 17- IFN-γ levels in the (CF-Ag) stimulated culture supernatants of lymphocytes of SN cases of tuberculosis

CF-Ag pool	Median IFN- γ levels (pg mL^{-1})	P value
H1	50.6	0.0003
L1	37.32	0.0043
H2	44.22	0.0004
L2	44.58	0.0011
H3	48.9	0.0004
L3	42.8	0.0000
H4	55.1	0.0409
L4	59.7	0.0175
H5	56.18	0.0110
L5	50.1	0.0007
PPD	59.2	0.0739

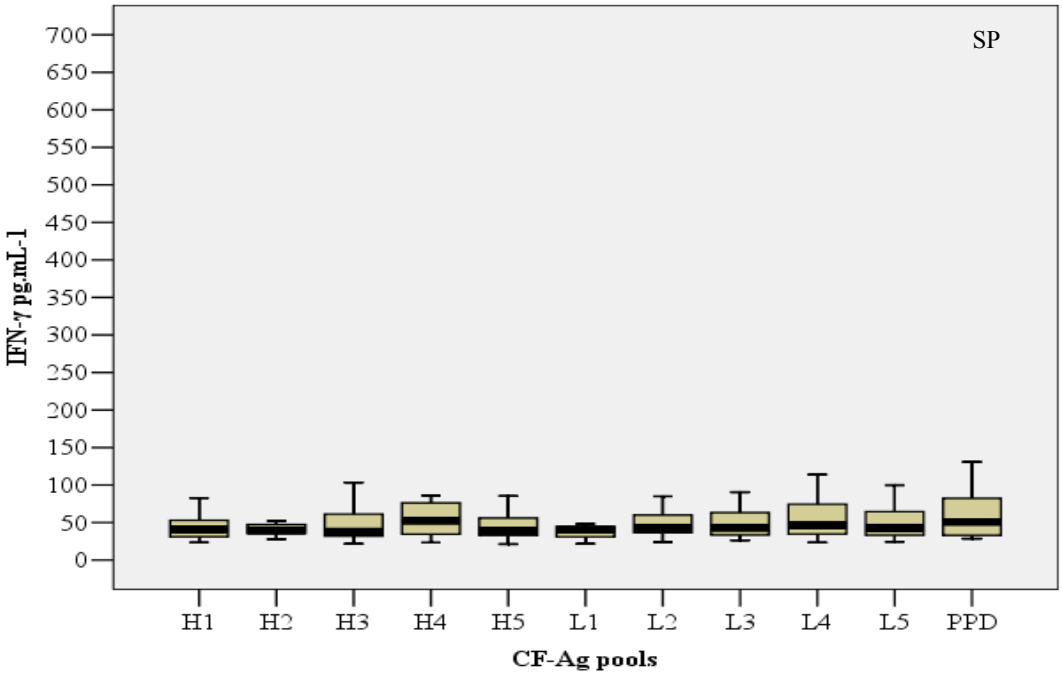


Fig. 3.26 - IFN-γ levels (pg. mL^{-1}) of the smear positive individuals in response to CF-Ag pools and PPD. The box plots represent the IFN- γ levels expressed in pg.mL^{-1} of 22 smear positive (SP) individuals to CF-Ag pools (H1 –H5) and (L1 – L5), prepared from high and low grown organisms respectively. The median value is represented as a line in the box plots.

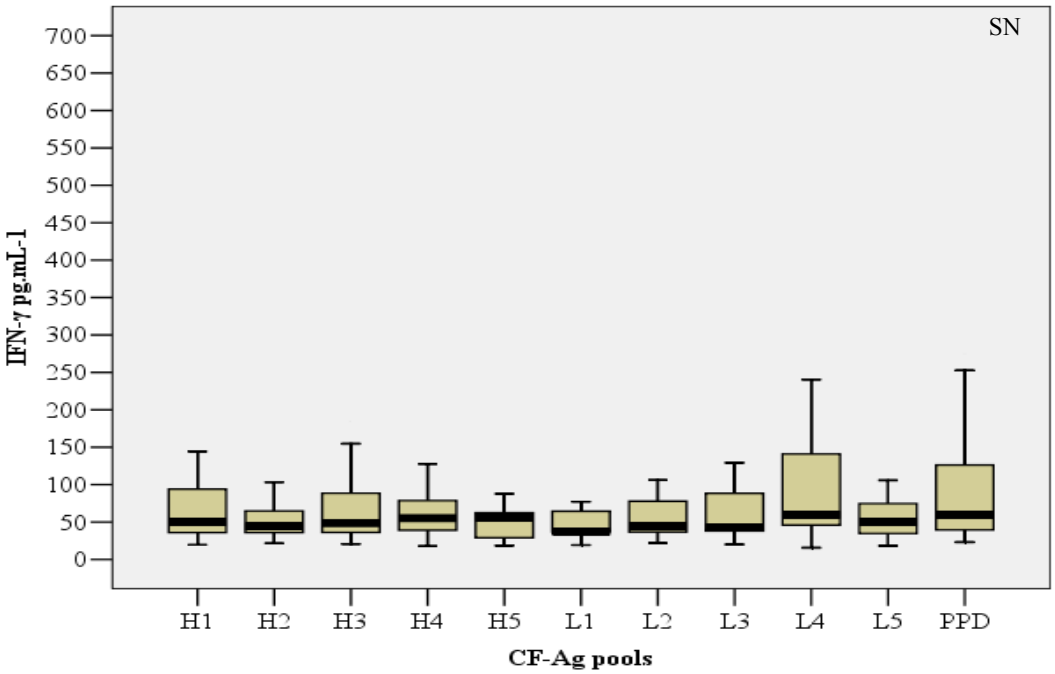


Fig. 3.27 - IFN-γ levels (pg. mL^{-1}) of the smear negative individuals to CF- Ag pools and PPD. The box plots represent the IFN- γ levels expressed in pg.mL^{-1} of 13 smear negative (SN) individuals to CF-Ag pools (H1 –H5) and (L1 –L5), prepared from high and low grown organisms respectively. The median value is represented as a line in the box plots.

3.4.4.4. Extra-pulmonary tuberculosis patients

When stimulated with PPD, IFN-γ level was 95.1 pg.mL^{-1} with a lower level with CF-Ag pools (< 50 pg.mL^{-1}) (Fig.3.28, Table 18). The IFN-γ levels of this group were low compared to the above two groups and was not statistically significant.

Table 18 - IFN- γ levels in the (CF-Ag) stimulated culture supernatants of lymphocytes of extra-pulmonary cases of tuberculosis

CF-Ag pool	Median IFN- γ levels (pg mL^{-1})	P value
H2	38.23	0.0000
L2	37.4	0.0017
H3	43.04	0.0002
L3	39.77	0.0003
H4	43.5	0.0008
L4	48.8	0.0002
H5	42.48	0.0165
L5	44.7	0.0006
H6	40.06	0.0017
L6	39.4	0.0001
PPD	95.1	0.0990

P < 0.05 was considered as statistically significant

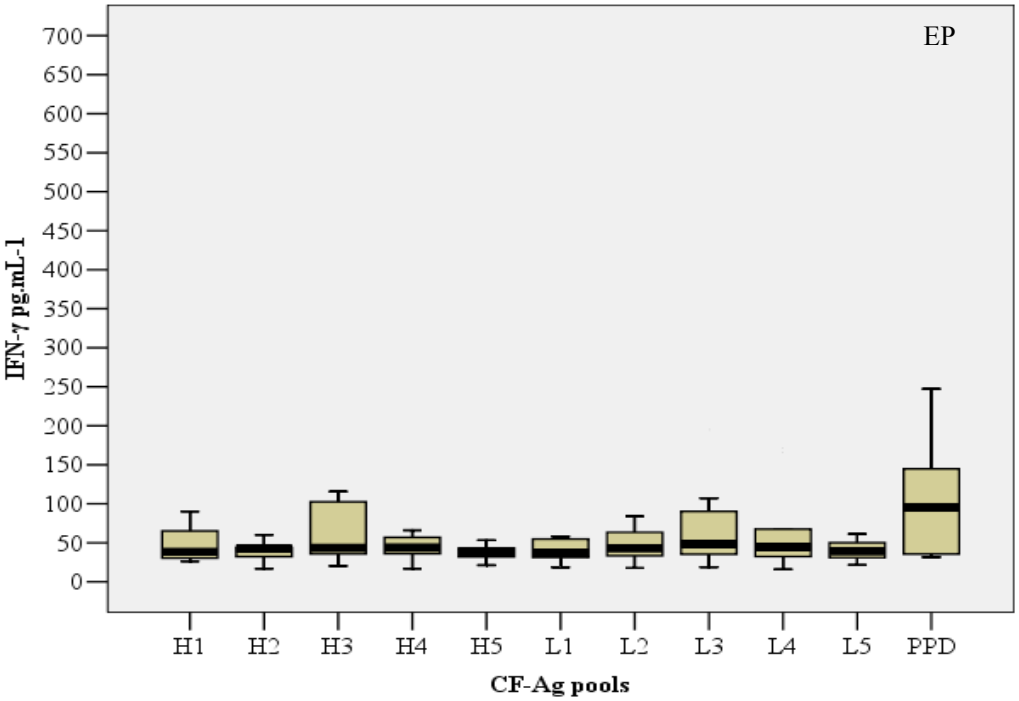


Fig. 3.28 - IFN-γ levels (pg. mL^{-1}) of the extra-pulmonary individuals to CF- Ag pools and PPD. The box plots represent the IFN- γ levels expressed in pg.mL^{-1} of 10 extra-pulmonary (EP) individuals in response to CF-Ag pools (H1 –H5) and (L1 –L5), prepared from high and low grown organisms respectively. The median value is represented as a line in the box plots.

3.4.5. Antibody-based detection of mycobacterial culture filtrate antigens

3.4.5.1. Screening of serum samples using Mycotest, a commercial lateral flow device.

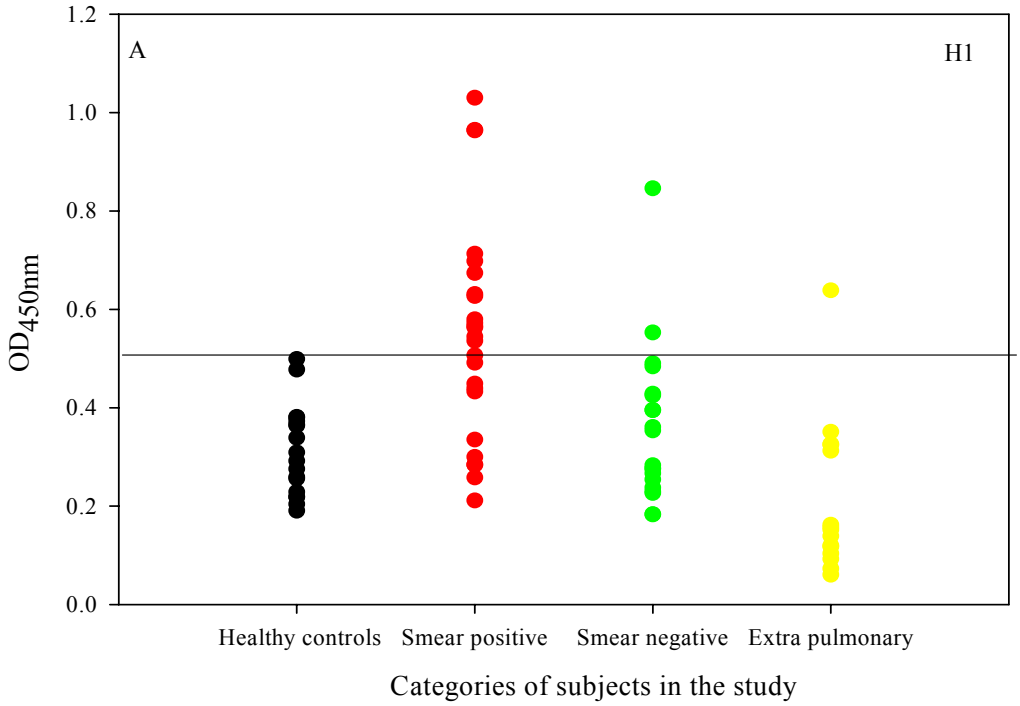
Mycotest, a lateral flow device developed based on culture filtrate antigens was used to screen serum samples in this study. Upon addition of about 80-100 µl of serum, the development of two bands, one representing the control and the second the test was considered as positive. Among the SP cases, 44% of the samples tested positive, with significant colour development of the bands. None of the normal healthy control subjects tested positive, and in the SN and EP group, positivity was 20% and 6% respectively.

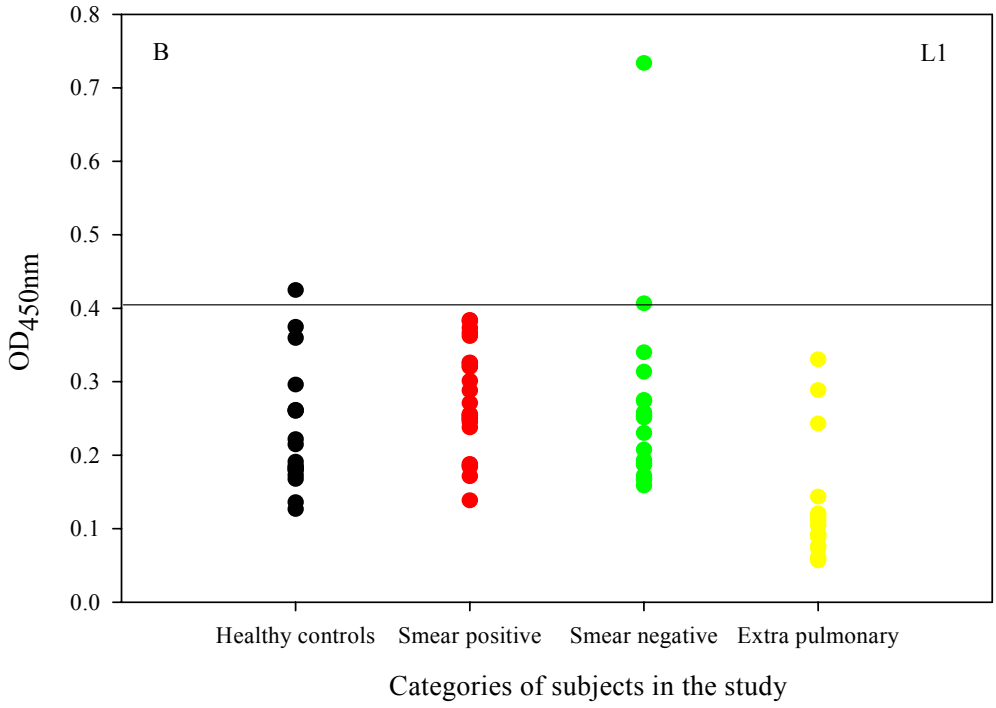
3.4.5.2. ELISA-based screening of serum samples using CF-Ag pools as antigens.

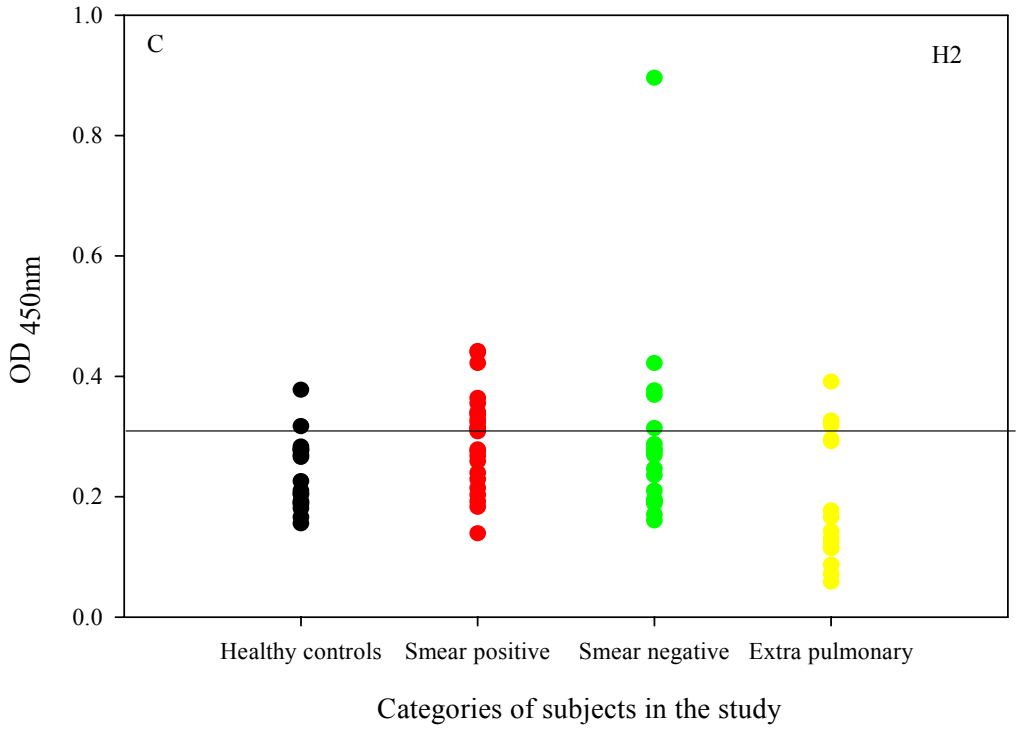
The different CF-Ag pools used for studying the T cell response were employed as antigens in ELISA for the detection of antibodies against them in the serum of all the three categories of tuberculosis patients. PPD was used as a control antigen. ELISA was done in duplicates for each sample. Screening of the serum from normal individuals was done and the cut-off for each CF-Ag pool was calculated by taking the mean OD plus two times the standard deviation of mean of these control samples. The cut-off values for each antigen pool is represented in Figures

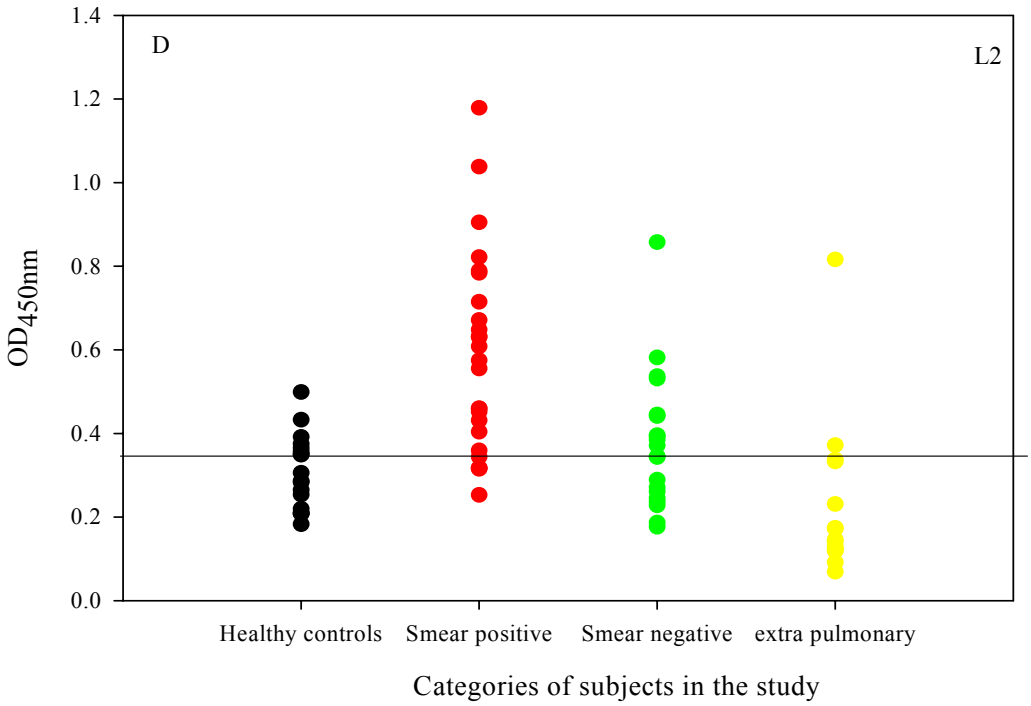
It was observed that PPD served as a poor antigen in ELISA-based tests and did not show much reactivity with serum from normal subjects and the different categories of tuberculosis patients in this study, except in SN cases with a positive detection of about 16% compared to about 4% with SP and EP cases of tuberculosis.

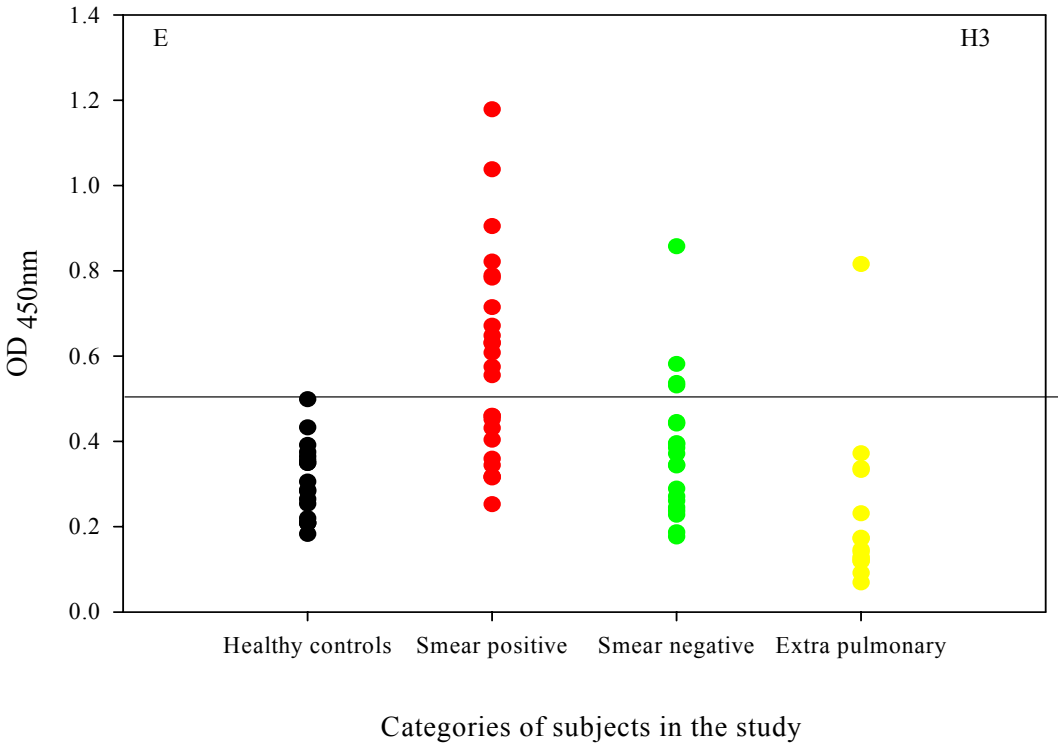
Figures 3.29 A to H represent the serum antibodies against the different antigens employed against the 4 categories of subjects, including the SP, SN and EP cases of tuberculosis and the normal healthy controls. The cut-off, calculated as mentioned above, is approximately 0.3 to 0.45 for the different antigens. Considering values above the cut-off values, the CF-Ags recognised the antibodies in the serum of the majority of SP patients, with a considerably lower level of detection in SN cases. The EP patients elaborated very low levels of antibodies against the mycobacterial CF-Ags.

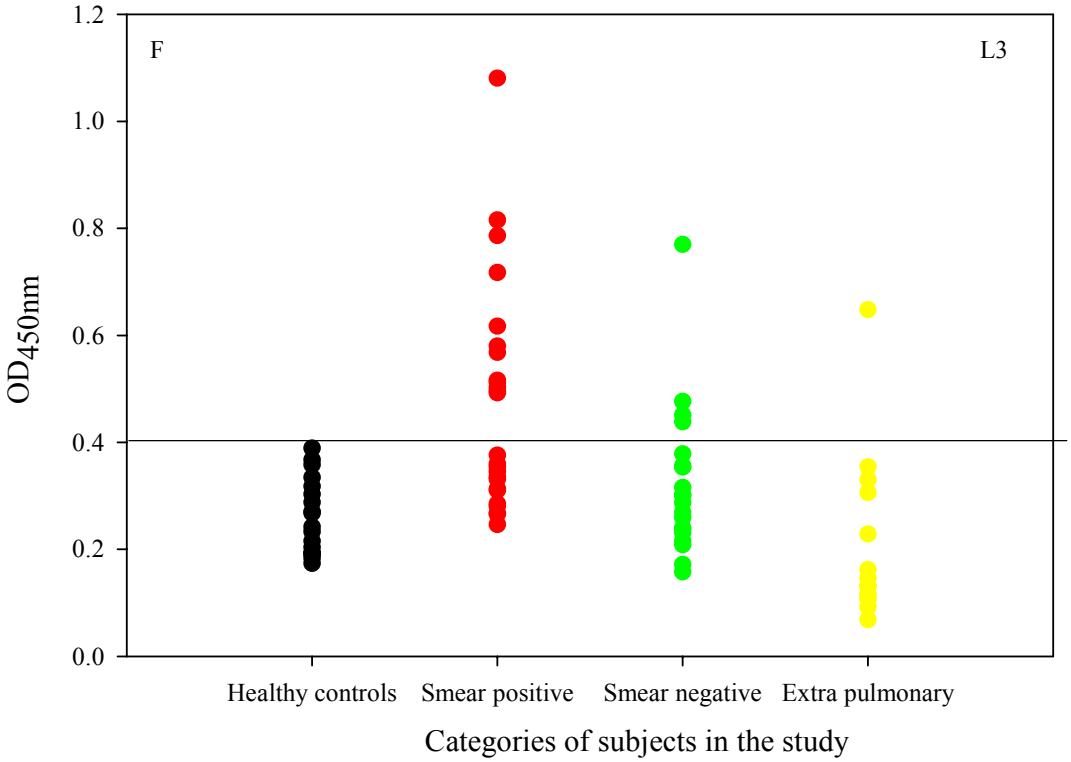

A
H1
1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD450nm
Healthy controls
Smear positive
Smear negative
Extra pulmonary
Categories of subjects in the study

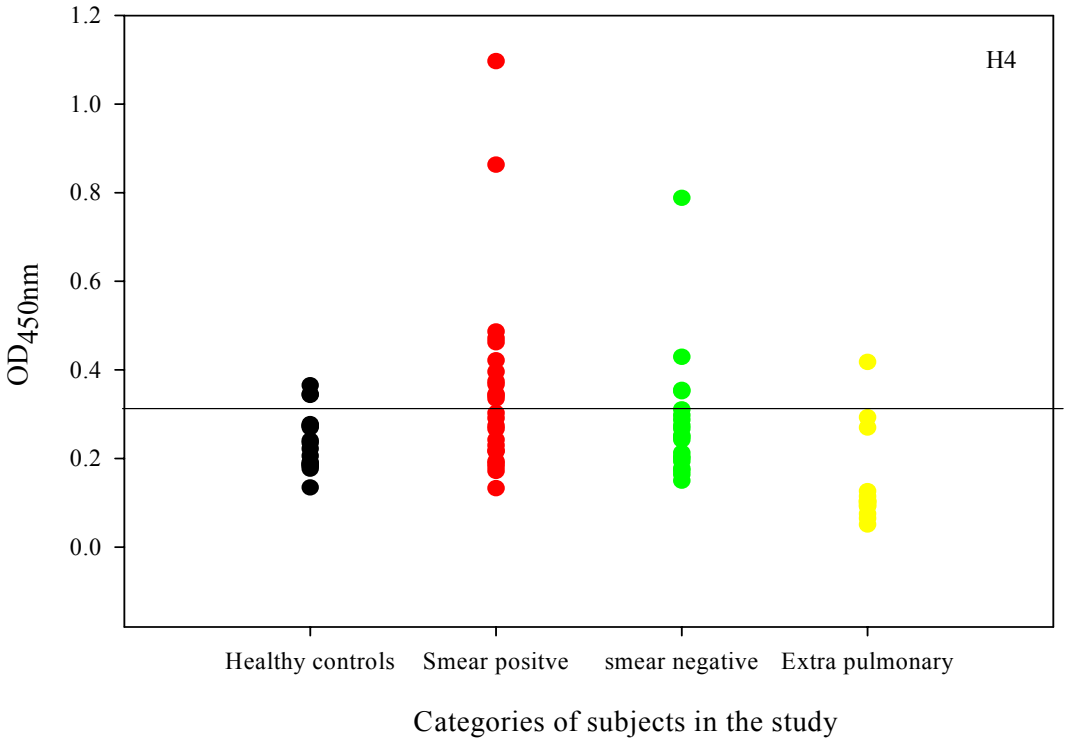

B
L1
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
OD450nm
Healthy controls
Smear positive
Smear negative
Extra pulmonary
Categories of subjects in the study

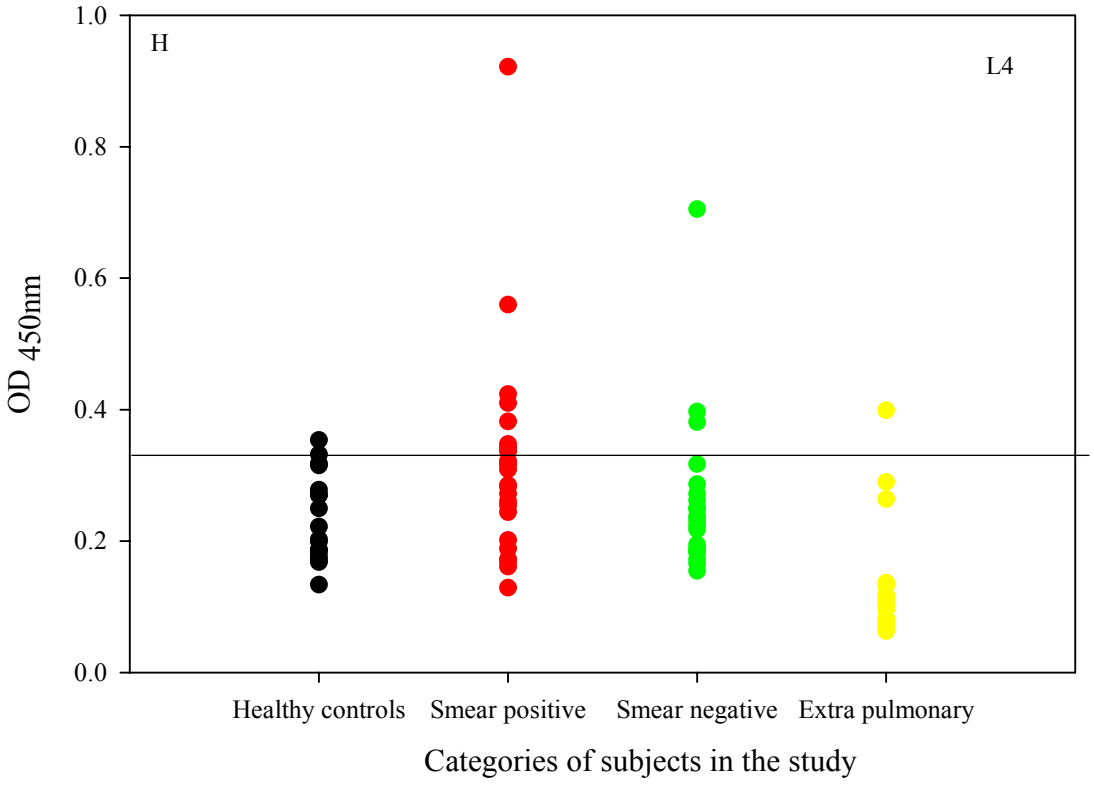

C
H2
1.0
0.8
0.6
0.4
0.2
0.0
OD 450nm
Healthy controls
Smear positive
Smear negative
Extra pulmonary
Categories of subjects in the study

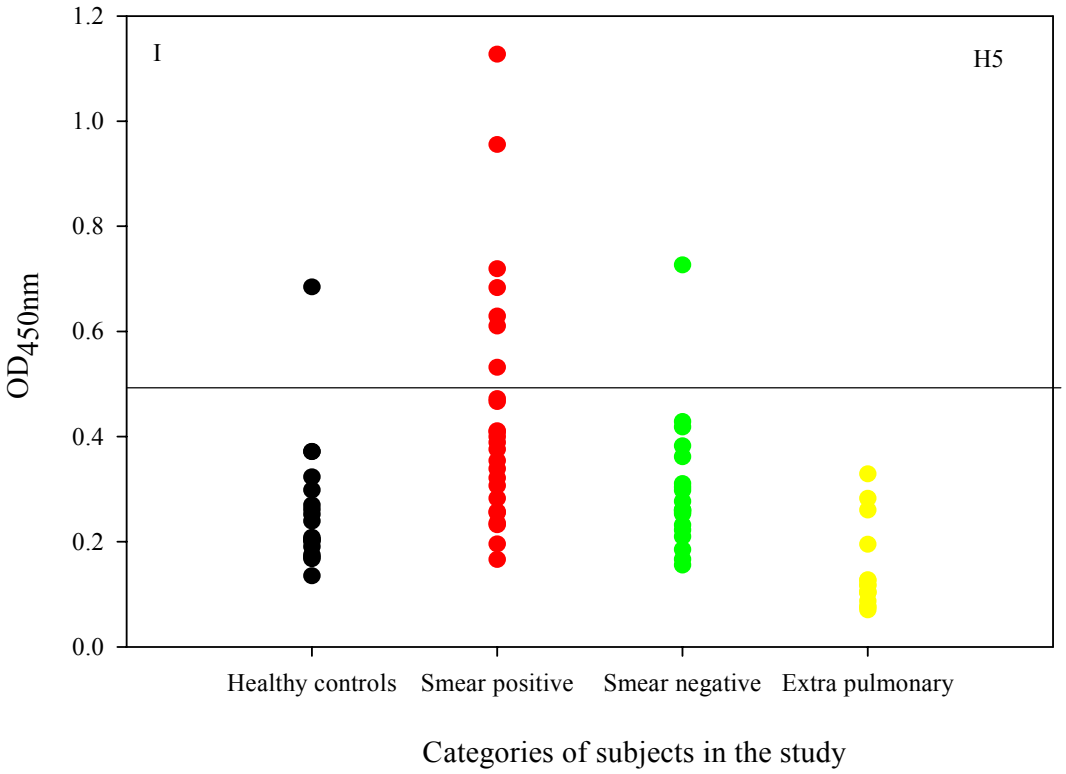

D
L2
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD450nm
Healthy controls
Smear positive
Smear negative
extra pulmonary
Categories of subjects in the study

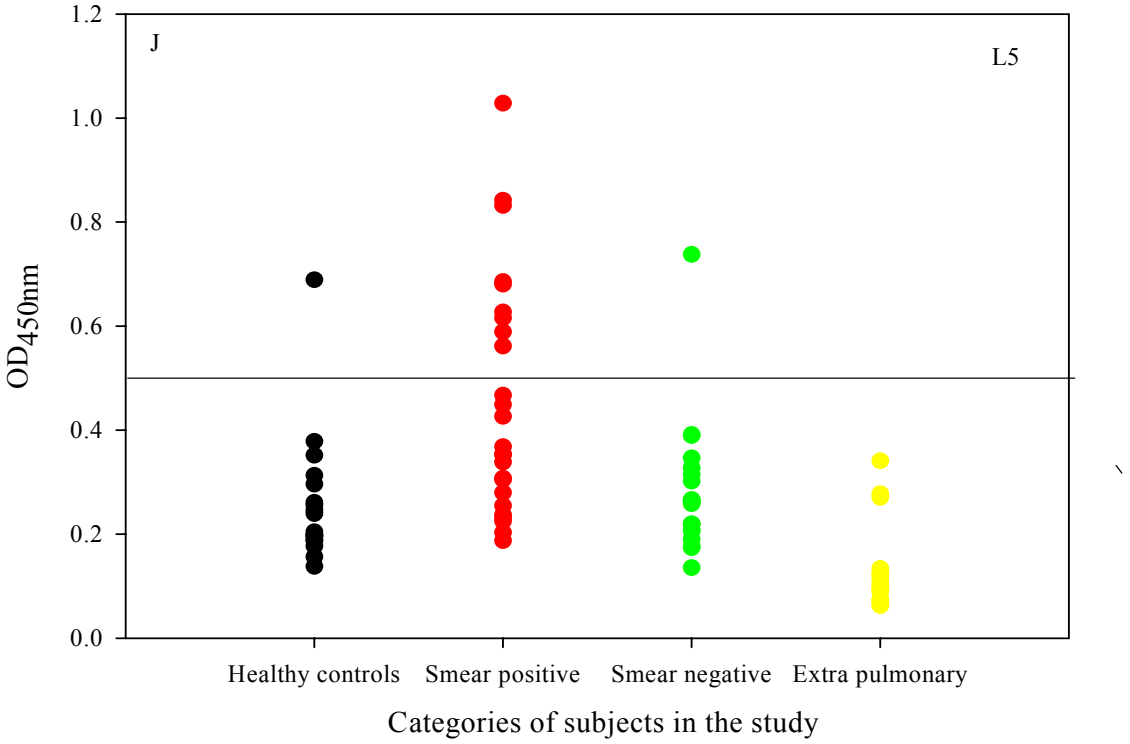

E
H3
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD 450nm
Healthy controls
Smear positive
Smear negative
Extra pulmonary
Categories of subjects in the study


F
L3
1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD450nm
Healthy controls
Smear positive
Smear negative
Extra pulmonary
Categories of subjects in the study


H4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD450nm
Healthy controls
Smear positve
smear negative
Extra pulmonary
Categories of subjects in the study


H
L4
1.0
0.8
0.6
0.4
0.2
0.0
OD 450nm
Healthy controls
Smear positive
Smear negative
Extra pulmonary
Categories of subjects in the study


I
H5
1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD450nm
Healthy controls
Smear positive
Smear negative
Extra pulmonary
Categories of subjects in the study


J
L5
1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD450nm
Healthy controls
Smear positive
Smear negative
Extra pulmonary
Categories of subjects in the study

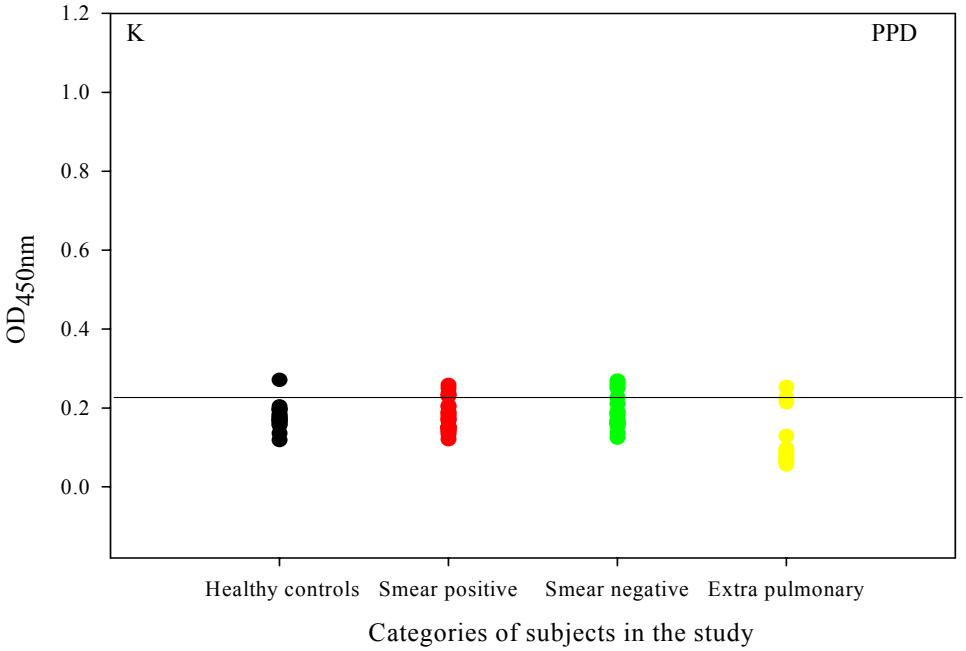


Fig. 3.29 - Detection of anti-CFP antibodies in sera of four study groups. Panels A – J represent antibody titres of the four groups of individuals to the CF –Ag pools (H1-H5) and L1-L5. Panel K indicates the antibody titres in response to PPD. X-axis indicates the four study groups smear positive, smear negative, extra-pulmonary and normal healthy individuals. Y- axis represents the OD_{450nm} for the samples. The cut-off was taken as mean and two times the standard deviation. It is represented as a line in the graph.

With CF-Ags H1, % of cases above the cut-off of 0.494 was found to be 64, 4 and 4 for SP, SN and EP cases respectively. On the other hand, CF-Ags L1 was not promising (Fig. 3.29 A, B and Table 19), with almost negligible detection of cases. The situation was reversed with CF-Ags H2 and L2, with the latter showing higher positivity of 60 and 16% respectively in SP and SN cases than the former. The EP group fared poorly with both the antigens.

With H3/L3 and H5/L5 antigen sets, the cut-off value was found to be high. The cut-off for CF-Ag H3 and L3 was 0.483 and 0.402 respectively and with CF-Ag H5 and L5, the values were higher (0.513 and 0.511 respectively). The serum of SP tuberculosis patients showed high levels of antibodies against the former group of antigens, with a detection level of 60 and 52% seen with both high and low iron preparations. This set of antigens along with H2 / L2 CF-Ags served as relatively better antigens for this category of tuberculosis patients. The CF-Ags H5 and L5, on

the other hand showed only 28 and 36%, perhaps due to the high levels of antibodies even with the control group.

The H4/L4 antigen set had a lower cut-off value of 0.370 and 0.366 and detected 20% of SP cases, while the low iron L4 preparation served as a better antigen preparation for the SN group, with about 12% positivity. As already mentioned, these antigens failed to detect the EP cases (Table 19)

Table 19. ELISA-based detection of antibodies against CF-Ags in the serum of tuberculosis patients

CF-Ag	% positivity in the 3 categories of tuberculosis patients		
	SP	**SN**	**EP**
H1	64	4	4
L1	0	8	0
H2	12	16	0
L2	60	16	4
H3	60	16	4
L3	52	16	4
H4	20	4	4
L4	20	12	4
H5	28	4	0
L5	36	4	0
PPD	4	16	4
Mycotest	44	20	6

Percentage positivity for smear positive (SP), smear negative (SN), extra-pulmonary (EP) was calculated based on the cut – off obtained for each antigen pool.

3.4.5.3. Performance of Mycotest and ELISA-based detection as a diagnostic test in smear positive cases of tuberculosis

The CF-Ags H1, L2, H3 and L3, detecting 50% of SP cases were compared with Mycotest and smear test for evaluation as potential diagnostic antigens. Using smear positivity as standard, the sensitivity and specificity of these antigen pools were determined (Table 20). The sensitivity obtained with these four antigen preparations was not high. The specificity was high with CF-Ag H1 when compared to smear test.

The specificity obtained with CF-Ags H3 and L3 was relatively lower and showed higher specificity when compared with Mycotest.

Table 20 - Evaluation of the sensitivity and specificity of defined CF-Ag pools.

I. AFB smear testing as standard						
CF-Ag	*No. of cases identified as				Sensitivity	Specificity
	TP	FN	FP	TN		
H1	16	9	1	19	64.000	95.000
L2	15	10	6	14	60.000	70.000
H3	15	10	4	16	60.000	80.000
L3	13	12	4	16	52.000	80.000
II. Commercial lateral flow device Mycotest as standard						
	*No. of cases identified as					
CF-Ag	TP	FN	FP	TN	Sensitivity	Specificity
H1	9	8	8	55	52.941	87.302
L2	11	6	8	55	64.706	87.302
H3	11	6	8	55	64.706	87.302
L3	11	6	8	55	64.706	87.302

TP- true positive (samples positive by both ELISA and Mycotest), TN – true negative (samples negative by both), FP – false positive (samples positive by standards and negative by ELISA), FN – false negative (samples negative by standards and positive by ELISA).

CHAPTER 4

DISCUSSION

Iron acquisition systems and the regulatory role of iron on the expression of specific virulence determinants / toxins are well understood in several bacterial systems (Sritharan, 2006; Ratledge, 2004; Sritharan, 2000). Of special importance is the regulation by iron of virulence determinants and toxins, as seen in *E. coli*, *C. diphtheriae* and others. Pathogenic mycobacteria, including *M. tuberculosis* do not produce any known virulence factors / toxins. However, it is highly likely that proteins, which play an important role in the host-pathogen interactions that facilitate the survival of the pathogen in the environment of the host are influenced by iron levels. The objective of this study was to study the influence of iron levels on the secreted proteins in *M. tuberculosis* and study the immune response of the lymphocytes from tuberculosis patients to the CFPs grown under high and low iron conditions. In the first part of the study, efforts were made to identify iron-regulated culture filtrate proteins expressed by *M. tuberculosis* grown under high (8 μg Fe / mL) and low iron (0.02 μg Fe / mL) conditions. The proteins in the spent growth medium, referred to as culture filtrate proteins (CFPs), due to their low levels were initially concentrated by ammonium sulphate precipitation and ultrafiltration. Single dimension SDS-PAGE was done to separate and analyse the fractions. However, no notable differences were appreciated between the CFPs grown under high and low iron organisms. They were then analysed on 2D-PAGE, which resolved the proteins into discrete spots; several proteins were up regulated under high and low iron conditions of growth. Identification of several of these proteins was done by techniques including MALDI-TOF MS and immunoblotting. In the second part of the study, defined culture filtrate antigen pools (CF-Ag pools) were prepared and the immune response of the peripheral blood mononuclear cells from tuberculosis patients was assessed and compared with that of control healthy individuals with no evidence of the disease. Immune proliferation response and the expression of IFN-γ in response to these antigens, highlighting the role of IFN-γ in the protection against disease development is presented in this study.

There is increasing evidence to show that the mammalian host limits iron (Kochan 1976, Gold *et al*. 2001) and iron is one of the contributing factors in determining the outcome of an infection by *M. tuberculosis*. Within the intracellular environment of the macrophage, *M. tuberculosis* is exposed to conditions of iron limitation due to the presence of lactoferrin within the macrophage that can hold iron even at low pH. Iron is essential for the growth of *M. tuberculosis*. The genome

analysis shows that as many as 40 enzymes are iron-dependant, some of which play vital roles in electron transport and other metabolic processes (Sritharan, 2000). Like other bacteria, pathogenic mycobacteria face conditions of iron limitation within the mammalian host. Very little free iron is present as most of it is held as protein-bound iron. Transferrin in the human serum has been demonstrated to be tuberculostatic, and this effect can be reversed by the addition of iron (Kochan, 1976). Thus the administration of iron to tuberculosis patients should be done with caution as it can exacerbate the disease (Ratledge, 2004).

The siderophore-mediated iron acquisition machinery in mycobacteria is well delineated from studies in *in vitro* grown cultures. Mycobacteria are unique in that they produce two forms of siderophores, the intracellular mycobactin and the extracellular carboxymycobactin / exochelins. These siderophores have a high affinity for the ferric iron and can remove iron from the insoluble ferric hydroxide and from iron-withholding proteins. The carboxymycobactins scavenge the iron and deliver it to the inside, though the mechanism of internalization is not understood. According to Ratledge (2004), mycobactin serves as a temporary store for the acquired iron. Iron is ultimately held in bacterioferritin. Mycobactin biosynthesis by the polyketide pathway is well understood (Quadri *et al.* 1998). The components for the biosynthesis of the siderophore are encoded in the *mbt* gene cluster. De Voss *et al*. (2000) developed a mycobactin-deficient *mbtB* mutant and showed that the inability to synthesise mycobactin resulted in the poor infectivity of the macrophage and low growth of the *M. tuberculosis* mutant, thus establishing that this is a virulence determinant.

Iron regulation is perhaps complex in mycobacteria, as more than one regulator is seen. The genome shows four regulators, among which the *IdeR* (iron dependant regulator) is well characterised. Based on its homology to *DtxR*, *IdeR* was initially predicted to function as an iron-dependant transcriptional repressor. Transcritpional profiling of the mutant vs the parental strain showed that about one third of the iron-regulated genes in *M. tuberculosis* are regulated by IdeR (Rodriguez *et al*., 2002). IdeR controls the genes involved in siderophore production and iron storage and in addition, controls genes encoding putative transporters, transcriptional regulators, proteins involved in general metabolism, members of the PE/PPE family of conserved mycobacterial proteins. The *furA* gene (encoding the FurA regulator) is located upstream of the katG gene and its expression is negatively regulated. The

katG gene expression is regulated by iron levels, as shown by other studies in our lab (Yeruva *et al*., 2005; Sritharan *et al*., 2006).

The influence of iron levels on cell surface proteins was first studied in *M. smegmatis* (Hall *et al*., 1987). At least five IREPs were detected in envelope extracts from *M. smegmatis* grown in iron-deficient conditions, which also included the 29 kDa protein. This protein can associate directly *in vitro* with ferri-exochelin, and the addition of a polyclonal antiserum generated against it to *M. smegmatis* cells significantly inhibits ferri-exochelin mediated iron uptake. Based on these observations, the 29 kDa protein has been postulated to be a ferri-exochelin receptor in *M. smegmatis*. IREPs were also identified in other mycobacterial species not only under defined lab conditions of established iron status but also under *in vivo* conditions. The 21 kDa IREP was expressed in *M. leprae* and *M. avium* recovered from experimentally infected animals (Sritharan & Ratledge, 1990). Recent studies on *M. tuberculosis* in our lab showed the coordinated expression of mycobactin, carboxymycobactin and Irep-28 upon iron limitation (Yeruva *et al*., 2006). The latter was identified as HupB, the DNA-binding HU homologue. The clinical correlation of the detection of anti-HupB antibodies with culture and radiological confirmation showed the significance of this protein.

In the light of the observations on the influence of iron on several proteins, the effect of iron on the expression of CFPs was studied. As established earlier (Yeruva *et al*., 2006), maximal levels of carboxymycobactin and mycobactin were found to be expressed in *M. tuberculosis* grown under low iron conditions (0.02 μg Fe / mL), which was about 5 - 6 times higher than organisms grown with iron added at 8 μg Fe / mL (high iron). The spent growth medium, recovered by centrifugation followed by filtration through 0.45 μm membrane to ensure the complete removal of all bacterial cells was processed further. The CFPs were concentrated by precipitation with ammonium sulphate followed by ultrafiltration. Electrophoretic separation by single dimension SDS-PAGE did not reveal any differences in the protein profile of the CFPs of high and low iron grown organisms, harvested after about 14 days of growth, when the cells were in the log phase of growth. However, when allowed to grow till day 20, differences in the protein profile was observed. Proteins of approximate molecular mass of 60, 27, 17 and 8 kDa proteins were expressed by low iron

organisms. Further analysis of these proteins was not pursued as they could be cytoplasmic proteins released from dead cells and /or stress proteins.

2D-PAGE analysis of CFPs of *M. tuberculosis* was attempted by Young and Garbe (1991); they identified DnaK, GroES, GroEL and four novel proteins, recognized as heat shock proteins. Simultaneously, Nagai *et al.* (1991), using 2D - PAGE and N-terminal sequencing identified the 12 most abundant proteins from the spent growth medium of *M. tuberculosis*. Subsequently, 2D – PAGE coupled with peptide mass printing identified ORFs, which are not revealed by genome sequencing. A number of techniques can be used for the identification of proteins from 2D - PAGE gels; they include N-terminal sequence analysis, mass spectrometry, amino acid composition analysis, co-migration studies and immunodetection, the latter techniques being applicable for small concentration of the protein samples, while the first three techniques were considered applicable for large-scale samples (Rosenkrands *et al.,* 2000; Malen *et al*., 2007). 2D maps have been generated by these groups and it is now possible to assign, with certain degree of accuracy the details of the protein(s) by comigrational analysis, based on the pI and molecular mass of the protein(s).

In this study, several iron-regulated proteins were identified by 2D – PAGE. They are represented as CFP with their respective molecular mass written as superscript. Iron induced the expression of several proteins (Table 9), including CFP^{65}, CFP^{44}, $CFP^{40a, 40b}$, CFP^{35}, CFP^{30c}, CFP^{23}, CFP^{22b}, CFP^{13}, $CFP^{12.5}$, CFP^{9a} and CFP^{9b}. The CFP^{30c} and CFP^{23} protein were subjected to MALDI-TOF analysis and shown to be MCE1B (Rv0170) and Rv0660 respectively. The former also reported by Malen *et al.* (2007) is a member of the MCE family of proteins, with a putative role in host cell invasion. The latter is a conserved hypothetical protein, possibly an antitoxin that is part of toxin-antitoxin (TA) operon along with Rv0659c, encoding the toxin. Iron limitation induced the expression of CFP^{70}, CFP^{55}, $CFP^{30a, 30b}$, CFP^{31}, $CFP^{28a, 28b}$, $CFP^{22a, 22c}$, $CFP^{11a, 11b}$, CFP^{10} and CFP^{8}. The CFP^{70} is DnaK, as also seen in the 2D maps of Statens Serum Institute and Max Plank Institute. It is a chaperone protein with ATPase activity and thought to be induced by stress conditions. Of the three proteins spots of about 22 kDa, CFP^{22a} and CFP^{22c} were induced upon iron limitation; the functions of these proteins are unclear with the former belonging to rRNA methylase family of proteins while the latter is a hypothetical protein. The Sec pathway is highly conserved and is the primary pathway for protein transport across

the cytoplasm of mycobacteria. Proteins transported by the Sec pathway are synthesized as precursors with sec sequence at their amino terminal. Sec precursor proteins are transported in an unfolded state through the core of the translocase, which is the membrane embedded channel, composed of SecY and SecE proteins (Braunstein *et al.,* 2001). CFP^{10}, identified as the pre-protein translocase SecE1 subunit and CFP^{8} as a probable 50 S ribosomal proteins L10RP need further analysis to understand the influence of iron on their expression as well as their role under in vivo conditions of growth. Kitaura *et al.* (1999), in their studies on PPD showed that two of the secretory proteins were the ribosomal proteins L7 and L12. Further, a combination of these two proteins L7/L12 induced a strong delayed-type hypersensitivity reaction in guinea pigs. Another subunit of the 50S ribosome is L22 (Rv0706), which is an IdeR- independent iron induced gene (Rodriguez *et al.,* 2003). As mentioned earlier, regulation by iron is complex in mycobacteria with more than one regulator and it is likely that the iron machinery and oxidative stress machinery are associated and gene regulation in these bacteria are so finely tuned to adapt them to the intracellular hostile environment of the macrophage. CFP^{55} and CFP^{11a} were recognized as glutamine synthetase and the GroES respectively by comigrational analysis. Of special significance is the ESAT-6 family of proteins, some members of which are found to iron-regulated.

There is a wealth of information on the ESAT-6 family of proteins (Brandt *et al.,* 2000, 1996, Brodin *et al*., 2006). The significance of these proteins stems from the observation that they are restricted to the pathogenic *M. tuberculosis* and they are lost in the attenuated *M. bovis* BCG, as a part of the RD1 region not seen in the latter as well in a majority of environmental mycobacteria (Maharis *et al.,* 1996). Genome analysis data and 2D map data, coupled with immuno-detection using specific monoclonal antibodies have shed light on this important group of mycobacterial proteins. Certain isoforms of the ESAT-6 molecule from *M. tuberculosis*, separated by two-dimensional electrophoresis, were identified as carrying a post translational modification, namely an acetylation of the threonine residue at position 2, and it was observed that only the unacetylated form of ESAT-6 interacted with CFP-10 (Okkels *et al.,* 2004). The implications of ESAT-6 in virulence and the diagnostic potential of these proteins for sero-diagnosis are gaining strength with experimental evidence from several reports. It is useful as a diagnostic marker to differentiate between the BCG vaccinated healthy individuals and the tuberculosis patients, with high

specificity and sensitivity (Ravn *et al*., 1999). Also, it is used to evaluate the T cell immune response as it stimulates T cells from tuberculosis patients to proliferate and produce IFN-γ. It is thus used in the IFN-γ commercial kits currently used in several clinical laboratories.

The first preparation of ESAT-6 available for biochemical characterization was purified from *M. tuberculosis* culture filtrate and the protein was expected to have a pI of 4.48 and a mass of 9.8 kDa. Initially studies by Sorensen *et al.* (1995) had identified 2 reactive spots with ESAT-specific monoclonal antibodies HYB 76.8 between the pI 4 - 4.8. Modification of the protocol by Okkels *et al*. (2004) identified as many as 8 reactive spots with HYB76.8. In our study here, we used HYB 76.8 for immunoblotting analysis. Two 9 kDa protein spots, recognized by HYB 76.8 were expressed constitutively while the 8 kDa protein was repressed upon iron limitation. The time course study also showed increased expression of ESAT-6 (it was possible to identify only one band of 9 kDa) in high iron organisms when compared to low limited cells. These observations need further analysis to understand the role of iron in this important class of proteins implicated in virulence. It may shed light on post-translational modifications, if any, of some of the members that perhaps could play a significant role in host-pathogen interactions.

In this study, we also analysed the different strains of *Mycobacterium bovis* BCG, which was first obtained by serial passages as an attenuated strain of the virulent *M. bovis* in 1921. Since then, it has moved to several countries around the world, resulting in several strains. Reports on the variations among these strains are available. IS6110-based restriction-fragment-length- polymorphism (RFLP) showed two patterns for BCG strains (Bher *et al.,* 1999). Some BCG strains contain two copies of this transposable element and other strains of BCG only have one copy. The strains Japan Russia, Moreau contain two copies while the others contain one copy of IS6110. MPT64 was another genetic variant in BCG. Genetic analysis documented that the gene encoding this antigen, *mpt64*, was absent in certain strains of BCG. Using the historical record of strain dissemination as reference, it was noted that two copies of IS6110 are only seen in strains obtained before 1925 (strains Russia, Moreau and Japan) and that absence of the *mpt64* gene coincides exactly with BCG strains obtained from the Pasteur Institute after 1927.

In this study, we included *M. bovis* BCG Danish, Birkhaug, Phipps, Moreau, Sweden, Phipps and Pasteur to analyse the effect of iron concentration on the CFP

profile of the different BCG strains. All the BCG strains responded to iron deprivation and expressed significant levels of both mycobactin and carboxymycobactin. Maximal level of mycobactin was expressed by Danish strain, with the lowest expression in Russia. Several iron-regulated CFPs were seen, as mentioned in results. However, further work is required to understand the role of these proteins.

The objective in the second half of this study was to look at the immune response of the lymphocytes from tuberculosis patients to the CFPs of *M. tuberculosis*. Due to the low levels of these iron-regulated proteins, and also due to the fact that antigen pools are better candidates for study, we prepared culture filtrate antigen (CF-Ag) pools from high and low iron grown organisms of *M. tuberculosis*. The selection of these antigen pools was done based on molecular weight cut-offs. The CFPs were separated by preparative gel electrophoresis and the gel slices between specified markers were cut out and the proteins eluted, as detailed in methods. The eluted antigen pools were re-run, both by single dimension PAGE and 2D – PAGE and compared with the crude CFPs. Our protocols were designed based on published data and strategies followed by other researchers to identify CFPs for studies in T-cell proliferation. In some of the methods, the CFPs were initially eluted from gel slices (carrying specific protein bands / spots) using SDS-containing buffer followed by the removal of SDS by high-pressure dialysis. In the T-cell Western blot method, nitrocellulose membrane carrying the CFPs (after transfer from gel subjected to SDS-PAGE) was cut into small pieces carrying closely associated proteins and then adding these nitrocellulose strips to T cell cultures (Abou-Zeid *et al.,* 1987). This technique minimised the SDS in the cell cultures, but it had the disadvantage that proteins added could not be quantified and characterized. Mehra *et al.* (1989) used the T-cell western blot method to identify a low molecular mass fraction of *M. leprae* sonicate recognised by a high frequency of reactive *M. leprae* reactive human T-cell lines. Another method included the elution of the protein spots from 2D gels and the subsequent stimulation of lymphocytes using these gel-eluted proteins (Wallis *et al.,* 1993). This technique helped to identify a 58 kDa protein in *M. tuberculosis* which induced the production of TNF-α in cultures of human monocytes. This technique however had the disadvantage of low recovery of protein(s), thereby limiting quantitative and qualitative analysis. Recent studies have adopted the procedure of Andersen and Heron (1993), in which the CFPs were separated by SDS-PAGE and the gel was cut into slices and the proteins eluted from them. A modification of this

procedure is to electrophoretically elute out the protein fractions. The net objective of these procedures is to obtain a panel of antigens, representing proteins over a narrow molecular mass range.

All these efforts are being made to identify potential diagnostic antigens and proteins that are immuno-protective, with the objective of preparing effective vaccines (Abebe *et al.,* 2007; Demisse *et al*., 1999). Till date, BCG is the only available tuberculosis vaccine. Since its discovery in 1921 by Calmette and Guerin in France, it has been distributed to several countries and is widely used for the control of tuberculosis, with more than three billion people receiving the vaccine. However, there is controversy about its efficacy, with the 10 year old trial in Chingleput, India (Tuberculosis Research Centre, 1999) showing it is ineffective in the population studied.

The outcome of an infection by *M. tuberculosis* is determined by a complex interplay of both the host and pathogen related factors. In tuberculosis, the cell-mediated immunity plays an important role. This involves a varied population of immune cells, including the macrophages, different subset of T-cells and interplay among them mediated by several cytokines. Phagocytes are activated to produce pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-12. TNF-α is a monocyte-activating cytokine which stimulates anti-mycobacterial activity and helps to maintain the integrity of the tuberculous granulomas in which *M. tuberculosis* is contained (Tufariello *et al.,* 2003). IL-12 links innate and adaptive immunity, drives T-cells and NK cells to produce Th1 pro-inflammatory cytokines, including IFN-γ and TNF-α (Hoeve *et al.,* 2006). In synergy with TNF-α, IFN-γ activates infected macrophages and tries to eliminate intracellular pathogens as a major effector mechanism of CMI. T-lymphocytes, sensitized by an antigen undergo blastic transformation and produce IFN-γ when stimulated by the same antigen. The profile of secreted cytokines *in vitro* is taken as an indication of T-lymphocyte function *in vivo*, but the possible relationships between antigen specificity and T-lymphocyte function have so far not been examined. Murine CD4+ T cells have been divided into at least two different subsets (Thl and Th2), based on the cytokine profiles that they secrete upon antigen stimulation. The Th1 cells characteristically secrete IL-2 and IFN-γ whereas the Th2 lymphocytes produce typically IL-4, IL-5 and IL-10, which enhance antibody synthesis of B cells. *In vitro* stimulation of leukocytes with mycobacteria or their products induces synthesis and release of several cytokines, including IL-1, IL-2, IL-

6, TNF-α and IFN-γ. Two approaches have been taken to identify the cytokines that mediate protective immunity: characterization of the cytokine milieu at a local site of effective immunity and identification of cytokines that activate intracellular killing by mononuclear phagocytes. The pleural fluid and the mononuclear phagocytes show an over expression of IFN-γ, IL-2, and IL-12 with lowered expression of IL-4 (Sable *et al.,* 2007).

As culture filtrate proteins are recognised by the host immune system, CFPs have been evaluated, either as individual proteins or as antigen pools (Abebe *et al.,* 2007, Pai *et al.,* 2004; Surekha *et al.,* 2005). In this study, the CF-Ag pools (mentioned above) were used to study the T cell immune response of peripheral blood mononuclear cells (PBMCs) from three categories of tuberculosis patients, namely the smear positive (SP), smear negative (SN) and extrapulmonary (EP) cases respectively and compared with normal healthy (NH) individuals. Both immuno-proliferation and IFN-γ levels were analysed to assess the immune response of T cells. The study population is from Hyderabad, Andhra Pradesh and consists of patients being screened and treated in the Government Chest Hospital, Hyderabad. As indicated in methods, specific exclusion criteria were adopted and clear cases of smear positive (n = 25) and smear negative (n = 20) confirmed cases of pulmonary tuberculosis with no prior treatment for tuberculosis and a group of extra pulmonary tuberculosis (n = 20) were major components of the study. The normal healthy individuals (n = 20) included student donors who were not associated with the hospital area.

Lymphocyte proliferation in the four study groups was done with the whole CFPs from high and low iron grown *M. tuberculosis* and compared with that of human PPD. The latter is used as a positive control in several studies, due to the high stimulation achieved with this mixture of culture filtrate proteins (Kori *et al.,* 2000, Van Crevel *et al*., 1999). The extrapulmonary group did not show notable proliferative response. In this study also, PPD evoked a strong proliferative response in cases of pulmonary tuberculosis, including both smear positive (SP) and negative (SN) group, unlike the control normal healthy (NH) individuals whose lymphocytes showed poor response. However, no differences were discerned between high and low iron preparations. This expected observation could be attributed to the large number of antigens in the crude preparations, resulting in a cumulative effect on proliferative response. Appreciable differences were however seen with defined CF-Ag pools, not only among the different pools from the respective high / low iron cells but also

between the corresponding CF-Ag pools from high and low iron grown cells. Statistical analysis of the data was done using SPSS software. The values are represented as medians since the data did not approximate normal distributions. This is consistent with the hypothesis that all the groups contain antigen responsive and antigen un-responsive individuals (Demissie *et al*., 1999).

The individual antigen pools, like the crude CFPs induced low levels of proliferation of the lymphocytes from the controls (NH). However, these values are relatively higher than expected, as this is an endemic area and most of the individuals are exposed to mycobacterial antigens. The extrapulmonary samples showed a similar effect, with a slightly higher response to PPD. Analysis of the different antigen pools on pulmonary tuberculosis cases showed significant findings. The stimulation index of the PBMCs of SP group in response to H1 and L1 CF-Ag pools showed a median SI of 2.3 and 2.47 respectively. This is close agreement with that seen with PPD (median SI of 2.5). Using PPD as antigen, case recognition in the SP group was 76%. A closer percentage of case recognition was seen with LI (68%) with a much lower percentage with H1 (60%), with case recognition of 60% as compared to 55% with both H1 and PPD. As mentioned above, low proliferation and poor recognition was observed with these two antigen pools; PPD showed a relatively significant response with 60% recognition of the extra-pulmonary cases. This first set of antigen pools contained proteins in the molecular weight range of 66 – 116 kDa.

In the second set of antigen pools, consisting of proteins in the molecular weight range of 45 – 65 kDa, observation with the L2 panel of antigens was significant, with a median SI < 2.25 and recognizing 72% of SP cases; the H2 panel recognized only 44% of cases. Similar findings were seen with the SN cases, with 65% recognition of SP by L2 antigen pool and 40% recognition by the H2 antigen pool. These results are notable as the L2 panel recognizes only 26% of control NH group. Results were poor with EP group, with only 35% of the extra-pulmonary individuals recognised by H2 and L2. Thus the L1 and L2 CF-Ag pools are promising candidates.

The next set of antigen pools, represented as H3 and L3 in the molecular weight range of 25-45 kDa showed poor response as explained in the results section. The L4 group of antigens, with proteins in the molecular weight range of 25-14 kDa performed better than the above group though lower than L1 and L2, with L4 showing 52% recognition of cases with a median SI of 2.06 in SP cases. The corresponding H4

pool showed a low response (28% recognition). The H5 CF-Ag pool, consisting of low molecular weight proteins, including the ESAT-6 group of proteins showed stimulation of the PBMCs from SP cases with a median SI of 2.01 while L5 showed a lower stimulation. However, the L5 fraction showed good promise for the EP cases, with a much higher % of recognition seen that is comparable to H3 and PPD.

Thus, the L1, L2 and L3 CF-Ag pools performed better than the corresponding high iron CF-Ag pools, with comparable results with PPD. The H5 CF-Ag pool, containing the ESAT group of antigens showed good proliferation though the recognition was relatively lower than PPD. The L1 and L2 CF-Ag pools proved to be significant in the SN group also and in addition, there was statistical significance of both SP and SN groups when compared with normal individuals. Though most of these CF - Ag pools failed to recognize the EP group of tuberculosis cases, L5 CF-Ag pool proved to be better when compared to other antigens, though the response was still lower than PPD.

It is now well established that higher levels of IFN-γ is promising as it correlates with lower incidence of the establishment of the disease. Individuals in an endemic area get infected but not all develop the disease. There are reports that suggest that activation pathways and induction of Th-1-like immune response pathways leading to the generation of IFN-γ is critical for protection against the disease (Cooper *et al.,* 1993). IFN-γ is a powerful activator of macrophages, thereby enabling the latter to kill the residing mycobacteria. Thus, the level of IFN-γ correlates with protection against disease development by the pathogen. However, as other studies have shown, it must be acting in concert with other cytokines and not on its own in affording protection to the host (Sahiratmadja *et al.,* 2007). In a study by Demisse *et al.* (1999), aimed at studying the immune response of tuberculosis patients and their close contacts, the latter showed high levels of this cytokine in comparison with the tuberculosis patients. In our study here, several fold higher levels of IFN-γ is seen in the healthy controls, with much lower levels of expression in patients with disease. This adds confirmatory evidence to the protective nature of this molecule. Among the antigen pools used in this study, the L5 CF-Ag pool (containing the ESAT-6) evoked the maximal response with markedly high levels of IFN-γ, higher than that triggered by PPD. The findings that ESAT-6 is present in members of *M. tuberculosis* complex but absent in *Mycobacterium bovis* BCG strains and most environmental mycobacterial species enable this molecule to be used both as a

potential vaccine candidate and a novel specific diagnostic reagent. ESAT-6 was consistently found to be a very important target for the T-cell response in the first phase of infection in humans, cattle and guinea pigs. ESAT-6 was recognized during the recall of protective immunity, and the T cells specific for ESAT-6 constituted as much as 25–35% of the mycobacterium-reactive T-cell repertoire recruited in the first phase of disease (Brodin *et al.,* 2004). H3 and L3 CF-Ag pool that caused low level of proliferation of the PBMCs from SP cases of tuberculosis stimulated the release of high levels of IFN-γ in normal individuals, thereby raising the possibility of further studying this group of antigens as potential protective antigens. This antigen pool presumably contains the Ag85 complex, which is comprised of three proteins that have been shown to induce strong T-cell proliferation and IFN-γ production in healthy TB patients (Lim *et al.,* 2004). Though we did not observe notable proliferation, high levels of IFN-γ were seen in the NH individuals. IFN-γ levels have also been shown to exert its influence on the cellular iron status of the macrophage. IFN-γ activation of human monocytes down-regulates (TfR) transferrin receptors numbers on the cell surface (Byrd & Horwitz, 1989) and the rate of macrophage iron acquisition from holotransferrin (Olakanmi *et al.,* 2002). IFN-γ decreases iron availability to intracellular microorganisms that utilize transferrin iron, such as *Legionella pneumophila* and *M. tuberculosis.*

Thus, in this study, we tested the efficacy of the CFPs of M. *tuberculosis* grown under high and low iron conditions to elicit appropriate immune response, so as to enable their application as diagnostic and vaccine candidates. As we could demonstrate differences only by 2D – PAGE, we could not obtain sufficient quantities of the individual antigens for immune proliferation studies. Hence, pools of antigen had to be used. The promising antigen pools have been identified and future work includes the scaling up of these antigen preparations and testing them on a bigger population of samples to establish their applicability as candidate antigens for both diagnosis and vaccine preparation. CFPs have been tested in various animal models of tuberculosis (Andersen *et al.,* 1991). One of the requirements of a candidate vaccine antigen is recognition by the immune system during the course of infection by the majority of individuals of the target population (Dietrich *et al.,* 2006). The parameters used as indicators of T-cell responses include antigen specific proliferation and the measurement of IFN- γ production to identify what are likely to be $CD4^+$ lymphocytes

of the Th1 phenotype that are considered to play an important role in the response against virulent *M. tuberculosis*.

Though antibody-based diagnostic tests have not shown much promise, there is increasing interest in the development of ELISA based tests, specifically with antigens like ESAT-6 that are expressed only by *M. tuberculosis*. Several CFP genes have been cloned, expressed and evaluated for their sero-diagnostic potential (Weldingh *et al*., 2005). Results with several different recombinant *M. tuberculosis* CFPs suggest that immune recognition varied randomly from patient to patient and there is no definite antigen or set of antigens that is recognized by all or a majority of patients (Gennaro, 2000). No commercially available serological test has so far shown useful levels of sensitivity and specificity, which may be due to the great heterogeneity of the antibody response in TB patients (Weldingh *et al*., 2005), and it is therefore generally accepted that it will be necessary to include several antigens in a future serodiagnostic assay (Lyashchenko *et al*., 1998). Through the use of both cocktails of single proteins and genetically engineered fusion molecules containing several antigens, it has been demonstrated that the necessary improvements in sensitivity can be achieved by combining the best antigens (Houghton *et al*., 2002).

In this study, the CF-Ag sets were used as antigens in ELISA to detect antibodies in the serum of tuberculosis patients. PPD was used as control, which however showed low reactivity. Sero-reactivity was seen only with the SP group with negligible reactivity in the SN and EP cases. In the SP group, the H1, L2, H3 and L3 showed prominent sero-reactivity. The results obtained with ELISA were correlated with Mycotest, a lateral flow device which was also useful only in the SP cases. The CF-Ag H1 showed 60% specificity and 90% sensitivity. Laal *et al.* (1997) showed that the high molecular weight proteins (88 kDa) elicited strong humoral response. Our data is in agreement with their studies that H1 and L2 had given prominent sero-reactivity with the smear positive group. The H3 and L3 antigen pools, containing the Ag85 complex had given about 60% and 50% positivity with the smear positive individuals; this was promising as earlier studies by Sable *et al.*(2005) showed substantial levels of antibodies against Ag85A and Ag85B were found in tuberculosis contacts.

In conclusion, this study has given significant observations on the influence of iron on the culture filtrate proteins. They have been shown to play a significant role in host-pathogen interactions when analysed with the PBMCs of tuberculosis patients.

Future work will focus on the further characterization and screening of a wider group of patients both for cellular immunity and humoral immunity and identify antigens of diagnostic and vaccine potential.

SUMMARY

Iron acquisition machinery has been the focus of research in our lab. Interest in this area has stemmed from the implications of iron acquisition under *in vivo* conditions. Culture filtrate proteins (CFPs) are the secretory / excretory proteins released into the immediate environment. Several factors like growth media, period of growth and aeration of cultures influence the profile of these proteins. Though there is a wealth of information on the culture filtrate proteins of mycobacteria, the influence of iron on the CFP profile has not been addressed. Since the secretory proteins have been shown to play an important role in the host T cell response in tuberculosis patients, the immune response to iron-regulated culture filtrate proteins formed an important objective of our study.

Mycobacterium tuberculosis was grown under high iron (8 μg Fe / mL) and low iron conditions (0.02 μg Fe / mL). As expected, there was notable expression of both the siderophores mycobactin and carboxymycobactin under low iron conditions. We used an actively growing culture of the organism to ensure that the cytoplasmic proteins from dead and degraded bacteria did not contaminate the sample. The spent growth medium was processed and the CFPs were analysed by SDS-PAGE / 2D – PAGE and several iron-regulated proteins were identified. MALDI-TOF MS analysis and immunoblotting with specific monoclonal antibodies helped to identify several of these proteins.

MCE1B (Rv0170), a member of the MCE family of proteins and Rv0660, a conserved hypothetical protein were expressed under high iron conditions. The former probably plays a putative role in host cell invasion and the function of the latter remains to be eludicated, though it is annotated as a possible antitoxin that is part of toxin-antitoxin operon along with Rv0659c, encoding the toxin. The ESAT-6 family of proteins appear to be influenced by iron levels. This needs further analysis to confirm if the additional protein seen in high iron conditions is a modified form of ESAT-6, as acetylation of this protein has been reported and which plays a role in its interaction with the CFP10 protein. Seven proteins expressed by *M. tuberculosis* upon iron limitation include DnaK, Rv2096c, Rv0881 with rRNA methyl transferase activity, pre-protein translocase SecE1 subunit and Rv0651, a probable 50 S ribosomal proteins L10RP and the remaining two were identified as hypothetical proteins.

The second major objective was to study the immune response of the human host to these proteins. Specific culture-filtrate antigen (CF-Ag) pools were prepared and the proliferation and assay of IFN-γ of the peripheral blood mononuclear cells of tuberculosis patients to these CF-Ags were done. Also, the serum of these patients was tested by ELISA for the presence of antibodies against these CF-Ags.

Mycobacterium bovis BCG obtained in 1921 was distributed globally and today a number of strains are available. Differences at the genome and protein levels have been demonstrated by several researchers. Here, the CFPs profile with reference to iron levels was analysed. All of them expressed the siderophores mycobactin and carboxymycobactin. The CFP profile of the different strains of *M. bovis* BCG showed several proteins influenced by iron.

In the second part of the study, the immune response of the lymphocytes from tuberculosis patients and normal individuals to CFPs was assessed. The CFPs were fractionated into defined culture filtrate antigen (CF-Ag) pools. Patients included smear positive (SP), smear negative (SN) and extra-pulmonary (EP) groups and the control group was normal healthy (NH) individuals. Maximal lymphocyte proliferation response was seen in the SP group with all the CF-Ag pools in general when compared to the other groups of tuberculosis patients and normal healthy individuals. Among the CF-Ag pools, the L1, L2 and L3 produced significant proliferation and recognised maximal number of cases. With the L5 fraction, encompassing the ESAT-6 antigens, proliferation was not as high but good recognition of cases was observed. The IFN-γ levels in response to these CF-Ag pools was highly indicative in these tuberculosis patients. Among the antigen pools used in this study, the L5 CF-Ag pool (containing the ESAT-6) evoked the maximal response with markedly high levels of IFN-γ, higher than that triggered by PPD.

To conclude, the influence of iron levels on the CFP profile was analysed and several iron-regulated proteins were identified. The clinical significance of these proteins was evident from the immune response studies. Pulmonary tuberculosis patients, both smear positive and smear negative cases were easily identified with these CF-Ags. However, these antigen pools were not useful in extrapulmonary cases, though the L5 pool proved relatively promising. In addition, ELISA-based detection of antibodies against these CF-Ag pools was promising in pulmonary cases and more samples should

be analysed for the practical application of screening for antibodies for diagnosis. The observations in the different strains of BCG also warrant further investigations, especially to identify those iron-regulated proteins that are not expressed in BCG strains but are seen in *M. tuberculosis*, so as to develop tests to distinguish between individuals who are infected with the pathogen and BCG – vaccinated individuals.

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