MOLECULAR AND IMMUNOLOGICAL APPROACHES IN THE DIAGNOSIS OF HUMAN TUBERCULOSIS

SUMI S

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MOLECULAR AND IMMUNOLOGICAL APPROACHES IN THE DIAGNOSIS OF HUMAN TUBERCULOSIS

A thesis presented

by

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DEPARTMENT OF PATHOLOGY

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CERTIFICATE

I, Sumi S, hereby certify that I had personally carried out the work depicted in the thesis entitled "*Molecular and immunological approaches in the diagnosis of human tuberculosis*" under the direct supervision of Dr. V.V. Radhakrishnan, Professor and Head, Department of Pathology, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala, India, except where external help was sought and is acknowledged.

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This is to certify that **Ms. Sumi S**, in the Department of Pathology of this institute, has fulfilled the requirements of the regulations relating to the nature and prescribed period of research for the PhD degree of the Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram. The work relating to her thesis entitled "***Molecular and immunological approaches in the diagnosis of human tuberculosis***" was carried out under my direct supervision.

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Dr. V.V.Radhakrishnan

(Guide)

The thesis entitled

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Sumi.S

LIST OF FIGURES AND TABLES

ABBREVIATIONS

ADA: adenosine deaminase

AFB: acid fast bacilli

Ag: antigen

ATT: antituberculosis chemotherapy

BCG: bacille Calmette-Guérin

bp: base pair

CFA: culture filtrate antigen

CMI: cell mediated immunity

DNA: deoxyribonucleic acid

DTH: delayed type hypersensitivity

EDTA: ethylenediaminetetraacetic acid

ELISA: enzyme-linked immunosorbent assay

ESAT-6: 6 kDa early secretory antigenic target

FFPE: formalin-fixed paraffin embedded

H&E: Haematoxylin and Eosin

His: Histidine

HIV: human immunodeficiency virus

Hsp: heat-shock protein

IFN-γ: interferon-gamma

Ig: immunoglobulin

IHC: immunohistochemistry

IL: interleukin

KDa: kilodalton

LJ: Lowenstein-Jensen

LTB: latent tuberculosis

MDR-TB: multidrug-resistant tuberculosis

MTT: 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide

Ni-NTA: Nickel- nitrilotriacetic acid

NTM: non-tuberculous mycobacteria

PAGE: Polyacrylamide gel electrophoresis

PBMC: peripheral blood mononuclear cells

PCR: polymerase chain reaction

PPD: purified protein derivative

PTB: pleural tuberculosis

RD: regions of difference

TB: tuberculosis

TBL: tuberculous lymphadenitis

TBGL: tuberculous glycolipid antigen

TNF: tumor necrosis factor alpha

TPE: tuberculous pleural effusion

Trx: thioredoxin

TST: tuberculin skin test

WHO: World Health Organization

XDR-TB: extensively drug resistant tuberculosis

ZN: Ziehl-Neelsen

SYNOPSIS

Human tuberculosis (TB) is a re-emerging infectious disease in many parts of the world. Prompt detection, isolation, and identification of the causative organism, *Mycobacterium tuberculosis,* in clinical specimens from patients with tuberculosis is absolutely essential in the overall management of patients suffering from TB. Currently available methods including microscopic examination of sputum smears stained with ZN method, culturing of *M. tuberculosis* from clinical specimens, PCR- based detection systems, etc, are unable to meet the requirement of an ideal diagnostic test that possesses high sensitivity and specificity.

Detection of active TB as well as individuals with latent TB (LTB) infection will play an important role in the control of TB epidemic. It is estimated that 2 billion people are latently infected with *M. tuberculosis*, which causes active disease in 5 to 10% of infected individuals. The most common test used to determine if a person has been infected by *M. tuberculosis* is the tuberculin skin test (TST) with PPD antigen, which is limited by its low specificity. An attractive methodology that continues to be explored is the detection of *M. tuberculosis*-specific antibody in patient sera. Several mycobacterial antigens have been used as antigens in the immunodiagnostic assays including secretory and excretory protein antigens, lipid antigens etc. The complex antigenic composition of mycobacteria and the presence of cross-reactive epitopes throughout the group have made it difficult to develop ideal immunological methods for the diagnosis of tuberculosis.

Recent reports indicated that recombinant mycobacterial antigens usually accessible in high purity and large amounts are being used to enhance the specificity of immunoassays such as ELISA and Western-blotting. Hence a diagnostic approach using

highly pure immunologic reagents, in the form of 'recombinant antigens' was found suitable in the context.

Broad objective of this study:

- To develop immunological and molecular methods for the early laboratory diagnosis of human tuberculosis.

Initial studies on the existing methods of TB diagnosis and evaluation of their efficiency in our population hold significance in the designing of any new method. Hence the following specific targets were set for the study to fulfill the above broad objective.

Specific aims of this study:

1. To evaluate existing diagnostic methods in patients with extra-pulmonary tuberculosis
2. To develop a diagnostic system for the accurate diagnosis of human tuberculosis
3. To study the host cell- mediated response against the recombinant mycobacterial antigens, and to assess their application to distinguish individuals with latent TB infection from individuals with BCG vaccination.

Study subjects: For the evaluation of existing methods, pleural fluid specimens and formalin fixed paraffin embedded lymph node specimens from tuberculosis and disease control patients were collected. For serodiagnostic studies sera samples were collected from healthy, disease control and TB patients. For cell mediated cytokine release assays, whole blood samples were collected from healthy controls and TB patients.

Basic procedures and findings: Basic cytology/histology studies did not yield more information other than a broad distinction between inflammatory and malignant

aetiologies. Bacteriological studies like Ziehl-Neelsen staining and culture was less sensitive and time consuming.

Standard PCR targeting insertion element *IS6110* was applied on these paucibacillary specimens. *IS6110* PCR yielded more sensitivity than conventional methods in detecting TB cases, but was found to be less specific with many false positive results. This led to the selection of *M. tuberculosis* specific sequence, *mtp40*, for standard PCR. *mtp40* PCR was able to eliminate many false positive results identified by *IS6110* PCR but was less sensitive. To attain high sensitivity with less false positive results, a nested PCR amplifying *mtp40* genomic sequence was performed. Nested PCR was more sensitive, with less false positive cases. Nested PCR was able to distinguish between malignant and tuberculous aetiologies.

Standard and nested PCR could not distinguish between active, treated, and latent cases of TB. The stringent conditions needed for DNA isolation as well as PCR would be very difficult to achieve in laboratories of developing countries. Concern regarding false positive results due to cross-contamination prevails.

In situ PCR was applied in TB lesions to demonstrate *M. tuberculosis* DNA within tuberculous granuloma. It was found that i*n situ* PCR could be applied to very less number of samples. The adhesion of different tissues on to the slide varied greatly depending on the histological variations of particular tissue. Different slides were used for the study, with very little success rate.

None of the methods evaluated or modified till this was found to be adequate for meeting the objective of the study. Detection of latent infection was not achieved. Reports suggest that the existing methods like TST are inefficient in detecting these

cases. An alternative method which is sensitive and specific like PCR and devoid of cross contamination issues evoked by PCR was hence to be attempted. For easy detection of active cases and latent cases, serodiagnosis was found to be a better method. An approach using highly pure immunologic reagents, i.e., recombinant antigens, was undertaken to develop a diagnostic system for the rapid detection of TB. Studies were also planned so as to point out some direction in distinguishing active and latent cases. Four mycobacterial genes were hence selected for this study.

The first gene selected was *Rv2031c (hspX)* which is expressed during infection, as recognized by sera from a majority of TB patients. Production appears to increase as the bacteria go into the metabolically resting stage and decrease as they revert to exponential growth. Second gene was *Rv1174c* (*tb8.4*) which was suggested to be involved in reactivation of dormant mycobacteria and also is considered to be an immuno-reactive T-cell antigen. Third gene was *Rv2351c* (*plcA*) coding for a probable membrane-associated phospholipase C 1. Sequence, *mtp40* was originally identified as a short genomic region in *plcA*. This gene was found in strains of *M. tuberculosis* but not in *M. bovis* and *M. bovis* BCG. Last gene selected was the highly studied Rv3875 (*esat-6*) coding for a 6 KDA early secretory antigenic target which is a RD1 encoded protein. Region of difference 1 (RD1), is absent from every strain of BCG and present in every virulent strain tested so far.

Primers were designed for these genes, and their prevalence in Kerala population was studied by PCR in respiratory and non-respiratory specimen DNA. After finding that these genes were present in our population, these genes were cloned and expressed in *Escherichia coli* using pET expression vector system. A 6X Histidine tag sequence was

placed on the N-terminal of the target protein by the cloning vector. Recombinant proteins were purified to near- homogeneity by affinity chromatography using Ni-NTA agarose columns which have strong affinity to Histidine residues. Purification was performed in native and denaturing conditions depending on the solubility of proteins. Urea used in denaturing mode of purification was removed by PD10 columns and also by dialysis by decreasing gradient of urea. Protein was quantified using Bradford's method.

The specific role of recombinant mycobacterial antigens in humoral as well as cell-mediated immunity was characterized, so that their specific properties will be incorporated in developing an efficient diagnostic method for fulfilling the above aims. Using ELISA, IgG antibodies against these recombinant antigens in sera and pleural fluid of patients were studied. Due to diverse antibody response in TB patients, it was essential to combine several immuno-reactive mycobacterial antigens in diagnostic assay to achieve desired levels of sensitivity and specificity. Hence a multiantigen cocktail consisting of all four antigens were mixed in equal concentration, and an ELISA was performed. Multiantigen cocktail was more sensitive with acceptable specificity. HspX was more sensitive among individual antigens. The study proved that PlcA, HspX and Tb8.4 antigens are immunoreactive like ESAT-6.

In-house polyclonal antibody against these antigens was effective in detecting the mycobacterial antigens in TB infected lymph node sections in immunohistochemical staining. These antibodies were compared with other in-house polyclonal antibodies like anti-tuberculous glycolipid antigen- antibody and anti-culture filtrate antigen-antibody.

Antibodies against recombinant antigens were more reactive and specific when compared to others.

Major findings of serological assays:

1. Multiantigen cocktail was found to be very sensitive in detecting active TB cases.
2. HspX, although renowned as a latency associated antigen, was found very useful in detection of active cases.
3. The multiantigen formulation is providing acceptable sensitivity without much loss of specificity.

Provided such assays as well as other conventional assays aid in the active TB detection, it becomes imperative to find some theme for LTB diagnosis as well. Accurate diagnosis of LTB is scientifically challenging because of the low burden of dormant tubercle bacilli. The strong cellular immune response triggered by LTB serves as an amplified signal for the presence of these dormant bacilli. T-cell-based interferon-gamma (IFN- γ) release assays represent a 100-year upgrade of existing TST. ESAT-6 and CFP10 are the predominant antigens used in IFN- γ tests. Although the diagnostic sensitivity of these tests is higher than that of the TST, their clinical use demands higher sensitivity.

Increasing the diagnostic sensitivity of such assays by the incorporation of additional antigens of established high specificity was considered a useful approach. A second approach was to infer additional useful clinical and biological information about our patients by measuring immunological mediators other than IFN-γ. Hence it became essential to study human peripheral blood mononuclear cells (PBMC) responses to recombinant ESAT-6, HspX, Tb8.4 and PlcA in our population. IFN-γ, Tumor Necrosis

Factor (TNF), Interleukin 4 (IL-4) and Interleukin 10 (IL-10) released *in vitro* was studied in BCG vaccinated healthy donors; healthy house-hold contacts of TB patients and in active TB patients.

Whole blood samples were collected from each group and PBMC was isolated, cultured with and without protein stimulus. After 5 days, supernatant was checked for the release of cytokines, while the cells were checked for proliferation using MTT assay. A commercial PPD was also evaluated with the four mycobacterial proteins.

Findings of MTT assay: Positive proliferative response was seen in most of the individuals with a total of 91.6% responders. Mantoux positive cases in TB and non-TB category had an increased proliferation. House-hold contacts of patients (without TB), but who are sensitized, showed maximum proliferation against mycobacterial antigens, in the healthy category.

Major findings of cytokine assays:

i. Pro-inflammatory cytokines like IFN-γ and TNF was found to be down-regulated in active TB patients.

ii. Anti-inflammatory cytokine, IL-10 was released in increased quantities in active TB cases.

iii. All the four antigens could distinguish the house-hold contact individuals of TB patients from BCG sensitized healthy donors.

iv. IFN-γ and TNF assay showed a very clear distinction between healthy BCG vaccinated subjects and healthy house-hold contacts.

v. IL-10 in healthy house-hold contacts was up-regulated as in active TB cases.

vi. All the other three antigens were useful in the detection process when compared to the highly studied 'ESAT-6'.

vii. Stimulation of PBMC with antigens did not induce the production of IL-4.

viii. TB patients undergoing optimal treatment showed increased IFN-γ and TNF release.

ix. These proteins are found to elicit strong TH1 response, i.e. elevated IFN- γ expression associated with lower expression of IL-4.

x. IL-10, which is another TH2 cytokine, was found increased which is in correlation with previous studies on prominent mycobacterial antigens.

xi. It has been reported that IL-10 inhibits the synthesis of cytokines by TH1 cells (i.e., IFN-γ and TNF). This may attribute, at least partially, to the decreased amounts of protective cytokines, IFN-γ and TNF.

Significance of this study:

- PlcA, HspX, and Tb8.4 were found to be as immunodominant as ESAT-6, and specific in serological studies. They can be used in development of different immunodiagnostic assays for the specific detection of TB. Heterogeneity in antibody production in TB patients can be compensated by using multiantigen cocktails where component antigens have acceptable sensitivity and high specificity.
- The pilot study on CMI responses against these antigens can be made useful in detection of latent TB.

- The study proves that T-cell based TNF and IL-10 release assays can be used as adjuncts to IFN-γ assays in diagnosis of both active and latent TB.

Further evaluation of this pilot study in large populations may help in the development of a diagnostic modality for latent tuberculosis.

I. INTRODUCTION

I.1. TUBERCULOSIS

Human tuberculosis (TB) is regarded as one of the major public health hazard in many parts of the world. Obligate aerobic bacillus-*Mycobacterium tuberculosis* is the causative agent of TB, and the disease is transmitted through air-borne droplets. As per a recent World Health Organisation (WHO) report, about one-third of the world population is exposed to *M. tuberculosis* and is at the risk of developing active disease (1). This report indicated that approximately 8.8 million new TB cases are being added every year. Much of the increase in the incidence of TB is as a result of world- wide human immunodeficiency virus (HIV) epidemic, which re-activates the latent phase of TB. HIV infected patients are also at an increased risk of acquiring multi-drug resistant (MDR) and extensively drug-resistant (XDR) tuberculosis (2). As long as HIV epidemic continue to dominate the scene, TB will remain a serious health problem throughout the world.

Due to the alarmingly high magnitude of the disease in terms of mortality and morbidity, WHO has launched several innovative TB control programs. The advances in mycobacterial research in terms of diagnostics, vaccine development as well as newer therapeutic measures have emerged from the above endeavours.

I.2. HISTORY OF TUBERCULOSIS

The history of human tuberculosis is rich in insight and enlightment. It is an ancient disease, evidences of which have been documented in Egyptian mummies dated 1500 BC (3). Tuberculosis has been described as early as 600 BC in the 'Sushruta samhita', a compendium of medicine and surgery of ancient India. In Sanskrit, the disease is called 'Kshayaroga' which means 'wasting disease'. In the past, tuberculosis was called 'consumption' as it seemed to consume people from

within, with repeated bouts of haemoptysis, productive cough, pyrexia, pallor, and persistent malaise.

After centuries of controversies and speculation regarding the aetiology of tuberculosis, on the evening of March 24, 1882, a German microbiologist, Robert Koch described the morphological characteristics of *M. tuberculosis*, in his famous presentation "*Die Aetiologie der Tuberculose"* at a conference of physiological society of Berlin. He also developed newer methods for staining bacteria and invented new methods for obtaining pure cultures of *M tuberculosis.*

I.3. *MYCOBACTERIUM TUBERCULOSIS*

Taxonomy:

Kingdom	Bacteria
Phylum	Actinobacteria
Class	Actinobacteria
Subclass	Actinobacteridae
Order	Actinomycetales
Suborder	Corynebacterineae
Family	Mycobacteriaceae
Genus	*Mycobacterium*
Species	*M. tuberculosis*

Etymologically 'mycobacterium' is derived from Greek for 'fungus' (*myces*) and small rods (*bakterion*). The 'fungus' component of this nomenclature derives from the tendency of this microbe to spread diffusely over the liquid medium in a mold-like growth pattern. Bacteria of the genus *Mycobacterium* are non-motile and non-sporulated rods. *M. tuberculosis* is typically curved or straight rod- shaped microbe. *In vitro,* it measures 1 to 4 µm in length and 0.3 to 0.6 µm in diameter. In tuberculous lesion it may assume different characteristics. *M. tuberculosis* when inoculated in human cell culture assumes larger and curved forms with more prominent beadings.

More consistently, mycobacteria have been identified by their unique tinctorial properties and to be more specifically, their 'acid-fastness'. Mycobacteria resist staining with conventional 'gram-stain', used for staining the bacteria in most clinical specimens. The resistance to standard gram-stain and the avidity with which *M. tuberculosis* retain certain dyes, are due to the high lipid content in their cell wall. Mycobacteria are basically acid-fast microbes on staining. However, not all acid-fast microbes are mycobacteria such as *Nocardia* and some strains of *Actinomyces* and *Corynebacteria* portray weak acid-fast staining characteristics.

M. tuberculosis is a slow-growing bacterium with a generation time ranging between 12-24 hours depending on the growth conditions. One characteristic property of *M. tuberculosis* is their tendency to form serpentine cords in cultures (4). This distinctive characteristic observed in the virulent *M. tuberculosis* has been attributed to presence of cord factor (trehalose 6, 6'-dimycolate) in the cell wall of the bacilli (5).

I.4. TRANSMISSION OF TUBERCULOSIS

Human tuberculosis is transmitted from patients with active disease to susceptible subjects through 'droplet nuclei' (6). A pulmonary TB patient with an open- cavity lesion in the lung generates aerosols that contain viable tubercle bacilli. In the atmosphere, the aerosol gets dehydrated and thus 'droplet-nuclei' are formed. High tuberculosis endemic zones more often promote the droplet-nuclei mode of transmission than in non-endemic zones. When droplet-nuclei containing viable tubercle bacilli are inhaled by a susceptible host, tubercle bacilli gain entry through the respiratory tract and subsequently the tubercle bacilli get lodged in the bronchioles or within the alveolar spaces of the lungs.

I.5. PATHOLOGY AND IMMUNOPATHOGENESIS OF TUBERCULOSIS

In most patients, due to the portal of entry of *M. tuberculosis*, respiratory tract face the brunt of TB infection. Inhaled tubercle bacilli are lodged in the lung parenchyma where they come in contact with the host immunocompetant system i.e., macrophages and T-lymphocytes. The alveolar macrophages are the first line of defence against *M. tuberculosis*. This initial response, in an immunocompetant host will result in the elimination of the pathogen through a sequence of inherent phagocytic mechanisms played by the macrophages. On the other hand, if the alveolar macrophages are not capable of arresting bacterial growth at this initial stage of infection, a localized inflammatory response is formed through the activity of Toll-like receptor agonists, abundant on the surface of bacteria. Tumour necrosis factor (TNF) and other pro-inflammatory chemokines produced by the host macrophages recruit more monocytes and macrophages to the site and they phagocytose bacilli.

The recruited cells produce additional chemokines and cytokines that amplify cellular recruitment and remodel the infection site into a cellular mass, and a tuberculous granuloma is formed. A typical tuberculous granuloma is composed of a central zone of caseous necrosis bordered by concentric layers of macrophages and lymphocytes. In patients with adequate immunological surveillance, tubercle bacilli in these granulomatous lesions are phagocytosed, and consequently the granulomas are replaced by fibrosis and calcification.

In an immunocompromised patient, tubercle bacilli continue to proliferate within the lesions resulting in the progression of the disease. In these circumstances, tuberculous lesions are not replaced by fibrosis and calcification. This would promote haematogenous dissemination of tubercle bacilli to other visceral organs like, central nervous system, kidney, bones etc. At times, in pulmonary TB patients, tuberculous granulomatous lesion expands and communicates through the bronchial tree and a cavity is formed. Besides, there are instances in which the tuberculous lesions can spread to the opposite uninvolved lung, resulting in 'trans-bronchial spread' of the infection (7). In a fraction of such patients, tuberculous lesions can extend to involve the pleura, as a result of which patients may develop massive pleural effusion. Tuberculous pleural effusion can also occur due to the hypersensitivity reaction mediated by the host immune system as a result of discharge of mycobacterial proteins from the underlying caseous lesions (8).

I.6. DIAGNOSIS OF HUMAN TUBERCULOSIS

Tuberculosis is a potentially curable disease. The high mortality rate in TB patients could be minimized by an early and accurate diagnosis and also by the initiation of appropriate antituberculosis chemotherapy (ATT). In general, TB is

diagnosed based on relevant clinical features. However, the clinical manifestations in most patients are not classical and more often may present with atypical clinical signs and symptoms. Prior to the introduction of sophisticated investigative procedures for TB diagnosis, direct microscopy of sputum smears using Ziehl- Neelsen (ZN) method was the only way for making a rapid diagnosis of pulmonary tuberculosis. But a positive smear for acid-fast bacilli (AFB) requires the presence of approximately 10^4 acid-fast bacilli /ml in clinical specimen (9). Although the sensitivity can be improved by concentrating the clinical specimens, especially sputum sediments, and substantiated by applying auramine-O and rhodamine fluorescent stain, direct microscopy cannot distinguish between *M. tuberculosis* and non-tuberculous mycobacterium (NTM) (10). Direct microscopy is also reported to be less sensitive in extra-pulmonary TB specimens (11).

A positive culture of *M. tuberculosis* from clinical specimens remains the 'gold standard' for TB diagnosis (12). The most affordable solid culture media, Lowenstein-Jensen (LJ) media, is considered useful, but requires at least 6-8 weeks for isolating tubercle bacilli from the clinical specimen. Certain specimens, such as, sputum and bronchoalveolar lavages require proper decontamination procedures prior to culture. This will invariably cause a significant reduction in the colony forming units of *M. tuberculosis*. Hence conventional bacteriological cultures are less sensitive and time-consuming (10).

Alternate methods like automated rapid culture techniques (BACTEC system) have been shown to be more sensitive than conventional methods. They possess high sensitivity and specificity in smear-positive samples (13). But the sensitivity of these methods in smear-negative pulmonary and extra-pulmonary patients is not significant when compared to those in smear positive patients (14). Owing to the cost

prohibitive nature of these methods, their application for routine diagnosis in developing countries has become impractical and hence could not be implemented.

Tuberculosis Skin Test (TST) has been in use for TB diagnosis since 1910. TST is based on the detection of delayed-type hypersensitivity (DTH) to purified protein derivative (PPD) of *M. tuberculosis.* Since PPD is a crude mixture containing several antigens shared by *M. tuberculosis*, *M. bovis* BCG (Bacillus Calmette- Guerin) and several non- tuberculous mycobacteria, a positive TST result could be due to latent TB infection, previous BCG vaccination, or exposure to environmental non-tuberculous mycobacteria. Hence TST cannot be regarded as a specific method in TB diagnosis (15).

One of the major goals in mycobacterial research is the development of newer methods for the early diagnosis of TB. It needs to be emphasized that any newly introduced test for the diagnosis of TB must be examined with respect to sensitivity of Ziehl-Neelsen staining and culture methods. It is also very essential to critically evaluate the specificity of the newly introduced assay because a false positive result may not only lead to the incorrect therapy but also will lead to the cessation of further diagnostic efforts. Besides these criteria, the assay should be rapid, reproducible, user-friendly and suitable for application in routine clinical laboratories, particularly in developing countries from where most of the TB cases are reported. During the past two decades, several indirect diagnostic approaches have been described for the laboratory diagnosis of TB. They can be classified as:

(a) Biochemical: Several sophisticated biochemical tests such as estimation of tuberculostearic acid (16), radioactive bromide partition test (17), and adenosine deaminase test (18) have been described in the literature. These assays are based on biochemical analysis of either constituents or the metabolic products of the

tubercle bacilli that are usually present in the tuberculous lesions or in body fluids. These biochemical tests are extremely valuable for diagnosis, but to perform these assays technical expertise is very essential. Issues of low sensitivity and specificity and the need of complex instrumentation like HPLC, restrict their application for the routine diagnostic purposes.

(b) Immunological: Demonstration of either mycobacterial antigen or antibodies in clinical specimens with a suitable immunoassay has been studied extensively. These approaches do not appear very promising from a clinical perspective especially in those patients in whom neither direct microscopy nor do cultures establish the diagnosis. In several published reports Enzyme linked immunosorbent assays (ELISA) have been standardized to detect antigens like 38KDa (19), Ag5 (20), Ag85 (21), and cord factor (22) of *M. tuberculosis* from clinical specimens. Reports indicate that sensitivity and specificity of these antigens vastly differ from one study to other.

Sensitivity and specificity of serological tests depend on the types of antigens being used. Currently available antigens appear neither significantly sensitive nor specific to function as a primary first-line screening test for TB diagnosis. It is also a fact that these assays perform reasonably well with smear positive AFB positive pulmonary TB patients but do not satisfactorily yield positive results in smear negative pulmonary and extra-pulmonary tuberculosis patients. Moreover, HIV positive patients, in whom TB is more frequent, antibody responses are more often, sub-optimal and hence cannot be detected in the immunoassays. Despite these limitations, immunodiagnostic assays still finds a place as an adjunct for the laboratory diagnosis of TB.

(c) Molecular biological: Recent advances in the field of molecular biology have provided new tools for the rapid diagnosis of TB and also enhanced the understanding of the molecular basis of drug resistance in *M. tuberculosis*.

Commercial and several in-house Polymerase chain reaction (PCR) methods (23-26) are being introduced from time to time as an important avenue for TB diagnosis. Initially sensitivity of these nucleic acid amplification assays was evaluated in smear-positive, culture-positive TB patients. The lack of specificity in these methods was a matter of serious concern than sensitivity. PCR-based sequencing techniques (27), PCR-restriction fragment length polymorphism analysis (28), DNA microarrays (29) are some other methods which are currently under evaluation for application under different clinical settings. The high cost of these techniques, and their requisite for advanced equipment or highly proficient skilled personnel have precluded their execution on a routine basis, especially in low-income countries.

Efficiency of PCR depends greatly on the type of specimen, technical protocol adopted in the laboratories as well as the target amplified. Since PCR is able to detect small number of organisms, cross-contamination during specimen collection may yield significant of false-positive results (30). Different variants of standard PCR, i.e., nested PCR (31), multiplex PCR (32), *in situ* PCR (33), and real-time PCR (34) are being currently used for the laboratory diagnosis of TB. Majority of the methods described, are still under evaluation for their application in large population. These techniques need to be critically evaluated in paucibacillary specimens collected from patients with extra-pulmonary TB. More specific targets needs be identified, characterized and employed for accurate detection of *M. tuberculosis* from clinical specimens.

I.7. BROAD OBJECTIVE OF THE STUDY

This study focused mainly on the development of immunological and molecular methods for the early and accurate laboratory diagnosis of human tuberculosis. An

effective test needs to be validated in the paucibacillary tuberculosis group rather than in smear positive tuberculosis patients. Existing as well as new *M. tuberculosis* specific markers had to be assessed in the quest of efficient diagnostic approach.

1.7.1. Specific objectives of this study

I.7.1.1. To evaluate existing diagnostic methods in TB patients in extra-pulmonary tuberculosis cases

Different methods for effective diagnosis of TB have been proposed by research groups all over the world. Majority of these methods and detection targets were reported to be useful in the diagnosis of smear-positive pulmonary TB. The first objective of this study is to evaluate the reproducibility and the efficiency of the existing methods for the diagnosis of extra-pulmonary TB.

I.7.1.2. To develop a diagnostic system for the accurate diagnosis of human tuberculosis

Serological tests, both antibody mediated and cytokine based methods can be used as simple, user-friendly and accurate diagnostic tests for TB. There are still many unsolved untenanted zones in this diagnostic approach. The sensitivity of these assays needs to be evaluated in the actual target population, i.e., smear-negative as well as smear-positive TB patients. It becomes imperative to include immunoreactive and specific antigens of *M. tuberculosis* in assays to increase the sensitivity and specificity. Development of molecular biology and proteomics has led to the large-scale preparation of highly pure immunological reagents in the form of 'recombinant antigens' and they can be used in the early diagnosis as well as monitoring of therapy in TB patients. To meet this objective, following genes of *M. tuberculosis* were selected in this study.

(i) *esat-6* (6 KDA early secretory antigenic target / Rv3875) is a *M. tuberculosis* gene, which encodes a potent T-cell antigen (ESAT-6) that is reported to be having a clear but as yet undefined role in the pathogenesis of tuberculosis (35).

(ii) *hspX* (Rv2031c) codes for a heat shock protein (HspX) induced during anoxic conditions. This gene has a role in maintenance of long-term viability during latent tuberculosis infections, and also in replication during initial infection (36).

(iii) *plcA* (Rv2351c) is a gene encoding a probable virulence factor (Probable membrane-associated phospholipase C1) that have been implicated in the pathogenesis of *M. tuberculosis* at the level of intracellular survival within the macrophages (37).

(iv) *tb8.4* (Rv1174c) is a *M. tuberculosis* gene, which encodes a hypothetical secretory protein. It is one of the most expressed genes of *M. tuberculosis* in log-phase growth (38).

For developing a diagnostic method based on the above genes, the following studies were proposed:

- Prevalence of the selected genes in clinical specimens from patients with tuberculosis
- Cloning, expression and purification of four mycobacterial antigens
- Immunological properties of individual recombinant antigens
- Immunological analysis of their multiantigenic cocktail

I.7.1.3. To study the host cell- mediated response against the recombinant mycobacterial antigens, and to assess their application to distinguish individuals with latent TB infection from individuals with BCG vaccination

Latently infected individuals comprise an important reservoir of *M. tuberculosis,* and these individuals should be identified and preventive measures should be designed. Gamma interferon (IFN-γ) release assays have been found to be useful in the detection of individuals with latent tuberculosis (39). The antigens being used in such assays have a great bearing in the outcome of the assays. The ability of the four recombinant mycobacterial antigens in eliciting specific cytokines in TB patients and individuals with latent tuberculosis will be evaluated.

II. REVIEW OF LITERATURE

II.1. HISTORY OF TUBERCULOSIS

Mycobacterium tuberculosis existed in human beings since antiquity. Fragments of the spinal cord from Egyptian mummies (2400 BC) revealed distinct pathological evidences of tuberculous decay. Around 460 BC, Hippocrates termed tuberculosis as 'phthisis' (meaning 'consumption', to 'waste away') and considered it fatal (40).

Pathological and anatomical descriptions of TB began to appear in the literature as early as seventeenth century. Franciscus Sylvius de la Boe of Amsterdam was the first to describe the presence of tubercles as the consistent and characteristic features in the lungs and other organs of consumptive patients. In *Opera Medica,* published in 1679, he described the progression of lung lesions from tubercles to ulcers and cavities. The name 'tuberculosis' was coined by Johann Lukas Schonlein in 1839 to describe diseases with tubercles. In Latin, '*tuber'* meant all kinds of degenerative protuberances.

Rene Theophile Hyacinthe Laennec, a French physician, occupied a pivotal position in the history of tuberculosis as being the pioneer for his outstanding descriptions of pulmonary lesions that really marked the beginning of our concept and understanding of pathology and pathogenesis of TB. He illustrated tuberculous cavities and described the classical pathological lesions that are now familiar as caseous necrosis. His autopsy studies established that pathological lesions in other visceral organs were in fact due to the same tuberculous disease (41).

In his publication, *A New Theory of Consumptions,* in 1720, Benjamin Marten was the first to describe that TB could be caused by 'minute living creatures', and

once they had gained entry to a susceptible host, could generate the lesions and symptoms of phthisis.

On the evening of March 24, 1882, in Berlin, Robert Koch made his famous presentation *Die Aetiologie der Tuberculose* before the Physiological Society of Berlin. Using solid media made of potato and agar, Koch invented new methods of obtaining pure cultures of *M. tuberculosis*. Koch also described a new staining method for *M. tuberculosis*, based on methylene blue, a dye developed by Paul Ehrlich, and counterstained with vesuvin. In 1886, Jorgen Lehmann and Ernst Neumann named this organism as *Mycobacterium tuberculosis*, probably because of the slow-growing nature of bacilli and its carpet-like appearance in culture resembling fungus.

The introduction of the sanatorium provided the first step in the treatment of TB. Hermann Brehmer in 1854 presented his doctoral dissertation bearing the title, "*Tuberculosis is a Curable Disease*". Later, physicians with vast experience with TB were unanimous in their opinion that open-air treatment can bring an improvement in the management of TB patients (42). Also, an Italian Physician, Forlanini, reported that lung collapse that occurred during the course of the study have a favourable impact on the outcome of the disease.

Another significant advance came in 1895 when Wilhelm Konrad von Rontgen discovered the radiation that bears his name. With the help of radiology, the progress and severity of tuberculosis could be accurately followed and reviewed. A further development was provided by the French bacteriologist Calmette, who, together with Guerin, used specific culture media to reduce the virulence of the bovine TB bacterium, and thus creating the basis for the development of BCG vaccine.

In the middle of World War II, came the final breakthrough, the greatest challenge to the *M. tuberculosis* that had threatened humanity for thousands of years. In 1943, streptomycin, a compound with antibiotic activity, was purified from *Streptomyces griseus* by Selman A. Waksman (43). Jorgen Lehmann in 1943 introduced the use of para-aminosalicylic acid for the treatment of tuberculosis (44) and the era of ATT.

II.2. TUBERCULOSIS - CURRENT STATUS

WHO estimated that approximately nine million people suffer from TB every year, of which 95% are reported to occur in developing countries. Most TB cases occur in the most populated nations such as India and China, but highest incidence rates of TB were seen in sub Saharan Africa, Indonesia, Afghanistan, Bolivia and Peru (45). HIV pandemic aggravates this situation and the fatality in HIV-infected patients with TB is twice that of HIV-infected patients without TB (46).

Resurgence of TB is associated with increasing numbers of MDR-TB and XDR-TB. MDR-TB is a form of TB that is resistant to two or more of the primary drugs (isoniazid and rifampicin) used for TB treatment. XDR-TB occur when resistance to at least isoniazid and rifampicin among the first-line anti-TB drugs and any fluoroquinolone among second-line drugs and at least one of three injectable drugs.

India is contributing nearly one third of the world's TB cases and has the highest rate of new TB cases (47). Prevalence of MDR-TB cases is on the rise in India, and new cases of MDR-TB have been observed in most of the reported studies (48). XDR-TB has also reported to occur in India. 5 XDR-TB patients were recognized from 68 MDR-TB strains during a preliminary study conducted recently (49).

While developed countries with good public health care systems are expected to keep TB under control, in much of the developing world a catastrophe awaits. It

becomes crucial that support should be given to research programs devoted to developing an effective TB vaccine, shortening the amount of time required to ascertain drug sensitivities, rapid assays for the diagnosis of TB, and creating new, highly effective anti-TB medications.

II.3. DIAGNOSIS OF TUBERCULOSIS – CONVENTIONAL METHODS AND NEWER AVENUES

II.3.1. Diagnosis by direct microscopy and culture methods in tuberculosis patients

TB diagnosis is based on salient clinical and radiological features, sputum smear microscopy, and cultivation of *M. tuberculosis*. In developing world, diagnosis of TB is usually made by microscopic examination of sputum smear by Ziehl-Neelsen (ZN) method. Although easy to perform and specific, it lacks sensitivity, requiring approximately 10^4 bacilli/ml of specimen to become positive. The use of auramine as a fluorescent method to detect mycobacteria in sputum was proposed many years ago and evaluated later using a combination of auramine-O and rhodamine (50). In a recent proficiency testing study performed in 167 laboratories, the auramine/rhodamine yielded better results than the ZN staining or its Kinyoun modification (51). It is, cost prohibitive than the conventional ZN staining as it requires a fluorescent microscope (52).

Isolation of *M. tuberculosis* from clinical samples is the 'gold standard' for the diagnosis of active TB. It can detect 100 bacilli/ml of sputum in comparison with 10^4 bacilli/ml needed for microscopy (53). Culture methods also provide material for further identification and drug susceptibility testing. Conventional methods of culture have relied on egg-based and agar-based media, such as LJ medium and

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Middlebrook agar (54). This method is laborious and time consuming requiring from 3–8 weeks for results.

The introduction of the BACTEC radiometric system (BACTEC TB-460) allowed the detection of *M. tuberculosis* within few days (approximately 12 days) compared with 3-8 weeks in the conventional culture media (55). However, the use of radioisotopes and the cost of the equipment precluded its use on a routine basis, except in reference laboratories predominantly in developed countries.

Another advancement in bacteriological method was the introduction of mycobacteria growth indicator tube (MGIT) system based on fluorescence detection of mycobacterial growth (56). More recently the MGIT system has been fully automated and turned into the BACTEC MGIT 960 system, which is a non-radiometric, non-invasive system with the tubes incubated in a compact system that reads them automatically. In a study, Idigoras *et al* (57) BACTEC MGIT 960 system showed a sensitivity of 93% and a turn-around time of 12.7 days. Operational and cost-effectiveness studies that assess the real impact of these systems in low- and middle-income countries are still lacking.

II.3.2. Diagnosis in smear-negative culture-negative pulmonary and extra-pulmonary tuberculosis

Majority of pulmonary TB patients, are not confirmed bacteriologically and are only diagnosed on the basis of high clinical suspicion and response to anti-TB drugs (58). The diagnosis of extra-pulmonary tuberculosis is especially challenging for a number of reasons: the lack of adequate sample volumes, the apportioning of the sample for various diagnostic tests resulting in non-uniform distribution of microbes,

paucibacillary nature of specimens, presence of inhibitors that undermine the performance of nucleic acid amplification (NAA) techniques and the lack of an efficient sample processing technique universally applicable on all types of extra-pulmonary samples (11). The performances of most of the methods described were inefficient in sputum-smear negative pulmonary cases and in extra-pulmonary cases. Hence any method used for the diagnosis should be validated in paucibacillary extra-pulmonary samples.

II.3.3. Nucleic acid amplification methods in TB diagnosis

A significant improvement in TB diagnosis was the development of different NAA techniques, such as PCR, that has been extensively evaluated for the rapid TB diagnosis. Several in-house PCR methods have been developed and tested during past decades and multiple studies have been published on the PCR based diagnosis of TB. Some of the important PCR studies on pulmonary and extra-pulmonary samples are represented in **Table: 1**.

Table1: Evaluation of PCR for detecting *M. tuberculosis* DNA in clinical specimens

Reference	Target Fragment	Amplification system	Specimens		Sensitivity (%)	Specificity (%)
			Number	Type		
59	*IS6110*	Standard PCR	75	NR+R	97.3	100
60	*IS6110*	Standard PCR	162	R	98	99

61	*IS6110*	Nested PCR	41	NR	95	89
62	*IS6110*	Standard PCR	286	NR	94	100
63	*IS6110*	Standard PCR	45	NR	90	100
64	*IS1081*	Standard PCR	78	NR	94.1	55.7
65	*MPB64*	Nested PCR	19	NR	70	88
66	*Ag85A*	Standard PCR	206	NR+R	93.9	94.3
67	*38 kDa*	Nested PCR	417	NR+R	97	92
68	*16S rRNA*	Standard PCR	729	NR+R	84.5	99.5
69	*mtp40*	Standard PCR	172	NR+R	98.8	98.9
70	*mtp40*	Nested PCR	116	R	97.7%,	95.9%

where R = respiratory samples and NR= non-respiratory samples

Most of these studies found that lack of specificity was more of a problem than lack of sensitivity. It was also found that many laboratories did not use adequate quality controls in their study (71, 72).

The parameters used, such as detection targets, primers, amplification, detection and specimen pre-treatment systems, vary considerably, resulting in a wide range of assays. For diagnosing TB, different DNA amplification targets have been used, such as gene encoding 65 KDa protein antigen (73), *secA1* (74), *MPB64* (75) etc.

The target most frequently amplified is the *IS6110* repetitive element, which is present in multiple copies (15 - 20) in most strains M *tuberculosis* complex (59). However, Kent *et al* (76) recently reported that 24 of 31 strains of non-tuberculous mycobacteria were positive by PCR amplification using primers based on IS*6110.* Some isolates of *M tuberculosis* from South India have been reported to lack this sequence in their genome leading to false negative results (77).

PCR with species-specific *mtp40* genomic fragment, which is exclusively detected within the *M. tuberculosis* has also found importance in TB diagnosis (69, 70). Their efficiency in paucibacillary specimens needs to be evaluated.

Hernandez-Pando R *et al* (78) demonstrated the persistence of *M. tuberculosis* DNA in the macroscopically-normal lung sections of individuals from TB endemic region using an *in situ* PCR. The efficiency of such variants of PCR should be evaluated in other specimens, to detect latent TB and to reduce the number of false positive results obtained due to cross-contamination occurring through DNA extraction procedures.

In general, molecular methods offer several advantages over conventional techniques such as rapid detection and identification of *M. tuberculosis*, better turn-around time for results, reliability, and reproducibility. Due to the requirement of additional equipment and trained personnel, most of these methods have not yet gained easy access to the routine procedures performed in clinical mycobacterial laboratories in low-income countries where TB is a more important health problem. Thorough evaluation of these methods in paucibacillary tuberculosis specimens are needed before implementing them in routine diagnostics. Even when such methods are highly sensitive, a concern regarding false positive results prevails.

II.3.4. Immunodiagnosis of TB

II.3.4.1. Antigen-detection tests in diagnosis of TB

Mycobacterial antigens have been detected by ELISA in sputum (79) and by latex agglutination assay in cerebrospinal fluid (80). Lipoarabinomannan, a major component of the mycobacterial cell wall, has been detected in the serum (81) and sputum (82) of patients with tuberculosis. None of these tests to detect mycobacterial antigens has achieved widespread use for the diagnosis of active tuberculosis.

Detection of mycobacterial antigens by immunohistochemistry (IHC) using polyclonal and monoclonal antibodies is an alternative to conventional acid-fast staining. A large number of different mycobacterial antigens including BCG (83), have been detected with varying results in tissues. These are all common mycobacterial antigens and thus cannot discriminate *M. tuberculosis* from non-tuberculous mycobacteria. Recently, IHC based on the detection of specific mycobacterial antigens have been reported, such as *M. tuberculosis* complex specific antigen

MPT64 (84). IHC is found to be a simple and user-friendly method and has immense scope in detection of *M. tuberculosis* in infected tissues.

II.3.4.2. Antibody-detection tests in diagnosis of TB

It is generally considered that detection of antibody is a poor indicator of TB infection. The humoral immune response rises towards the end stage of the disease process when the host is most infectious, and although many tests for antibody were trialled in the 1980s and 1990s, these tests have not had a role in eradication or control programmes to date. ELISA have been developed and evaluated in a number of studies. Even then serological tests to detect antibodies to *M. tuberculosis* has immense potential to make an optimal and low-cost diagnostic test for TB in developing countries, especially under field conditions (85).

Earlier studies used partially purified antigens, which allowed the detection of anti-mycobacterial antibodies in TB patients, but the tests showed poor specificity (86). Identification of an appropriate *M. tuberculosis* antigen suitable for serodiagnosis that can offer high specificity, ease of detection, and specificity that can distinguish active TB patients from BCG-vaccinated controls is highly desirable for developing suitable control measures and early treatment of the disease. It has also been found that the degree of humoral response to TB is heterogeneous (87). For this reason, the use of serodiagnostic tests based on mixtures of multiple *M. tuberculosis* antigens has been proposed (88).

II.3.4.3. Recombinant antigens of *M. tuberculosis* in serodiagnosis

The extremely long doubling time and pathogenicity of mycobacteria has delayed the identification of antigens and virulence factors. Gene cloning and

expression, DNA and protein sequencing, PCR, comparative genomics, bioinformatics, proteomics and DNA and peptide synthesis coupled with the application of cellular immunology techniques have led to the identification of several antigens of *M. tuberculosis*, which have potential for diagnosis applications (89).

M. tuberculosis genome contains about 4000 genes, of which approximately one-third code for proteins of unknown functions or are classified as conserved hypothetical proteins. Mycobacteria secrete several proteins (90-92), many of which are antigens.

In recent years, several defined antigens of *M. tuberculosis* have become available by producing large quantities of recombinant antigens using molecular biology procedures. This has led to the identification of several major antigens of *M. tuberculosis* such as heat shock proteins (e.g. hsp60, hsp70) and secreted antigens, e.g. Ag85, MPT64, MPB70, etc (93). However, most of these antigens were found to be shared between the virulent strains of *M. tuberculosis,* the vaccine strains of BCG and other environmental mycobacteria (93, 94), and therefore cannot be used for the specific diagnosis of TB.

Even though numerous mycobacterial antigens have been described, no clear immunodominant antigen covering all the stages of the disease has been found (95, 96). This fact has caused research to be directed to an antigenic cocktail instead of a unique protective or dominant antigen. It is essential that each antigen component must contribute in increasing the sensitivity of the diagnostic test while maintaining a high degree of specificity for inclusion in a multi-antigen diagnostic test for TB. It is equally important to select such antigens, which can distinguish between active and latent phases of TB.

Subtractive DNA hybridization of pathogenic *M. bovis* and *M. bovis* BCG (97) and DNA microarray analysis of *M. tuberculosis* H_{37}Rv and BCG (98) have led to the

identification of several 'regions of difference', one of which was designated RD1 and was found to be present in all *M. tuberculosis* and pathogenic *M. bovis* strains but lacking in all BCG strains and most environmental mycobacteria. RD1 antigens are being evaluated for their serodiagnostic potential.

II.3.5. Mycobacterial antigens and cell mediated immune responses

Since one-third of the world's population is infected with *M. tuberculosis*, it would be important to be able to predict who among the latently infected will develop the disease, so as to treat them before active TB is developed. The only available test to detect infection was, until recently, the tuberculin skin test (TST).

PPD of *M. tuberculosis* is used to detect DTH reaction to *M. tuberculosis* antigens, in TST. But a positive TST result may be detected in patients with active disease in healthy donors following BCG vaccination and in healthy subjects exposed to cross-sensitization by other mycobacterial species (99). The strong cellular immune response triggered by latent TB serves as an amplified signal for the presence of dormant bacilli. T-cell-based interferon-gamma (IFN-γ) release assays represent a 100-year upgrade of existing TST. Sensitivity and specificity of such assays depends on the antigen being used. There is a need to identify and evaluate *M. tuberculosis* antigens for the specific diagnosis of TB and also to develop effective and safer vaccines.

II.3.5.1. Recombinant mycobacterial antigens in assays based on CMI

Many *in vitro* T-cell-based methods have been proposed recently (100 -102). One such assay, the gamma interferon (IFN-γ) assays, is based on the fact that T-cells sensitised with tuberculous antigens will produce IFN-γ when they are re-

exposed to mycobacterial antigens. The first IFN-γ assays made use of purified protein derivative (PPD) as the stimulating antigen; more recent assays, use antigens that are specific to *M. tuberculosis*, such as the early secretory antigen target-6 (ESAT-6), and culture filtrate protein 10 (CFP-10) (103).

In general, the studies performed have shown that IFN-γ assays using RD1 antigens have some advantages over PPD, such as a higher specificity, a better correlation with previous exposure to *M. tuberculosis*, and low cross-reaction due to BCG vaccination or previous exposure to NTM. It has also been found that IFN-γ assays that use cocktails of antigens rather than individual antigens have a better accuracy (104). In-depth study into the various cytokine profiles, other than IFN-γ evoked by different mycobacterial antigens can contribute in better understanding of CMI against tuberculosis. This understanding will pave the way for the development of better vaccines and diagnostics in near future.

II.4. Recombinant antigens in this study

II.4.1. ESAT-6

The *esat-6* gene (Rv3875) encoding the early-secreted antigenic target 6 kDa protein (ESAT-6) is found within RD1 where it was provisionally referred to as *orf1C* (105). ESAT-6 is specific for *M. tuberculosis* complex, and is reported to be absent from *Mycobacterium bovis* BCG (106). It is a major T-cell antigen, which has been purified from *M. tuberculosis* short-term culture filtrates (ST-CFs) (107, 108). ESAT-6 has been reported to elicit strong antibody responses and DTH skin reactions in guinea pigs (109). ESAT-6 is therefore a promising candidate antigen for inclusion in a novel immunodiagnostic assay.

II.4.2. PlcA

Phospholipase C (Plc) and phospholipase D (Pld) activities have been described in several mycobacterial species. Although Pld activity has been detected in both virulent and saprophytic species, PlcA and sphingomyelinase activities seem to be restricted to pathogenic *Mycobacterium* subsp. (110). PlcA is probable membrane associated phospholipase C1 of *M. tuberculosis*, which is also referred to as Mtp40 antigen (37). The sequence called *mtp40* actually constitutes only a part of the *plcA* gene (Rv2351c) (111). *plcA* is found to present in *M. tuberculosis* but not in other members of the *M. tuberculosis* complex (112).

II.4.3. HspX

Typically, in latent infection, bacteria are present only in low numbers. The physical microenvironment where the bacteria survive has not been characterized in detail, but is thought to include restricted access to nutrients and to oxygen and low pH, together with elevated levels of hydrolytic enzymes, and reactive nitrogen and oxygen species released by the host's immune response. The abundance of regulatory proteins in the *M. tuberculosis* genome (113), may explain the ability of the pathogen to adapt to this hostile environment (114) by up-regulating so-called latency genes. One of the most prominent genes among these is *hspX* (Rv2031c) whose importance is demonstrated by the reduced ability of bacteria deficient for this gene to grow in macrophages (36). It is reported to be expressed during infection in humans, as it is recognized by sera from a majority of TB patients (115). Moreover, production of HspX protein (*acr* /16-kDa antigen / alpha-crystallin) appears to increase as the bacteria go into the metabolically resting stage (116) and decrease as they revert to exponential growth (117). It therefore serves as the prototypic 'latency-associated antigen'.

II.4.4. Tb8.4

M. tuberculosis tb8.4 gene (Rv1174c) encodes a secreted protein of 8.4 kDa (Tb8.4) which has been suggested to be involved in reactivation of dormant mycobacteria (118). Coler RN *et al* (38) reported that Tb8.4 is an immunoreactive T cell antigen in individuals with latent *M. tuberculosis* infection. They suggested that this gene may play an important role in determining the outcome of infection as it elicits abundant levels of the TH1 cytokine, IFN-γ. As resistance to TB depends on antigen-specific T cell activation of macrophages, and as the IFN-γ pathway has been shown to be crucial in the human response to mycobacterial infection, the elicitation of high levels of IFN-γ by Tb8.4 in tuberculosis-sensitized donors is significant. More importantly, immunization of mice with either plasmid DNA encoding *tb8.4* or Tb8.4 recombinant protein elicited strong T cell responses and induced protection on challenge with virulent *M. tuberculosis*. These results suggest that *tb8.4* is a potential candidate for inclusion in a subunit vaccine against tuberculosis (119). The potential role of Tb8.4 as a diagnostic parameter is yet to be studied.

III. MATERIALS AND METHODS

III.1. MATERIALS

III.1.1. Routine Chemicals

Acrylamide, N N' – Methylenebisacrylamide, L-Asparagine, EDTA, Trizma base, Xylene cyanol FF, Sodium acetate, Tween-20, Agarose, Bromophenol blue, Ethidium bromide, TEMED, Triton X100, Sodium azide were purchased from (Sigma-Aldrich, MO, USA). Potassium dihydrogen phosphate, Magnesium Citrate, Glycerol, Citric acid, Dipotassium hydrogen phosphate, Ferric Ammonium Citrate, Potassium chloride, Potassium acetate, SDS, Chloroform, Ethanol, Iso-amyl alcohol, Hydrogen Chloride, Rubidium Chloride, D-Formamide, Diethanolamine, Sodium hydroxide were purchased from Merck, Mumbai, India. LB culture media, Agar-agar and Milk powder were purchased from Hi-Media, Mumbai, India. Sodium chloride, Boric acid, Glacial acetic acid and Isobutanol were obtained from Qualigens, Mumbai, India. CTAB powder and saturated phenol were from Bangalore Genei, India. Urea was purchased from Promega Corporation, Madison, WI. Calcium chloride, Manganese chloride, Ammonium sulphate, Citric acid, Magnesium chloride, Maleic acid, Sodium bicarbonate, Magnesium sulphate, Tris hydrochloride and Bovine serum albumin were purchased from SISCO research laboratories, Mumbai, India.

III.1.2. Fine chemicals

100bp and 500bp molecular ladder (Bangalore Genei, India), 100 bp Marker, lambda DNA-*BstE*II marker (New England Biolabs, MA, USA), Nucleotides, $MgCl_2$ for PCR, protein molecular markers, Ni-NTA Magnetic agarose beads (Promega Corporation, Madison, WI), Ficoll - Hypaque, ampicillin, Kanamycin sulphate, Isopropyl-β-D-thio galactopyranoside, X-Gal, Incomplete Freund's adjuvant, Protein-Sepharose A, Goat Anti-human IgG-alkaline phosphatase conjugate, Anti-rabbit IgG-biotin, Streptavidin

horseradish peroxidise, Extr-avidin alkaline phosphatise, Poly-L-Lysine, Concanavalin A, Para-nitrophenyl phosphate, NBT, BCIP, Nuclear fast red, Haematoxylin, Eosin, Toluidine blue, Malachite green, Coomassie brilliant blue, Imidazole, Streptomycin, Anti-mouse IgG alkaline phosphatase conjugate and PPD (Sigma Aldrich, MO, USA), DIG-labelled UTP, Anti- Digoxigenin - alkaline phosphatase, Self-seal reagent (Roche Diagnostics, Mannheim, Germany), MOPS buffer (SISCO research laboratories, Mumbai, India), Anti- HIS antibody (GE healthcare biosciences, NJ,USA), Foetal Bovine Serum (GIBCO-BRL, Gaithersburg, USA), Benzyl penicillin (Alembic, India), AEC and DAB (DAKO, Hamburg, Germany).

III.1.3. Membranes

Hibond PVDF membrane (Amersham Life Science, Little Chalfont, UK), 0.45µm pore-size filter (Millipore, MA, USA)

III.1.4.Enzymes

Lysozyme and Proteinase K (Bangalore Genei, India), Taq DNA polymerase in storage buffer B and T4 DNA Ligase (Promega Coorperation, Madison, WI), *Nde* I , *Hind* III, *Kpn* I (New England Biolabs Inc, MA, USA).

III.1.5. Kits

Ziehl- Neelsen acid-fast stain kit (Hi-Media, Mumbai, India), pGEM®-T Easy Vector kit (Promega Coorperation, Madison,WI), GFX PCR DNA and Gel band purification kit, Plasmid isolation kit and PD10 columns (GE healthcare Biosciences, NJ,USA), Bradford assay kit and MTT assay kit (Sigma Aldrich, MO, USA), 10X Sequence Buffer, Sequence mix, ABI PRISM Big Dye Terminator cycle sequencing Ready

Reaction kit, Version 2.0 (PE Applied Biosystems, CA, USA), Human TNF, IFN-γ, IL-10, IL-4, ELISA kits (BD Biosciences, San Jose, CA),

III.1.6. Software

Primer Premiere 5 (PREMIER Biosoft International, CA, USA), Sequence scanner (PE Applied Biosystems, CA, USA), Quantity one software (Bio-Rad Laboratories, CA, USA).

III.1.7. Lab-ware and cell culture-ware

Micropipettes, Microcentrifuge tubes - 0.5ml, 1.7ml, 2ml (Eppendorf, Germany), PCR tubes (Axygen, CA, USA), 96-well flat-bottom sterile tissue culture plates (Greiner Bio-One, Germany) and 96-well ELISA microplates (Dynatech Laboratories, Alexandria, VA), Histobond microslides (Superior Marienfeld, Germany).

III.1.8. Equipments

Centrifuge (Eppendorf, Germany), Thermocyclers (i -Cycler from Bio-Rad Laboratories, CA, USA and PTC100 with *in situ* adaptor from MJ Research Inc, USA), Electrophoresis power supply EPS 600 (Pharmacia Biotech, Uppsala, Sweden), UV transilluminator (Herolab, Germany), UV Torch (Mineralight UV-254/365 nm, Upland, USA), Fluor-S-Gel documentation System (Bio-Rad Laboratories, CA, USA), Sonifier (Branson, NY, USA), Biospec-1601 spectrophotometer (Shimadzu, Columbia, USA), CO_2 incubator (Sanyo, Japan), Laminar flow hood (CLAS, India), cytospin (Cytopro Wescor, USA), Light microscope (Leica Microsystems, Bensheim, Germany), Phase-contrast microscope (Nikon, Japan), Magnetic stirrer (Remi, India), Easy pure UV/UF compact reagent grade water system (Barnstead, USA), Electrophoresis unit and mini-blot apparatus (Bio-Rad laboratories, USA), pH meter

(Genei, India), Submarine electrophoresis unit (Biotech, Yercaud, India), ABI 310 Automated DNA sequencer (Applied Bio-System, Perkin Elmer, USA), Microplate reader (Bio-Tek instruments, USA) and Deep freezer vertical (Sanyo, Japan).

III.1.9. Bacterial strains

M tuberculosis $H_{37}R_v$ and *M tuberculosis* $H_{37}R_a$ strain (TRC, Chennai, India)

E. coli JM109 (Novagen, Madison, WI)

E. coli BL21 (DE3) (Novagen, Madison, WI)

E. coli JM109 (DE3) (Novagen, Madison, WI)

E. coli BL21 (DE3)pLysS (Novagen, Madison, WI)

III.1.10. Vectors

pGEM®-T Easy vector (Promega Coorperation, Madison, WI)

pET- 32a(+) (Novagen, Madison, WI)

pET- 28a(+) (Novagen, Madison, WI)

III.1.11. Primers (Sigma Aldrich, MO, USA)

IS6110

Forward: 5'CGT GAG GGC ATC GAG GTG GC 3' (INS1)

Reverse: 5'GCG TAG GCG TCG GTG ACA AA 3' (INS2)

mp40

Forward: 5′-CAA CGC GCC GTC GGT GG-3′ (PT1)

Reverse: 5′-CCC CCC ACG GCA CCG C-3′ (PT2)

Forward: 5'CACCACGTTAGGGATGCACTGC3' (PT3)

Reverse: 5'-CTGATGGTCTCCGACACGTTCG3' (PT4)

esat-6

*Forward: 5'GGGGTACCCCGATGACAGAGCAGCAGTGGAATTTC3'

***Forward: 5' GGAATTCCATATGACAGAGCAGCAGTGGAATTTC3'

**Reverse: 5'CCCAAGCTTGGGCTATGCGAACATCCCAGTGACG3'

plc A

*Forward: 5'GGGGTACCCCGATGTCACGTCGAGAGTTTTTG3'

**Reverse: 5'CCCAAGCTTGGGTCAGCTGCACAGCCCGC3'

hspX

*Forward: 5'GGGGTACCCCGATGGCCACCACCCTTCCCGTTC3'

**Reverse: 5'CCCAAGCTTGGGTCAGTTGGTGGACCGGATCTG3'

tb8.4

*Forward: 5'GGGGTACCCCGATGAGGCTGTCGTTGACCGCA3'

**Reverse: 5'CCCAAGCTTGGGTTAATAGTTGTTGCAGGAGC3'

*Forward primers designed with *Kpn*I restriction site

**Reverse primers designed with *Hind*III restriction site

***Primer designed with *Nde*I restriction site

T7 promoter primer

5' TAATACGACTCACTATAGGG 3'

SP6 promoter primer

5' ATTTAGGTGACACTATAGAAT 3'

T7 terminator primer

5' GCTAGTTATTGCTCAGCGG 3'

III.2. COMPOSITION OF MEDIA, REAGENTS AND BUFFERS

III.2.1. *M. TUBERCULOSIS* CULTURE MEDIA

III.2.1.1. L.J medium

i. Mineral salt solution

Potassium dihydrogen phosphate	2.4 g
Magnesium sulphate ($MgSO_4$. 7 H_2O)	0.24 g
Magnesium Citrate ($Mg_3C_6H_5O_7)_2$ .14H_2O	0.6 g
L-Asparagine	3.6 g
Glycerol	12 ml
Double distilled water	600 ml

Autoclaved for 15 minutes at 121°C.

ii.Medium

Mineral salt solution	600 ml
Beaten egg	1000 ml
Malachite green *	20 ml

* Malachite green solution – 2% in sterile water by dissolving the dye for 1-2 hours.

III.2.1.2. Sauton's medium

Asparagine	4 g
Citric acid	1 g
Magnesium Sulphate	0.5 g
Dipotassium hydrogen phosphate	2.5 g
Ferric Ammonium Citrate	0.05 g
Glycerol	35 ml
Double distilled Water	1000ml

III.2.2. *ESCHERICHIA COLI* CULTURE MEDIUM

III.2.2.1. Luria Bertani broth

LB culture media	2g
Double distilled water	100ml

Autoclaved at 121°C for 15 minutes.

III.2.2.2. Luria Bertani Agar

LB culture media	2g

Agar Agar	1.8g
Double distilled water	100ml

Autoclaved at 121°C for 15 minutes.

III.2.3. HUMAN PBMC GROWTH MEDIUM

RPMI 1640	10.4g
Sodium bicarbonate	2.2g
Sterile deionized water	1000ml

Autoclaved at 121°C for 15 minutes. Supplemented with 100U/ml penicillin, streptomycin (100μg/ml), and 10% heat-inactivated foetal bovine serum just before use.

III.2.4. COMPOSITION OF ROUTINE REAGENTS AND BUFFERS

III.2.4.1. Acrylamide solution

29.2g Acrylamide and 0.8g Bisacrylamide dissolved in 70ml of deionised water and made up to 100ml with distilled water and stored at 4° C in a dark bottle

III.2.4.2. Agarose gel (1%) for DNA electrophoresis

1g Agarose in 100ml of 0.5X TBE buffer

III.2.4.3. Ampicillin

60mg of ampicillin dissolved in 1ml sterile deionized water

III.2.4.4. 5-bromo-4-chloro-3-3 indolyl phosphate toluidine salt (BCIP)

6.05 mg BCIP in 1ml sterile deionized water

III.2.4.5. Blocking solution (ELISA)

1g BSA in 100ml PBS

III.2.4.6. Blocking solution (IHC)

1g BSA in 100ml TBS

III.2.4.7. Blocking solution (*In situ* PCR) -10X

5g blocking reagent (Roche Diagnostics, Mannheim, Germany) in maleic acid buffer

III.2.4.8. Blocking solution (Western blotting)

2.5% (w/v) skim milk in TBST containing 0.1% Tween-20

III.2.4.9. Carbonate bicarbonate buffer (pH 9.6)

1.59 g Sodium carbonate, 2.93 g Sodium bicarbonate and 0.2 g Sodium azide in 1000ml distilled water

III.2.4.10. Chloroform: Iso-amyl alcohol (24:1)

Chloroform and Iso-amyl alcohol was mixed in a proportion of 24:1, and was stored under 100mM Tris-Cl (pH8) at 4 °C in dark glass bottle

III.2.4.11. Citrate buffer (pH 6) - 0.01M

1.92g of citric acid in 1000ml double distilled water

III.2.4.12. DAB substrate (IHC)

6mg DAB in 10ml Tris (pH 7.6) containing 10μl of 30% H_2O_2

III.2.4.13. Developing solution (Western blotting)

1M Tris (pH 9.5), 5M NaCl and 500mM $MgCl_2$ in double distilled water

III.2.4.14. Destaining solution (SDS-PAGE)

30 ml Methanol and 7ml Acetic acid dissolved in 100ml deionized water

III.2.4.15. Diethanolamine buffer (pH 9.8)

97 ml Diethanolamine, 0.2g Sodium azide and 0.1g Magnesium chloride.$6H_2O$ dissolved in 1000ml double distilled water

III.2.4.16. Detection buffer (*in situ* PCR) (pH 9.5)

0.1M Tris Cl and 0.1M NaCl in double distilled water

III.2.4.17. DNA gel-loading dye

Bromophenol blue (0.25%); xylene cyanol FF (0.25%); EDTA (1mM); glycerol (50%) in deionized water

III.2.4.18. EDTA (pH 8) - 0.5M

930mg EDTA in 5 ml deionized water

III.2.4.19. Elution buffer (native Ni-NTA purification) (pH 8)

0.78g of $NaH_2PO_4.2H_2O$, 1.75g of NaCl and 1.7g of Imidazole in 100 ml deionized water

III.2.4.20. Elution buffers (denaturing Ni-NTA purification) (pH 5.9, 4.5 and 4)

0.78g of $NaH_2PO_4.2H_2O$, 0.06g of Tris-Cl and 24.025g of Urea was completely dissolved in 100 ml of deionized water.

III.2.4.21. Ethidium Bromide

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

III.2.4.22. Kanamycin

25mg of Kanamycin sulphate dissolved in 1ml sterile deionized water.

III.2.4.23. Isopropyl-β-D-thiogalactopyranoside (IPTG)

0.1M IPTG in 1ml distilled water

III.2.4.24. Lysozyme

50mg of Lysozyme dissolved in 1ml of sterile deionized water

III.2.4.25. Lysis buffer (native Ni-NTA purification) (pH 8)

0.78g of $NaH_2PO_4.2H_2O$, 1.76g of NaCl and 0.0136g of imidazole dissolved in 100 ml deionized water

III.2.4.26. Lysis buffer (denaturing Ni-NTA purification) (pH 8)

1.56g of $NaH_2PO_4.2H_2O$, 0.12g of Tris-Cl and 48.05g of Urea in 100ml of deionized water

III.2.4.27. Maleic acid buffer (pH 7.5)

0.1M Maleic acid and 0.15M NaCl in double distilled water

III.2.4.28. NBT (Tetrazolium nitroblue)

5% NBT in 70% Dimethyl formamide

III.2.4.29. Phenol: Chloroform (1:1)

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

III.2.4.30. Phosphate-buffered saline (PBS) (pH 7.4)

Sodium chloride (137mM), potassium chloride (2.7mM), disodium hydrogen phosphate (10.14mM), potassium dihydrogen phosphate (1.76mM)

III.2.4.31. Phosphate buffered saline -Tween 20 (PBS-T)

0.5 ml of Tween-20 dissolved in 1000ml of PBS.

III.2.4.32. Proteinase K

20 mg of Proteinase K in 1ml distilled water.

III.2.4.33. Resolving Gel for SDS – PAGE (12%)

6ml of 30% acrylamide, 3.8ml of 1.5M Tris (pH 8.8), 0.15ml of 10% SDS, 0.15ml of 10% ammonium per sulfate and 6μl of TEMED were added to 4.9ml of deionized water

III.2.4.34. SDS-electrophoresis buffer (pH 8.3)

Tris base (25mM), glycine (192mM), SDS (0.1%) in deionized water

III.2.4.35. SDS gel-loading buffer (1X)

SDS (2% w/v), bromophenol blue (0.03%), β-mercaptoethanol (0.3%), glycerol (10%) in Tris buffer (0.067M, pH 6.8)

III.2.4.36. Sodium acetate (pH 5.6) - 3M

1.23 g sodium acetate in 5 ml deionized water

III.2.4.37. Sodium Chloride -5M

29.22g NaCl in 100 ml sterile distilled water

III.2.4.38. Sodium dodecyl sulphate -10%

100 g of SDS was dissolved in 900 ml of water, heated at 68°C to assist dissolving and made upto 1000ml with double distilled water

III.2.4.39. Solution - I (plasmid isolation)

After adding 25mM Tris HCl and 10mM EDTA, solution was autoclaved and to that solution, filter sterilized 50mM glucose was added.

III.2.4.40. Solution - II (plasmid isolation)

Sodium hydroxide (0.2N) and SDS (1%) in sterile deionized water (freshly prepared)

III.2.4.41. Solution - III (plasmid isolation)

Potassium acetate (5M), Glacial acetic acid (11.5ml) in 28.5ml sterile deionized water

III.2.4.42. Stacking gel for SDS – PAGE (5%)

0.83ml of 30% acrylamide, 0.63ml of 1M Tris (pH 6.8), 0.05ml of 10% SDS, 0.05ml of 10% ammonium per sulfate and 5μl of TEMED were added to 3.4ml of deionized water.

III.2.4.43. Staining solution (SDS – PAGE)

0.1% Coomassie brilliant blue R-250, 20% Methanol, 0.5% Acetic acid in double distilled water.

III.2.4.44. TAE buffer (pH 8) - 50X

0.5M EDTA (200µl), Trisbase (242g), 57.1ml Glacial acetic, made up to 1000ml with double distilled water

III.2.4.45. Transformation buffer solution (TFB-1) (pH 5.8)

30mM Potassium acetate, 10mM Calcium chloride, 50mM Manganese chloride, 10mM Rubidium chloride and 15% glycerol in deionized water, filter sterilized.

III.2.4.46. Transformation buffer solution (TFB -2) (pH 6.5)

10mM MOPS, 75mM Calcium chloride, 10mM Rubidium chloride and 15% glycerol in deionized water, filter sterilized.

III.2.4.47. Tissue digestion buffer

50mM Tris Cl (pH 7.5), 10mM EDTA, 500mM NaCl in 100ml deionized water, sterilized by autoclaving and stored at 4°C. 1% SDS as well as Proteinase K (300µg/ml) was added at the time of use.

III.2.4.48. Towbin's buffer (Western blotting)

3.027g Tris base, 14.4g glycine, 200ml methanol made up to 1000ml with deionized water

III.2.4.49. Tris borate- EDTA buffer (10X TBE)

108g of Tris base, 58g of Boric acid and 40 ml of 0.5 M EDTA (pH8) in deionized water which was made up to 1000ml final volume.

III.2.4.50. Tris buffered saline (TBS) (pH 7.6)

6g of Tris base, 17.4g of NaCl in 1000ml of deionized water.

III.2.4.51. Tris-buffered saline - Tween-20 (TBST) (1X)

0.1% Tween-20 in 1X TBS

III.2.4.52. Tris Cl (pH 6.8) - 4X

6.05g Tris base in 100ml with sterile double distilled water after adjusting the pH. 0.4g SDS was added finally.

III.2.4.53. Tris Cl (pH 8.8) - 4X

18.2g Tris base in 100ml with sterile double distilled water after adjusting the pH. 0.4g SDS was added finally.

III.2.4.54. Tris EDTA (TE) buffer (pH 8)

10mM Tris-Cl (pH 8) and 1mM EDTA (pH 8) in 100ml deionized sterile water

III.2.4.55. Washing buffer (native Ni-NTA purification) (pH 8)

0.78g of $NaH_2PO_4.2H_2O$ and 1.75g of NaCl in 100 ml deionized water

III.2.4.56. Washing buffer (denaturing Ni-NTA purification) (pH 6.3)

0.78g of $NaH_2PO_4.2H_2O$, 0.06g of Tris-Cl and 24.025g of Urea in 100ml of deionized water

III.2.4.57. Washing buffer (*in situ* PCR) (pH 7.5)

0.1M Maleic acid, 0.15 NaCl, 0.3% Tween 20 in double distilled water

III.2.4.58. X-Gal

50mg/ml of X-Gal in N, N'-dimethylformamide

III.3. STUDY POPULATION

III.3.1. Pleural fluid specimens

In this study, 140 patients with clinical and radiological evidences of pleural effusion were selected. 8-10ml of pleural fluid sample was collected from each patient during thoracocentesis, and they were simultaneously subjected to cytological, bacteriologic, immunologic, and molecular investigations. The patients were admitted to the Hospital for Tuberculosis and Chest Diseases, Pulayanarkotta, Thiruvananthapuram, India (n = 129), and Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, India (n = 11). There were 93 male and 47 female patients, and the age of the patients ranged from 20 to 82 years. Relevant clinical and radiological features were recorded from the case records of these patients. The most common clinical symptoms were productive cough, breathlessness, chest pain, and pyrexia of more than 4 weeks duration. Constitutional symptoms such as loss of

appetite, reduction in body weight, and general malaise were also recorded in majority of these patients. At the time of admission, 106 of 140 patients showed positive intradermal tuberculin test (>12 mm) and elevated erythrocyte sedimentation rates (45–120 mm). The plain skiagrams of the thorax were suggestive of pleural effusion of varying degree, and the underlying lung lesions in the skiagrams were considerably masked because of the effusion. In 114 patients, the pleural effusion was unilateral, and 26 patients had bilateral effusions. Radiological features of the thorax alone could not define the precise aetiology of pleural effusion necessitating further analysis of pleural fluid in 140 patients. No pleural biopsies were performed during the period of their hospital stay.

III.3.2. Formalin-fixed paraffin embedded (FFPE) lymph node biopsy specimens

Specimens were collected from Department of Pathology, Medical College, Thiruvananthapuram. Paraffin blocks of 71 patients, (male = 39, female =32) with a clinical diagnosis of lymphadenopathy were selected. The relevant clinical features of these patients were recorded from respective case records. The age of these patients ranged between 19 and 65. The duration of lymphadenopathy at the time of biopsy ranged between 20 days to 24 months. (Mean duration = 3.2 months) The salient clinical features in most of these patients were pyrexia, weight-loss, anorexia, elevated erythrocyte sedimentation rate and cervical lymphadenopathy. Fine needle aspiration (FNA) of the involved lymph nodes was initially attempted, but the aspirated material in most cases was adequate only for undertaking ZN staining and cytological studies. The results of FNA were inconclusive in majority of patients, and hence these patients subsequently underwent excision biopsy of the lymph node to ascertain the precise etiological cause of lymphadenopathy. Lymph nodes from

different anatomical locations in the cervical region were biopsied. The most common location was supraclavicular followed by posterior cervical, anterior cervical and axilla.

III.3.3. Serum specimens

A total of 194 sera were collected from the following patient groups. The test group consisted of (a) 26 serum specimens from sputum culture positive patients with pulmonary TB (confirmed pulmonary TB) and (b) 34 serum specimens were collected from culture negative sputum smear negative TB patients. Since these patients showed optimum clinical response to anti-tuberculosis chemotherapy, they were classified as 'probable' cases of TB. The control group consisted of (i) a panel of 42 serum specimens collected from voluntary blood donors attending transfusion medicine of this institute and (ii) a panel of 92 specimens from patients with non-TB respiratory diseases like bronchial carcinoma (n=59), bacterial pneumonia (n=11), and bronchial asthma (n=22)

III.3.4. Whole blood samples

For this study, whole blood samples from seven pulmonary tuberculosis patients were collected. Five of them showed positive TST while negative TST were recorded in two patients. Venous blood (10 ml) was collected from these patients before and during treatment. Venous blood from six BCG vaccinated healthy individuals and three house-hold contacts of active TB patients was also collected for assessment of CMI responses.

III.4. METHODS

III.4.1. Cytology of pleural fluid specimens

Two cytospin smears were prepared from pleural fluid specimens from each patient using a cytocentrifuge. Smears were fixed immediately in absolute alcohol for one hour. Toluidine blue staining method was routinely performed for the assessment of cytological characteristics in pleural fluids. Briefly, re-hydrated cytospin smears were stained with 1% Toluidine blue for one minute, followed by thorough washing in tap water, and dehydration in ascending grades of ethanol. The slide was cleared in xylene. Cover slip was laid using a synthetic mounting medium. Smears were then examined under light microscope.

III.4.2. Histology of lymph node specimens

Lymph node biopsy specimens were fixed in 10% buffered formalin for 24 hours and processed using standard protocol followed in the surgical pathology laboratory. 5µm thick sections from paraffin blocks of lymph nodes were cut with a rotary microtome. Haematoxylin and eosin (H&E) staining was performed as per standard laboratory protocol. Briefly, hydrated paraffin sections were stained with Harris's haematoxylin for 10 minutes. Subsequently the paraffin sections were differentiated by acid-alcohol, treated with ammonia (pH 9.2) solution, counter-stained with 1% aqueous eosin solution for 2 minutes. Sections were dehydrated in graded ethanol and finally cleared twice in xylene. Cover slip was laid on the paraffin sections using a synthetic mounting medium. H&E stained sections were examined under light microscope.

III.4.3. Ziehl- Neelsen staining method

Cytospin smears of pleural fluid and deparaffinised paraffin sections of lymph nodes were hydrated and Ziehl-Neelsen staining was performed as per manufacturer's (Hi-media, Mumbai, India) instructions. Briefly, pleural fluid smears and paraffin sections of lymph nodes were layered with Carbol Fuchsin, and heated from lower side of the slide for about 8-10 minutes. Following this, the slides were washed, briefly decolourized with acid-fast decolourizer and counter-stained with 1% methylene blue for one minute. Finally the slides were washed thoroughly with tap water, dehydrated and observed under the microscope using oil immersion objective (100X magnification).

III.4.4. *M. tuberculosis* culture: Reference *M. tuberculosis* culture was obtained from Tuberculosis Research Centre, Chennai, India. From the stock culture, 'seed' cultures were periodically transferred on to solid LJ media and liquid Sauton's media. Thus the cultures of *M. tuberculosis* were maintained till the end of this study.

III.4.5. Polymerase Chain Reaction

III.4.5.1. Isolation of mycobacterial DNA from *M. tuberculosis* culture

- A loopful of *M. tuberculosis* bacilli was transferred into 2ml microcentrifuge tube containing 400µl TE (pH7) with 10-20 glass beads (0.5mm diameter)
- Mixed by vortexing, for two minutes, and centrifuged at 10000rpm for 5 minutes at room temperature (RT) and the supernatant was discarded
- TE buffer (400µl) was added to the pellet and incubated at 80-90°C for 20 minutes
- 100µl of lysozyme (20mg/ml) was added and vortexed for 30 seconds and incubated at 37°C for 2 hours

- 65µl of 10% SDS and 12µl of 10mg/ml Proteinase K was added and incubated at 65ºC for 30 minutes
- 5M NaCl (100µl) and 10% CTAB (120µl) was added and incubated at 65ºC for 1 hour
- Mixed with equal volume of chloroform and centrifuged at 10000rpm for 10 minutes at RT
- Aqueous layer was removed and mixed thoroughly with 1/10 (w/v) of 3M Sodium acetate (pH 5.2) and 2.5 volumes of absolute ethanol, and incubated overnight
- The pellet was rinsed in 70% ethanol, which was removed by short spinning at 4ºC
- Pellet was air-dried and dissolved in 1X TE (pH8)

III.4.5.2. Isolation of DNA from FFPE lymph node

- Three 20µm thick sections from FFPE lymph nodes were cut using a microtome.
- Paraffin sections were deparaffinised by incubating at 65˚C, followed by vortexing with xylene for 30 minutes at RT and tissue pellet retrieved by centrifugation at 10000 rpm for 20 minutes at RT.
- Xylene was removed by rinsing the pellet with 100% ethanol.
- Pellet treated with 300µl tissue digestion buffer at 37˚C for 24hours.
- Pellet was incubated at 94˚C for 10 minutes to inactivate Proteinase K present in digestion buffer.
- CTAB-NaCl treatment was performed to remove polysaccharides.

- Phenol-chloroform-isoamyl alcohol treatment was carried out to remove the proteins.
- Aqueous layer was removed and DNA was precipitated by 3M Sodium acetate and 100 % ethanol at -20˚C overnight.
- DNA pellet was rinsed with 70% ethanol.
- Air dried pellet dissolved in 20µl TE buffer.

III.4.5.3. Isolation of DNA from pleural fluid specimens

- Centrifuged the pleural fluid sample (5ml) at 15000 rpm for 30 minutes at 4˚C.
- Supernatant was discarded and pellet was mixed with 500µl TE buffer.
- 10% SDS (25µl) was added and mixed vigorously.
- 2.5µl Proteinase K (20mg/ml) was added, mixed and incubated at 37˚C overnight.
- CTAB-NaCl incubation was performed to remove polysaccharides.
- Phenol-chloroform-isoamyl alcohol treatment was carried out to remove the protein fraction.
- DNA was precipitated by 3M Sodium acetate and 100% ethanol at -20˚C overnight.
- DNA pellet was washed with 70% ethanol.
- Air dried pellet dissolved in 20µl TE buffer.

III.4.5.4. Standard PCR using *INS1* and *INS2* primers (*IS6110* amplification)

- Master mix consisted of sterile deionized water, 1X PCR buffer, 1.5mM $MgCl_2$, dNTPs 100µM each, 10 picomoles of each primer (*INS1* and *INS2*), 2U Taq polymerase and 1µl template DNA.

- PCR amplification conditions were as follows: Initial denaturation at 94°C for 5 minutes, 35 cycles, each consisting of denaturation at 94°C for 30 seconds, annealing at 65°C for 1 minute, extension at 72° for 1 minute, and a final extension at 72°C for 7 minutes.

III.4.5.5. Standard PCR using *PT1* and *PT2* primers (*mtp40* amplification)

- Master mix consisted of sterile deionized water, 1X PCR buffer, 1.5mM $MgCl_2$, dNTPs 200µM each, 10 picomoles of each primer (*PT1* and *PT2*), 2.5U Taq polymerase and 1µl template DNA.

- PCR amplification conditions were as follows: Initial denaturation at 94°C for 5 minutes, 35 cycles, each consisting of denaturation at 94°C for 30 seconds, annealing at 72°C for 1 minute, extension at 72° for 1 minute, and a final extension at 72°C for 7 minutes.

III.4.5.6. Nested PCR using *PT1*, *PT2*, *PT3* and *PT4* primers

- A standard PCR using *PT1* and *PT2* primers (*mtp40* amplification) was performed as described

- The first PCR product (PT1 and PT2) was diluted 100 times in sterile deionized water and used for the nested PCR as template DNA.

- Master-mix consisted of sterile deionized water, 1X PCR buffer, 1.5mM $MgCl_2$, dNTPs 100µM each, 10 picomoles of each primer (*PT3* and *PT4*), 2.5U Taq polymerase and 1µl diluted first-round product.

- PCR amplification conditions were as follows: Initial denaturation at 94°C for 5 minutes, 35 cycles, each consisting of denaturation at 94°C for 30 seconds,

annealing at 66˚C for 90 seconds, extension at 72˚ for 1 minute, and a final extension at 72˚C for 5 minutes.

III.4.5.7. Detection of PCR products using agarose-gel electrophoresis

- 1% agarose gel was prepared in1X TBE buffer containing ethidium bromide to a final concentration of 0.5µg/ml
- 4µl of each PCR product was loaded into each well and the gel was run at 60V until bands have migrated enough
- The gel was visualized under a UV Transilluminator, and documented

III.4.6. Detection of *M. tuberculosis* DNA in the FFPE lymph nodes sections using *in situ* PCR.

- 5µm paraffin sections were cut with a rotary microtome, incubated at 37ºC overnight on microslides, deparaffinised for 18 hours at 60ºC
- Treated with xylene to remove paraffin by incubating at 37ºC for 30 minute
- Rinsed in absolute, 75%, 50% and 25% ethanol followed by distilled water
- Sections were permeabilized in 0.02MHCl for 10 minutes at RT
- Washed slides in PBS twice for 5 minutes each
- 0.01% TritonX100 treatment performed for 90 seconds at RT, followed by washing in PBS
- Incubated with 10mg/L proteinase K for 30 minutes at 37ºC
- Proteinase K was deactivated by boiling the sections briefly
- Immediately plunged slides into 20% acetic acid for 15 seconds, washed thoroughly in PBS.

- Overlaid the section with 50µl of PCR mixture consisting of sterile deionized water, 10X PCR buffer, 1.5mM $MgCl_2$, dNTPs 100µM each, Primers (*INS1* and *INS2*) 60 picomoles each, 2.5U Taq polymerase and DIG-labelled UTP (10µm) and self sealing reagent.
- Cover slips were placed and slides were placed over the specific adapter of PCR machine
- PCR amplification conditions were as follows: Initial denaturation at 94°C for 5 minutes, 38 cycles, each consisting of denaturation at 94°C for 30 seconds, annealing at 65°C for 1 minute, extension at 72° for 1 minutes, and a final extension at 72°C for 10 minutes
- Rinsed slides in maleic acid washing buffer and cover slips were removed, and slides were incubated for 10-20 minutes in blocking solution
- Anti- Digoxigenin - alkaline phosphatase was applied over sections (1:5000 dilution in blocking solution) and incubated for 30 minutes
- Slides were rinsed with maleic acid washing buffer twice for 5 minutes
- Equilibrated sections in detection buffer for 5 minutes
- Freshly prepared substrate (NBT-BCIP) in detection buffer was added (1:50 dilution) and incubated overnight in dark
- Slides were rinsed in distilled water twice, counterstained with nuclear fast red, washed again in distilled water and mounted with an aqueous mountant

III.4.7. Amplification of *plcA*, *hspX*, *tb8.4* and *esat-6* genes from *M. tuberculosis*

III.4.7.1. Primer designing

Primer designing was done with Primer-Premiere 5 software. Primers were designed in such a way that the 'Histidine tag' of expression vector was present as a N terminal tag of the expressed protein. Restriction sites were provided for *Kpn*I and *Hind*III

restriction sites in forward and reverse primer respectively, for directional insertion of genes (*plcA, hspX, esat-6 and tb8.4*) into pET-32a vectors and *Nde*I and *Hind*III restriction sites in forward and reverse primer respectively for amplification of gene (*esat-6)* which was cloned into pET-28a vector.

III.4.7.2. PCR amplifying *plcA*, *hspX*, *tb8.4* and *esat-6* from pulmonary and extra-pulmonary tuberculosis DNA samples

- Sterile deionized water, 1X PCR buffer, 1.5mM $MgCl_2$, 100µM dNTP, 5pmol primers (each forward and reverse), 1.25U Taq DNA polymerase and 1µl of DNA
- DNA isolated from cultures of sputum samples from pulmonary tuberculosis patients (a kind gift from Dr. Sathish Mundayoor, Rajiv Gandhi Centre Biotechnology, Thiruvananthapuram) and pleural fluid DNA and FFPE lymph node DNA
- The thermal cycling was done in Bio-Rad iCycler. Initial denaturation at 94˚C for 4 minutes, 35 cycles, each consisting of denaturation at 94˚C for 30 seconds, annealing at 65˚C for 30 seconds, extension at 72˚ for 1 minute, and a final extension at 72˚C for 7 minutes
- PCR products were visualized by agarose-gel electrophoresis

III.4.8. Cloning of mycobacterial antigens into pET-32a/pET-28a vectors

III.4.8.1. PCR cloning into pGEM®-T-easy vector

III.4.8.1.1. Amplification of *plcA*, *hspX*, *tb8.4* and *esat-6* from *M. tuberculosis* $H_{37}Rv$ genome and desired gel band elution

- PCR was performed as described (section III.4.7.2.) with 10ng *M. tuberculosis* $H_{37}Rv$ genomic DNA as template, but with 28 cycles of amplification
- For gel elution, PCR products were electrophoresed in 1% agarose gel in 1X TAE buffer
- Purification of DNA was done using GFX PCR DNA and Gel band purification kit (GE Healthcare Biosciences) as per manufacture's instruction
- Briefly, slice of agarose containing the DNA band to be purified, was excised, weighed, and was further cut into smaller pieces
- To the gel slice, added 10µl of capture buffer for each 10mg of gel slice, vortexed and incubated at 60°C until the agarose was dissolved
- Transferred this mixture into GFX column, incubated at RT for 1 minute, centrifuged at full speed for 30 seconds, and the flow-through collected in the collection tube was discarded
- Column was rinsed with 500µl of wash buffer
- 50µl of elution buffer was added on top of the column, incubated for 1 minute, and centrifuged at full speed for 1 minute to collect the eluted DNA in a fresh collection tube.

III.4.8.1.2. Competent cell preparation

- A single colony of the *E. coli* strain was inoculated from LB plate into 5ml LB broth and incubated at 37°C with shaking overnight
- The entire overnight culture was inoculated into 250ml of LB broth
- Cells were grown until absorbance at 600 nm (A_{600}) reached 0.4-0.6
- Cells were pelleted by centrifugation at 4500 rpm for 5 minutes at 4^0C
- Cell pellets were resuspended in 0.4 volume of ice cold TFB-1 and incubated in ice for 5 minutes
- The cells were pelleted by centrifugation at 4500 rpm for 5 min at 4^0C and were resuspended in 1/25 of original culture volume of ice cold TFB-2
- The cells were incubated in ice for 60 minutes and aliquots were stored at -70°C.

Note: Competent cells were prepared for *E. coli* JM109, *E. coli* BL21 (DE3), *E. coli* JM109 (DE3), and *E. coli* BL21 (DE3) pLysS.

III.4.8.1.3. Ligation of eluted PCR products into pGEM®-T easy

- Briefly centrifuged the pGEM®-T easy vector and insert DNA tubes to collect contents at the bottom of the tube.
- Ligation reactions were prepared with 5μl of 2X Rapid Ligation Buffer, 1μl of pGEM®-T Easy Vector, 3 µl of PCR product, and 1μl T4 DNA Ligase.
- Mixed the reactions by pipetting and incubated overnight at 4°C.

III.4.8.1.4. Transformation

- Centrifuged the tubes containing the ligation reactions to collect contents at the bottom of tube.

- Removed tubes of frozen *E. coli* JM109 competent cells from –70°C storage and thawed in ice.
- Mixed the cells by gently flicking the tube and carefully transferred the whole of ligation mixture into respective vials of competent cells.
- Gently flicked the tubes to mix and placed them on ice for another 30 minutes.
- Cells were given heat-shock for 1 minute at exactly 42°C and immediately returned the tubes to ice for 2 minutes.
- 300μl of LB broth was added to the tubes containing cells transformed with ligation reactions, and incubated for 1.5 hours at 37°C with shaking (~150rpm).
- Transformed culture was plated onto LB/ 60µg/ml ampicillin/ IPTG/X-Gal plates, and incubated overnight at 37°C.

III.4.8.1.5. Colony PCR using standard T7 and SP6 promoter primers

- Colony PCR of the white and blue colonies was performed using standard T7 and SP6 promoter primers.
- 20µl reaction mixture was prepared with sterile deionized water, 1X PCR buffer, 1.5mM $MgCl_2$, 100µM dNTP, 10pmol primers (forward and reverse), 1.25U Taq DNA polymerase, and portion of single colony was used instead of template DNA.
- One negative control (no colony) was maintained with every set of PCR reaction.
- The thermal cycling was done in Bio-Rad iCycler. Initial denaturation at 95˚C for 5 minutes, 35 cycles, each consisting of denaturation at 94˚C for 30

seconds, annealing at 50˚C for 30 seconds, extension at 72˚ for 1minute, and a final extension at 72˚C for 7minutes.

- PCR products were visualized by agarose gel electrophoresis.

III.4.8.1.6. Glycerol stock preparation

- A positive colony was selected from each 'pGEM®-T easy - gene construct' transformed plates and were incubated in 3ml LB media with 60µg/ml ampicillin.
- 500µl of each culture (3-4 hr incubated) was mixed with 500µl of sterile 50% glycerol and was stored at -70^0 C.

III.4.8.2. Sequencing analysis

III.4.8.2.1. Recombinant plasmid isolation

- 4ml of overnight culture of 'pGEM®-T easy-gene' inserted colony was centrifuged at 10,000 rpm for 3 minutes at RT.
- Supernatant was discarded and the pellet was mixed with 200µl of solution I buffer by vigorous vortexing.
- 400µl of freshly prepared solution II was added and mixed by inverting the tube till the solution became clear and incubated in ice for 5 minutes.
- 300µl of solution III was added to the tube and mixed by inverting the tube slowly.
- Incubated in ice for 10 minutes and centrifuged at 12000 rpm for 10 minutes at 4^0 C.

- Supernatant was taken and equal volume of isopropanol added and mixed by inversion.
- Incubated for 20 minutes at RT and centrifuged at 10000rpm for 10 minutes at RT
- Supernatant was removed by pipetting and pellet was air-dried and resuspended in 150µl of Sephaglas FP slurry (GE Healthcare Biosciences), vortexed gently for 1 minute to dissolve the pellet
- Centrifuged at full speed for 15 seconds and carefully removed the supernatant without disturbing the Sephaglas pellet
- Washed the pellet by adding 200µl of wash buffer and spinned off the supernatant
- 300µl of 70% ethanol was used for another wash of the pellet, and the ethanol was discarded after a short spin
- Air-dried the pellet, and eluted the bound DNA from Sephaglas by incubating with 50µl of TE buffer for 5 minutes, and then by spinning at full speed for 1 minute

III.4.8.2.2. Standard PCR using T7 and SP6 promoter primers and gel band elution

- 50µl reaction mixture was prepared with sterile deionized water, 1X PCR buffer, 1.5mM $MgCl_2$, 100µM dNTP, 10pmol primers (each forward and reverse), 2.5U Taq DNA polymerase, 0.5µl of plasmid DNA.
- The thermal cycling as described earlier (section III.4.8.1.5.) for T7 and SP6 primers, except that only 30 cycles of amplification was performed.

- PCR products were run on 1% agarose gel in 1X TAE buffer and the desired DNA band was eluted.

III.4.8.2.3. Sequencing PCR

- For DNA sequencing, two reactions are kept, one with forward and one with reverse primer of particular gene.
- The master mix prepared consisted of 2µl of 10X Sequencing Buffer, 0.35µl of Sequence mix, 0.3µl Primer, 5.35µl of sterile deionized water and 2µl of DNA template.
- 10µl reaction was used for each sample. Sequencing was performed in a 96-well plate.
- After adding the master-mix, the plate was sealed properly with an aluminium foil.
- The conditions of the sequencing were:

Cycle 1 (25x)

- Ramp to 96.0°c at 1.0°C/sec : 96.0°c for 00.15
- Ramp to 55.0°c at 1.0°C/sec : 55.0°c for 00.05
- Ramp to 60.0°c at 1.0°C/sec : 60.0°c for 04.00

Cycle 2 (1x)

- Ramp to 4.0°c at 1.0°C/sec : 4.0°c for ∞

- After the sequencing PCR, the products were subjected to a post PCR clean-up process.

III.4.8.2.4. Post PCR clean-up process

- Post PCR clean-up is a series of chemical treatments for the removal of dye components, unwanted enzyme and buffer that would influence the quality of sequencing.
- 12μl of master mix -1 was prepared by 10μl of sterile deionized water and 2μl of 125mM EDTA.
- 52μl of master mix-2 was prepared by mixing 2μl of 3M Sodium acetate and 50μl of 100% Ethanol.
- 12μl of mastermix-1 was added first to the wells of the plate followed by 52μl of mastermix-2.
- The plate was incubated at RT for 15 minutes.
- Spun at 3000 rpm for 30 minutes at RT.
- Supernatant was discarded by inverting the plate over a paper towel.
- The plate was again spun for a few seconds in the inverted position to remove the remaining liquid completely.
- The plate was then washed twice with 100μl of 70% alcohol.
- Finally 12μl of D-formamide was added and DNA was denatured at 94°C for 10 minutes.

- Finally the plate was kept at 4°C and was subjected to DNA sequencing.
- The DNA sequencing was performed on ABI 310 Automated DNA sequencer (Applied Bio-System, Perkin Elmer, USA) using ABI PRISM Big Dye Terminator cycle sequencing Ready Reaction kit, Version 2.0.
- The Ready mix (sequence mix) used during sequence PCR includes, A-Dye Terminator labelled with dichloro (R6G), C-Dye Terminator labelled with dichloro (ROX), A-Dye Terminator labelled with dichloro (R110), A-Dye Terminator labelled with dichloro (TAMRA).

III.4.8.2.5. Sequence retrieval

- The complete sequences of genes were obtained from the Tuberculist web server - http://genolist.pasteur.fr/TubercuList/, and the sequences obtained from experiments were matched and analysed.

III.4.8.3. pET- 32a vector modification

III.4.8.3.1. Restriction, re-ligation and transformation

- pET-32a vector was digested with *Nde*I enzyme to remove the thioredoxin (trx) tag
- The reaction mixture consisted of 14μl of sterile water, 2μl of 10X digestion buffer, 3μl of undigested pET-32a and 1μl of *Nde*I enzyme.
- Mixture was vortexed, incubated at 37°C for 16 hours
- The digestion mixture was run on a 1% agarose gel in 1X TAE and the desired DNA band was subsequently eluted from the gel

- A ligation reaction was kept for overnight incubation at 16°C with 3μl of sterile deionized water, 1μl of 10X ligation buffer, 5μl of undigested pET-32a and 1μl of ligase enzyme
- 2μl of ligation mixture was used for transforming *E. coli* JM109 competent cells.
- Transformed cells were plated on LB media with ampicillin, and incubated overnight at 37°C
- The successful removal of tag and transformation was checked by colony PCR

III.4.8.3.2. Colony PCR with T7 promoter and T7 terminator primers

- A colony PCR was performed with T7 promoter and T7 terminator primers, the conditions were similar to T7 and Sp6 promoter PCR, except that the annealing reaction was set at 55°C
- Reaction products were detected on 1% agarose gel in 0.5XTBE

III.4.8.3.3. Isolation of modified pET-32a vector

- pET-32a minus 'trx' tag was isolated in high yield by culturing the positive transformed colonies overnight in LB media mixed with ampicillin
- Plasmid isolation was performed as described earlier

III.4.8.4. Cloning of individual genes into modified pET-32a expression vector

- 50µl of digestion reaction mixture was prepared by mixing 37.5µl sterile deionized water, 5µl of 10X Buffer (NEB Buffer 2), 0.5µl BSA, 5µl of pGEM®-T

easy- *plcA*/ pGEM®-T easy- *hspX*/ pGEM®-T easy- *tb8.4*/ pGEM®-T easy- *esat-6* and 1µl each of *Kpn*I and *Hind* III enzymes.

- Incubated at 37°C, for 3 hours.
- Digestion mixture was run on a 1% agarose gel in 1X TAE and the desired DNA band was subsequently eluted from the gel
- A ligation reaction was maintained overnight at 4°C with 2μl of sterile deionized water, 1.5μl of 10X ligation buffer, 3μl of digested pET-32a vector and 7μl of insert (*plcA / hspX / tb8.4 / esat-6)* and 1.5μl of ligase enzyme.
- 2μl of ligation mixture was used for transforming *E. coli* JM109 competent cells.
- Transformed cells were plated on LB media with ampicillin, and incubated overnight at 37°C
- The successful transformation was checked by colony PCR using T7 promoter and T7 terminator primers as described earlier.
- Plasmid isolation was carried out as described earlier.

Note: *esat-6* was also cloned into pET-28a besides pET-32a. The open reading frame for *esat-6* was amplified with *Nde*I and *Hind*III restriction sites so as to clone into pET-28a vector. Here the transformed cells were plated on LB media with 25 μg/ml kanamycin, and incubated overnight at 37°C. For plasmid isolation also, colonies were cultured in LB media with kanamycin.

III.4.9. Expression of mycobacterial proteins

III.4.9.1. Transformation of *E. coli* expression host strains

- 2μl of recombinant pET-32a plasmids were used in transforming competent cells of *E. coli* BL21(DE3), *E. coli* BL21(DE3) pLysS and *E. coli* JM109(DE3) strains
- The mixture was plated on LB / ampicillin agar plates, and incubated overnight at 37°C.

Note: pET-28a - *esat-6* plasmids were used in transformation, but recombinant colony selection was based on kanamycin resistance (25µg/ml).

III.4.9.2. Protein expression

- A colony of the host *E. coli* strain was inoculated into 3ml of LB both without ampicillin.
- Cells with vector alone and cells with recombinant vector (plasmid with insert) were inoculated into 3 ml LB broth with 60µg/ml ampicillin.
- Recombinant vector for each gene in all the three strains of *E.coli* like BL21(DE3), BL21(DE3)pLysS and JM109(DE3) was cultured separately with proper labelling.
- Tubes were incubated overnight at 37^0C.
- 150µl of overnight culture was inoculated in 6 ml of LB broth with 60µg/ml ampicillin and incubated for 2 hours at 37^0C

- 1 ml of the culture from each tube were taken and centrifuged at maximum speed for 2 minutes and cell pellets were re-suspended in 1X PBS and mixed vigorously and stored at -20^0 C, labelled as zero hour.
- To rest of the culture 150 µl of IPTG was added and incubated at 28° C for induction.
- Cells were pelleted and re-suspended in 1XPBS at different time intervals, i.e., 2 hours, 4 hours, 6 hours and 8 hours.

Note: pET-28a - *esat-6* plasmids were induced in same conditions except that antibiotic used was kanamycin (25µg/ml).

III.4.9.3. Analysis of protein expression by SDS-PAGE

- A Bio-Rad mini PAGE apparatus was used.
- The glass plates were cleaned, assembled and locked to the casting stand.
- Resolving gel was prepared and poured between the plates using pipette and water saturated isobutanol was poured above resolving gel to form a thin layer.
- The gel was allowed to polymerize for 1 ½hours.
- The layer of saturated isobutanol was poured off and washed with water, wiped with filter paper.
- Stacking gel was prepared and poured over the resolving gel and the comb was inserted.
- Stacking gel was allowed to polymerize for 1½ hours and samples with 6X loading buffer (3:1) and was kept in a boiling water bath for 10 minutes.

- The comb was removed and wells were cleaned with double distilled water.
- Plates were set in the tank and the tank was filled with Tris-Glycine buffer.
- Samples (cooled to RT) were loaded in wells. Protein marker was also loaded for reference.
- Voltage supplied during the run in the stacking gel was 80V, which was changed to 100V once the dye front reached the resolving gel.
- The power was switched off when the dye front reached the bottom of the resolving gel.
- Gel was stained with Coomassie brilliant blue for 1 hour in a shaker.
- Staining solution was poured off and the gel was de-stained in 7% acetic acid solution and kept on a shaker overnight.

III.4.10. Purification of recombinant mycobacterial proteins in high yield

III.4.10.1. Protein expression for purification procedures

- A single recombinant *E. Coli* BL(DE3)pLysS colony was inoculated into 4ml LB medium containing antibiotic, and grown overnight at 37 °C.
- The overnight culture was used to inoculate 100ml LB with antibiotic and grown at 28°C.
- When the A_{600} reached 0.6-0.7, IPTG was added to a final concentration of 1mM, and culture was incubated for 4 hours.
- Cells were harvested by centrifugation at 4500rpm for 5minutes at 4°C

- Cell pellet was lysed by sonicated on ice (6 times for 10 s each time with 5 s pulses), centrifuged at 10000rpm for 30 minutes at 4°C, supernatant and pellet was run separately in SDS-PAGE for each protein for checking the location of expressed protein (whether present in soluble or insoluble fraction).

III.4.10.2. His-tagged recombinant proteins purification

- Recombinant antigens were purified from cell extracts to near-homogeneity by Ni-NTA affinity method as per manufacturer's instruction (Promega Coorperation, Madison, WI).

- Soluble proteins were eluted under native conditions and insoluble proteins (inclusion bodies) were eluted under denaturing conditions.

- The conditions and buffer constituents were modified to suit the present purification study.

III.4.10.2.1. Purification under native conditions

1. Cell pellet of 100ml culture was thawed and resuspended in 4 ml lysis Buffer.

2. Lysozyme was added to 1 mg/ml final concentration and tubes were incubated at 4°C for 30 minutes with proper shaking.

3. Cell pellet was sonicated on ice to lyse cells (6 times for 10 s each time with 5 s pulses).
4. Cleared the lysate by centrifugation at 10,000rpm for 30 minutes at 4°C and supernatant was collected.
5. 20μl of the cleared lysate was saved for SDS-PAGE analysis.

6. Ni-NTA magnetic agarose beads were washed with deionized water and equilibrated with lysis buffer.
7. 500μl of 6xHis-tagged protein was added to 100μl of 5% Ni-NTA magnetic agarose beads suspension.
8. Incubated the suspension on a shaker for 20 minutes at 4°C, spinned and supernatant was removed.
9. Repeated steps 7& 8 till the whole of the 4ml protein was used.
10. 1ml of wash buffer was added to each tube, mixed, spinned and the supernatant removed.
11. Step 10 was repeated thrice
12. Added 500μl of elution buffer, mixed, incubated for 1 minute at 4°C, spinned and collected the elute.
13. Step 12 was repeated till most of the protein was eluted, as analyzed by a SDS-PAGE.

III.4.10.2.2. Purification under denaturing conditions

1. Thawed cells from 100ml culture and resuspended in 4 ml Urea lysis buffer (pH 8) and cells were allowed to shake for 1 hour at RT
2. The pellet was sonicated on ice to lyse cells (6 times for 10 s each time with 5 s pauses between).
3. Cleared the lysate by centrifugation at 10,000 x *g* for 30 min at 4°C and supernatant was collected.
4. 20μl of the cleared lysate was saved for SDS-PAGE analysis.
5. Ni-NTA magnetic agarose beads were rinsed with distilled water and equilibrated with urea lysis buffer.

6. 500μl of 6xHis-tagged protein was added to 5% Ni-NTA magnetic agarose beads suspension (100μl).
7. Incubated the suspension on a rotary shaker for 20 minutes at RT, and supernatant was removed.
8. Repeat steps 6& 7 till the whole of the 4ml protein was used.
9. Added 1ml of wash buffer (pH 6.3) to each tube, mixed, spinned and supernatant removed.
10. Step 9 was repeated thrice
11. Added 500μl of elution buffer (pH 5.9), mixed, incubated for 2 min at RT, spinned and elute was collected.
12. Step 11 was repeated thrice and eluted proteins were stored at -20°C.
13. 500μl of elution buffer (pH 4.5) added, mixed, incubated for 2 min at RT, spinned and collected elute.
14. Step 13 was repeated thrice and eluted proteins were stored at -20°C.
15. 500μl of elution buffer (pH 4), mixed, incubated for 2 minutes at RT, spinned and elute was collected.
16. Step 15 was repeated thrice and eluted proteins were stored at -20°C.

III.4.10.3. SDS-PAGE analysis and Western blotting

- Expression and size of expected protein was checked by a SDS-PAGE, followed by immunoblotting using anti-His antibody.
- Proteins separated by SDS-PAGE were transferred onto PVDF membrane with mini transblot electrophoretic transfer cell (Bio-Rad Laboratories) and blotting was performed at 100V for 2 hours at 4^0C.
- The membrane was removed and washed twice with ice cold TBST buffer.

- 10ml of 5% milk powder in ice-cold TBST buffer was poured on to the nitrocellulose membrane and incubated for 1 hour at RT on a shaker.
- Membrane was washed 4 times with 10ml ice cold TBST buffer.
- Primary antibody (anti-His antibody) was added and incubated at 4^0C overnight in a shaker.
- 2 minutes wash with 10ml TBST buffer was performed 4 times.
- Secondary antibody conjugated with alkaline phosphatase was added and incubated for 1 hour at RT on a shaker.
- Membrane was given 10 minutes washing with TBST buffer for 4 times.
- Membrane was kept in 10 ml of developing solution, mixed with 66µl of NBT (stock) and 33µl of BCIP, shaked until the band was observed.
- Developing solution was poured off and membrane was washed with sterile distilled water and air-dried.

III.4.10.4. Desalting of proteins

III.4.10.4.1. Desalting by dialysis

- Denaturing agents in eluted proteins was performed by concentration gradient dialysis of urea.
- The solution of denatured protein was dialyzed against 500ml of freshly made 6, 4, 2, 1, 0.5, and 0M urea, respectively, with 5mM Tris (pH 7.4).
- With each concentration, the protein was dialyzed 12 h at 4°C.

- Protein solution was concentrated by ultra filtration (Amicon-GMBH, Germany).

III.4.10.4.2. Desalting with PD10 columns

- Desalting of proteins was also performed with PD10 columns as per the manufacturer's (GE Healthcare Biosciences) instructions.
- Briefly, PD10 columns were equilibrated with urea buffer, following which the sample is allowed to enter the resin-bed.
- Eluted the protein with PBS (pH7.4) and stored in aliquots at -20°C.

III.4.10.5. Protein quantification

- Bradford method was performed, as per manufacturer's protocol (Bio-Rad Laboratories), to assay protein concentration
- Briefly, 200µl of 1:4 diluted Bradford reagent was mixed with 10μl sample, incubated for 5 minutes at RT and absorbance was determined at 595 nm
- A standard curve was established each time a protein assay was performed with BSA dilutions of known concentrations
- Using the standard curve, the concentration of each sample was determined

III.4.11. Immunization Schedule

- All the four recombinant antigens PlcA, HspX, Tb8.4 and ESAT-6 as well as tuberculosis glycolipid antigen (TBGL) and culture filtrate antigen (CFA) which were isolated and characterized in this laboratory were used in immunization.
- Two albino- rabbits were used as source of antisera for each antigen.

- 5ml venous blood was collected and the serum was separated and labelled as pre-immune serum.
- In the primary immunization each rabbit received an inoculum containing antigen (50µg in case of individual recombinant antigen or 2mg in the case of TBGL and CFA, mixed in 2ml of phosphate buffered saline (PBS) and 2ml incomplete Freund's adjuvant).
- The inoculum was injected into two intra muscular sites and two subcutaneous sites.
- The immunization schedule was repeated on the 14^{th}, 28^{th} and 42^{nd} day following the primary immunization.
- Gamma globulin fraction was isolated from the sera by 33% ammonium sulphate saturation followed by dialysis against PBS.
- IgG was isolated from the gamma globulin fraction by standard Protein-A Sepharose chromatography (Amersham-Pharmacia Biotech, NJ, USA).
- Protein concentration was estimated by Bradford's method (Bradford, 1976).
- Antibody titre was calculated by ELISA method (Engvall and Perlmann., 1972).
- Antibodies were dispensed in aliquots and stored at -20˚C.

III.4.12. Immunohistochemical demonstration of specific mycobacterial antigens in FFPE lymph node

- 5µm thick sections from paraffin blocks were cut using a microtome.

- Sections were deparaffinised by 15 minutes incubation at 65°C, and treated with xylene.
- Sections were rehydrated in 100%, 70%, 50% ethanol and double distilled water.
- Antigenic sites were retrieved by boiling in 0.01M sodium citrate buffer (pH 6).
- Endogenous peroxidase activity in tissues was removed by incubating in 3% Hydrogen peroxide for 5 minutes.
- Sections were incubated with normal goat serum (5% in 1% BSA / PBS) for 30 minutes to remove non-specific reaction in tissues.
- Sections were incubated overnight with primary antibody * (1μg/ml) at 4°C.
- Sections were treated with anti rabbit IgG-biotin for 40 minutes, washed in TBS-T followed by Streptavidin horse-radish peroxidase for 30 minutes.
- Sections were washed and incubated with substrate (AEC) for 5-10 minutes
- Counterstained with haematoxylin, dehydrated, cleared and mounted.

*Primary antibody was, anti-TBGL, anti-CFA, anti-PlcA, anti-HspX, anti-Tb8.4 or anti-ESAT-6, as the case may be.

III.4.13. Detection of antigen-specific IgG antibody level in sera and pleural fluids

- For detection of IgG antibodies against recombinant antigens*, an ELISA was used.

- Briefly, wells of round-bottom microtitre plates were coated with purified protein (500pg/well) for 2 hours following which the wells were quenched with 1% BSA in PBS.

- Samples from disease control and tuberculosis groups were serially diluted in 1% BSA in PBS and 100µl (1:5000 dilution) was added to each well and incubated overnight at 4˚C.
- Washed the plates with PBS- T and incubated for 2 hours with 1:1000 diluted anti-human IgG-alkaline phosphatase conjugate (100μl).
- Plates were washed with PBS- T seven times.
- Colour reaction was developed by the addition of a substrate containing para-nitrophenyl phosphate (1mg/ml in diethanolamine buffer) and the plates were incubated for 30 minutes.
- 3N Sodium hydroxide (25µl) was added to wells to stop the reaction.
- The absorbance was read at 405 nm.
- Whenever ELISA was performed in a batch of 10 specimens, a positive control from a culture-positive tuberculosis patient was used.
- Batch to batch variation of recombinant antigen, was checked by using two different batches of antigen with the same serum sample at two different occasions.
- In each batch, mean OD of disease control group plus 2 standard deviations was used to determine the 'cut-off' to confirm tuberculosis patients.
- PlcA, Tb8.4, HspX, ESAT-6 and multiantigen cocktail. ELISA was also performed using a multiantigen cocktail of all the four recombinant antigens in equal concentration (200pg each).

III.4.14. Human PBMC culture and protein stimulation

- PBMC were isolated from heparinised blood by density gradient centrifugation by Ficoll-Hypaque method.
- Briefly, 10ml blood was diluted in 10ml PBS, and was layered on 20ml histopaque.
- Centrifuged at 300g at 25ºC for 25 minutes, and the buffy-coat was collected.
- Buffy-coat was washed in PBS, and with RPMI media devoid of foetal bovine serum (FBS), by centrifugation and cells were counted on a cell cytometer. PBMC was suspended at 10^6 cells/ml concentration in RPMI 1640 growth medium supplemented with 10% heat-inactivated foetal bovine serum and antibiotics.
- 200μl PBMC suspension was distributed into 96-well round-bottom plates.
- Plates were incubated for 3 hours at 37°C in a 5% CO_2 incubator for cell adhesion.
- 50μl of protein in RPMI medium was added into wells and kept for incubation at 37°C in a 5% CO_2 incubator for 96 hours.
- Three different concentrations of proteins (3µg/ml, 5µg/ml, 10µg/ml respectively) were used in the study.
- For positive controls, cells were cultured in the presence of 10µg/ml Concanavalin A.
- Cultures incubated with medium alone served as negative controls.

- After 96 hours, the plates were taken out, media containing cytokines were aspirated and stored at -20°C and attached cells were used in MTT assay.

III.4.15. MTT assay

- Assay was performed using 3- (4, 5 dimethylthiazole-2-4) 2, 5-diphenyl tetrazolium bromide (MTT) dye using the method described by Mosmann (1983) with some modifications.
- 20µl of 5mg/ml MTT reagent was added into each well and incubated for 2 ½ hours at 37°C and 5% CO_2.
- Solution phase was aspirated out slowly and 100µl of HCl: Isopropanol (1:100) was added and incubated for 10 minutes
- Absorbance was read at 570nm.

III.4.16. Cytokine release assay

- Supernatants aspirated out were checked in ELISA for the presence of cytokines, IFN-γ, TNF, IL-4 and IL-10, using Human cytokine ELISA Kits (BD Biosciences-Pharmingen) as per the manufacturer's protocol.
- Briefly, a monoclonal antibody specific for specific cytokine was already coated on a 96-well plate.
- Standards (recombinant cytokine in known quantities as supplied by the manufacturer) and samples were added to wells, incubated.
- After thorough washing, Streptavidin-horseradish peroxidise conjugate mixed with biotinylated anti-human cytokine antibody was added and incubated.

- Wells were washed and TMB substrate solution was added, incubated and the reaction was stopped with a stop solution.
- Absorbance was read at 450nm.
- Using the standard curve, the concentration of each sample was determined.

III.5. DATA ANALYSIS

The diagnostic value of the ELISA was evaluated in terms of sensitivity and specificity. The sensitivity of a diagnostic test was interpreted as the proportion of TB patients correctly identified by the test as having tuberculosis; the specificity is the proportion of normal or disease control subjects correctly identified as not having tuberculosis by the test. Comparisons among groups for cytokine and proliferation profiles were performed using the Mann-Whitney U-test. Data were considered statistically significant when $P \leq 0.05$. Analysis in cytokine release assays and proliferation assay was performed using stimulation index values. Stimulation index was the ratio of absorbance of test to absorbance of negative control.

IV.RESULTS

IV.1. CYTOLOGICAL FEATURES OF PLEURAL EFFUSION IN TUBERCULOUS AND NON-TUBERCULOUS GROUPS

Cytological features in cytospin smears of pleural fluid specimens in 66 patients with tuberculous pleural effusions demonstrated 'lymphocytosis' in which lymphocytes appeared well- differentiated (Figure 1). Pleural fluids in fifteen patients predominantly showed neutrophils (Figure 2). Pleocytosis was visualized in 30 pleural fluids and this was composed by admixture of lymphocytes, monocytes and reactive mesothelial cells (Figure 3). The cytological features in the remaining 29 cases showed presence of malignant cells (Figure 4).

IV.2. HISTOPATHOLOGICAL FEATURES OF FFPE LYMPH NODE

In four patients, H&E stained paraffin sections of the lymph nodes showed caseating granulomatous lesions composed of macrophages with Langhan's giant cells. Dense fibroblastic proliferation surrounding the granulomatous lesions was seen. In 29 patients, lymph nodes showed less dominant caseous granulomatous lesions which were composed by epithelioid cells, and Langhan's giant cells (Figure 5). In 2 patients, lymph nodes showed extensive fibrosis, replacing granulomatous lesions. In 12 lymph node biopsies, the histopathological features suggested metastatic carcinoma and in 6 patients the features were suggestive of lymphoma. In 5 lymph node biopsies the histological features were due to fungal aspergillosis, (n=4) and filariasis (n=1). Histopathological characteristics in 9 biopsies were suggestive of reactive follicular hyperplasia. 4 cases were reported as 'non-specific' lymaphadenitis.

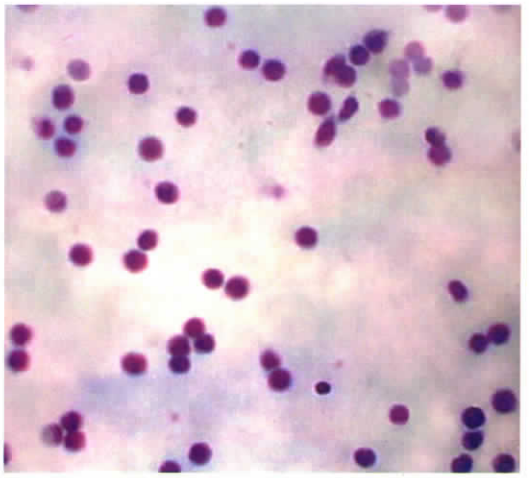


Figure 1: Photomicrograph showing lymphocytosis in pleural effusion (Toluidine blue stain (X 200)

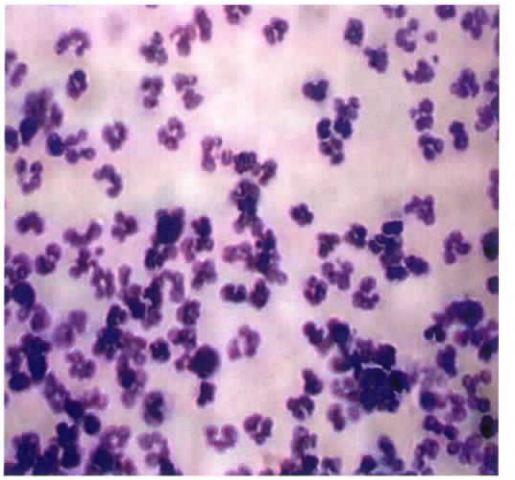


Figure 2: Photomicrograph showing neurophils in pleural effusion (Toluidine blue stain (X 200)

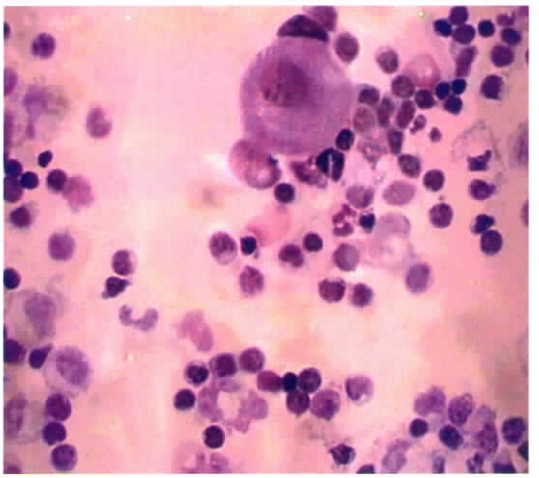


Figure 3: Photomicrograph showing pleocytosis in pleural effusion (Toluidine Blue stain X 200)

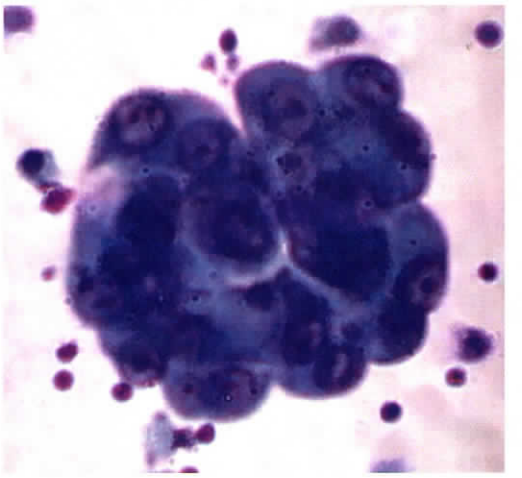


Figure 4: Photomicrograph showing malignant cells in pleural effusion (Toludine Blue stain X 200)

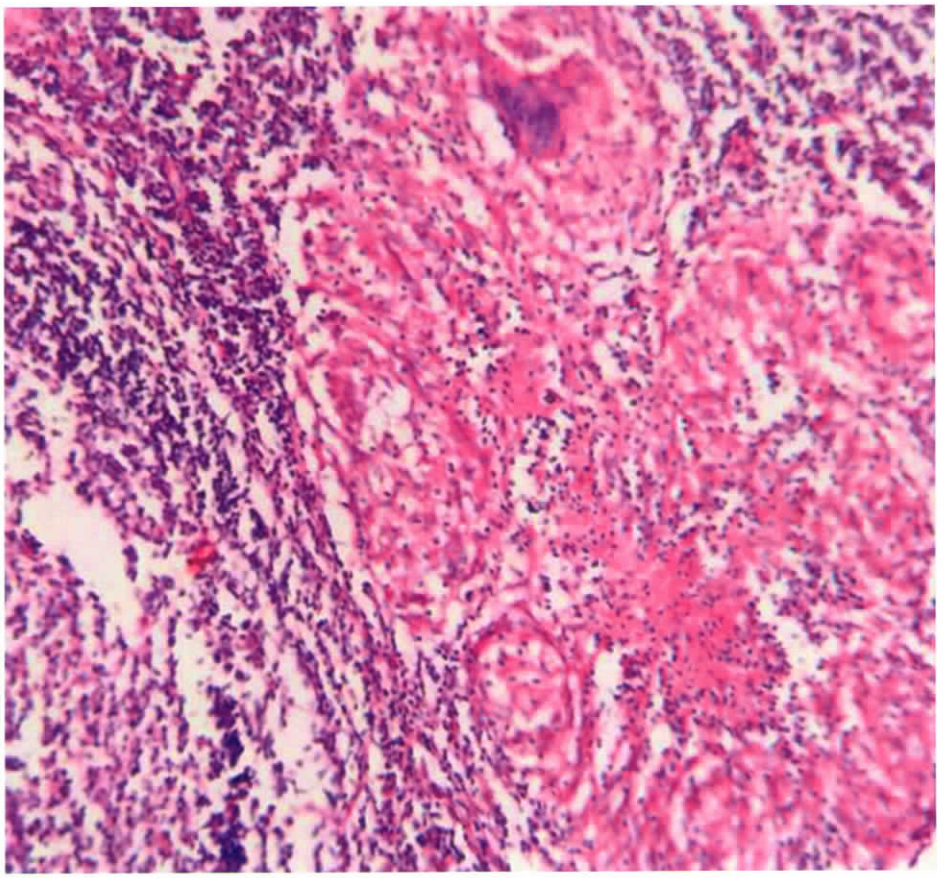


Figure 5: Photomicrograph showing granulomatous lesions composed of epithelioid cells, Langhan's giant cells and lymphomononuclear cells in lymph node (H&E stain X 200)

IV.3. BACTERIOLOGICAL METHODS FOR TB DIAGNOSIS

IV.3.1. Pleural fluid specimens

Ziehl- Neelsen (ZN) staining method demonstrated the presence of AFB in 3 pleural fluid specimens. *M. tuberculosis* was isolated in six pleural fluid specimens by culturing in L-J medium.

IV.3.2. Lymph node specimens

ZN staining method showed the presence of AFB in the section of 4 lymph node biopsies. Results of culture of in the lymph node biopsies were taken from the case records of the patients.

IV.4. CLASSIFICATION OF CLINICAL SPECIMENS

IV.4.1. Classification of pleural effusion specimens

Based on the results of the clinical, radiological cytological and bacteriological methods, patients with pleural effusion were classified into 3 groups:

a) Bacteriologic studies revealed the presence of *M. tuberculosis* in 6 pleural fluid specimens. They were regarded as 'confirmed' patients with pleural tuberculosis (PTB).

b) The bacteriologic cultures in the 63 pleural fluid specimens did not grow *M. tuberculosis* or any other microbial agents. However, the radiological and clinical features in these patients were suggestive of tuberculosis, and these patients were given a course of ATT. The patients showed optimal clinical recovery. Hence, these patients were classified as 'probable' PTB patients.

c) Seventy - one patients with pleural effusion were classified as malignant pleural effusion based on clinical data together with the results of cytological studies Patients with malignant pleural effusion were categorized as 'disease control'.

IV.4.2. Classification of lymph node specimens

(A) In four patients, *M. tuberculosis* bacilli were demonstrated in the paraffin sections of the lymph nodes by ZN method. H&E staining in these patients showed caseating granulomatous lesions. They were 'culture-positive' as per the case records. Hence these four patients were categorized as 'confirmed' TB-L cases.

(B) In thirty-one patients, there were histopathological evidences of caseating / non-caseating granulomatous lesions in lymph nodes but AFB was not demonstrated. Based on the relevant clinical features supported by compatible histopathological features and optimal clinical response to ATT, they were classified as 'probable TB-L'.

(C) In 12 lymph node biopsies, the histopathological features were suggestive of metastatic carcinoma and in 6 patients the histopathological features were regarded as lymphomas. In 5 lymph node biopsies the histopathological features were due to fungal aspergillosis (n=4) and filariasis (n= 1). Four cases were reported as 'non-specific' lymphadenitis of unknown aetiology. Nine biopsies showed features of reactive follicular hyperplasia.

Lymph node biopsy specimens in group (A) and (B) were regarded as TBL group (n = 35) and specimens in-group (C) were selected as 'disease control' for this study (n = 36).

IV.5. PCR METHODS IN DIAGNOSIS

IV.5.1. Results of standard *IS6110* PCR

IV.5.1.1. Standard *IS6110* PCR in pleural fluid specimens

Standard PCR using INS1 and INS2 primers amplifying the insertion sequence *IS6110* was able to detect mycobacterial DNA in all the 6 culture-positive pleural fluid

specimens. PCR was positive in 52 out of 63 probable PTB specimens. *IS6110* PCR gave an overall sensitivity of 84% (58/69) in PTB patients. False positive results were obtained in eleven patients with malignant pleural effusion, which reduced the specificity of *IS6110* PCR to 84.5%.

IV.5.1.2. Standard *IS6110* PCR in lymph node specimens

IS6110 PCR gave positive amplification in 22/31 'probable' TB-L patients. All the four 'confirmed' cases gave positive results. Positive results in the disease control were encountered in one patient with fungal granuloma, four patients with reactive follicular hyperplasia and in one patient with non-specific lymphadenitis of unknown aetiology. These positive results in disease controls were considered as false positive. Thus *IS6110* PCR gave an overall 74.3% (26/35) sensitivity and 83.3 % specificity in this study. A representative photograph showing amplification of 245bp segment of IS6110 from pleural fluid and lymph node are shown in Figure 6.

IV.5.2. Results of standard *mtp40* PCR

IV.5.2.1. Standard *mtp40* PCR in pleural fluid specimens

Standard PCR using PT1 and PT2 primers amplifying a 396bp fragment of *mtp40* genomic sequence was standardized. This method detected the presence of mycobacterial DNA in all the 6 culture positive pleural fluid specimens. PCR was positive in 24 out of 63 probable PTB specimens, thus giving a sensitivity of 43.5% (30/69) in pleural fluid specimens of PTB patients. Six false positive cases were detected in disease control group (specificity 91.5%).

IV.5.2.2. Standard *mtp40* PCR in lymph node specimens

PCR using specific primers designed against *mtp40* gave positive results in 14 out of 31 probable TBL cases. All the four confirmed TBL cases were positive. The overall sensitivity of the method was 43.5% (18/35). Two false - positive results were encountered in lymph nodes from reactive follicular hyperplasia and one from fungal granuloma. The specificity of standard *mtp40* PCR was 91.6%. A representative photograph showing amplification of 396bp segment of *mtp40* in pleural fluid and lymph node are shown in Figure 7.

IV.5.3. Results of nested PCR

IV.5.3.1. Nested PCR in pleural fluid specimens

Nested PCR was performed in all the 140 pleural fluid specimens and it gave positive results in 57/63 'probable' PTB patients. All the six 'confirmed' PTB cases gave positive results. Seven positive results were encountered in patients with malignant pleural effusion. Hence nested PCR gave 91.3% (63/69) sensitivity and 90.1 % specificity in this study.

IV.5.3.2. Nested PCR in lymph node specimens

Nested PCR using PT1, PT2, PT3 and PT4 primers detected the presence of*mtp40* genomic sequence in lymph nodes from all the 4 confirmed TBL. PCR was positive in 27 out of 31 probable TBL, giving an overall sensitivity of 88.6% (31/35). Two false positive results were encountered in lymph nodes with reactive follicular hyperplasia and in one aspergillosis patient. The specificity of nested PCR was 91.6%. A representative photograph showing nested PCR products (both first round and nested round products) in pleural fluid and lymph node DNA is represented in Figure 8.

Figure 6: Gel image of *IS6110* PCR products: lane 1: 100bp ladder, lanes 2-26: pleural fluid and lymph node specimen DNA, lanes 27&28: positive controls, lanes 29&30: negative control

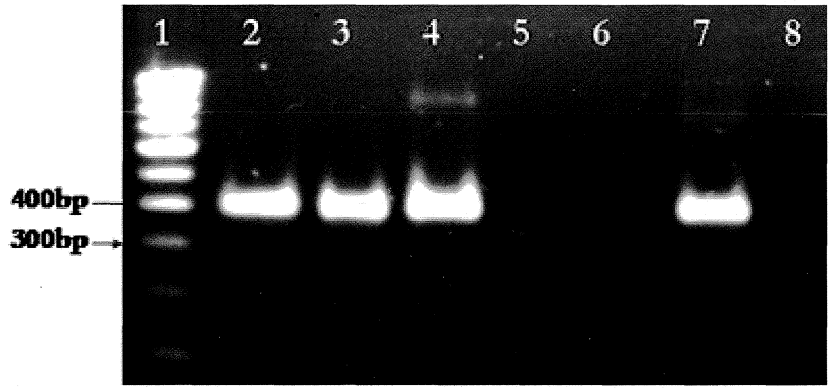


Figure 7: Gel image of *mtp40* standard PCR products: lane1: 100bp ladder, lanes 2-6: pleural fluid and lymph node specimen DNA, lane 7: positive control, lane 8: negative control

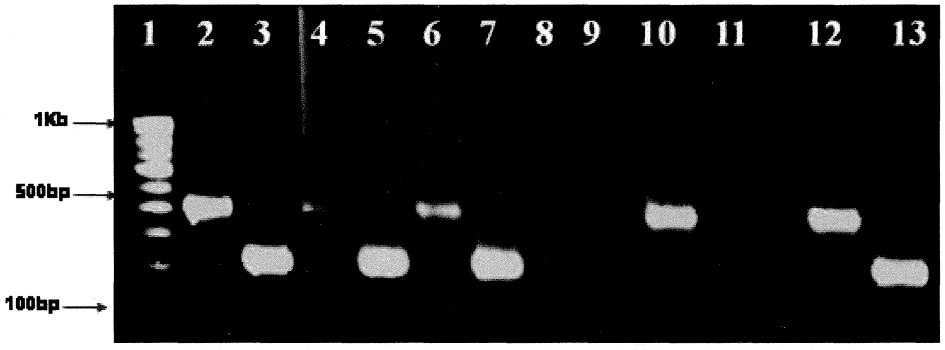


Figure 8: Gel image of nested PCR products: lane1: 100bp ladder, lanes 2, 4, 6, 8 & 10: first round products, lanes 3, 5, 7, 9 & 11: nested PCR products, lanes12&13: positive controls

IV.5.4. Results of *in situ* PCR

In situ PCR demonstrated the presence of mycobacterial DNA in the form of bluish black bodies within the tuberculous granulomatous lesions (Figure 9). However the assay was performed only in limited number of cases (4/13). Considerable technical difficulties were encountered during *in situ* PCR. The adhesions of tissues on to the slide were unsatisfactory and the sections got disintegrated during the procedure. In order to overcome this technical problem, slides were coated with poly-L-lysine, Histobond slides etc. Despite these, the results were not consistent and could not be reproduced. Hence this method was not evaluated further.

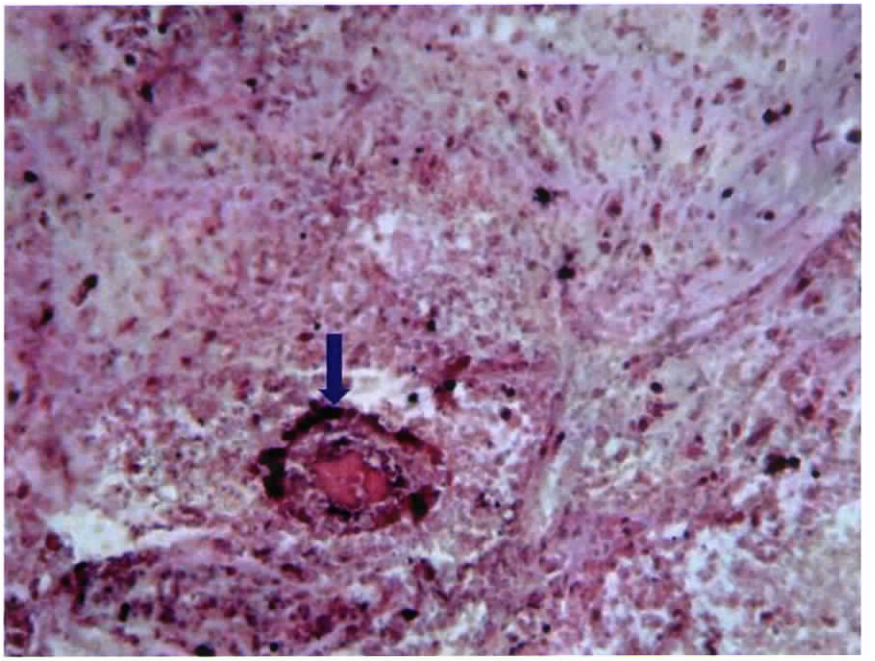
IV.6. CLONING OF *PLCA*, *HSPX*, *TB8.4* AND *ESAT-6* GENES

IV.6.1. PCR for amplifying *plcA*, *hspX*, *tb8.4* and *esat-6* genes

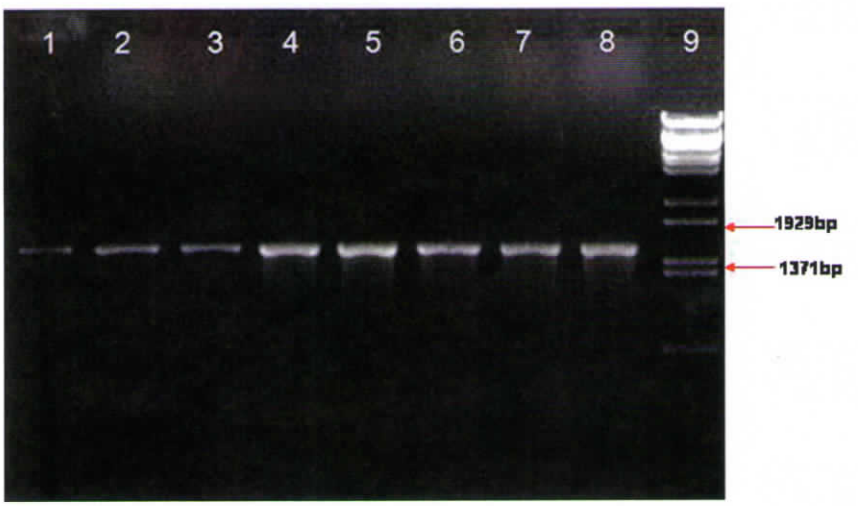
PCR was standardized with primers specific for *plcA (Rv2351c)*, *hspX (Rv2031c)*, *tb8.4 (Rv1174c)* and *esat-6* (*Rv3875)* genes. A gradient PCR was performed with temperatures ranging from 65˚C to 72˚C. Annealing temperature was selected at 65˚C for all the four genes.

IV.6.2. Standard PCR using *plcA*, *hspX*, *tb8.4* and *esat-6* in clinical samples

All the four mycobacterial genes- *plcA*, *hspX*, *tb8.4* and *esat-6* were found to be present in specimens from patients with pulmonary as well as extra-pulmonary tuberculosis. PCR was 100% sensitive in the DNA isolated from *M. tuberculosis* (cultured from sputum specimens from the patients with pulmonary tuberculosis). When PCR was performed with DNA isolated from extra-pulmonary specimens, there was a mild reduction in sensitivity. PCR amplifying *plcA* (Figure 10), *hspX* (Figure 11), *tb8.4* (Figure 12) and *esat-6* (Figure 13) gave a sensitivity of 88%, 92%, 90.5% and 93% respectively.



igure 9: Photomicrograph showing positive result in *in situ* PCR: mycobacterial NA in the form of bluish black bodies in and around tuberculous granuloma (X200)



igure 10: Gel image of amplified *plcA* from tuberculosis clinical specimens: ne 1-8: *plcA* amplified from tuberculous specimen, lane 9: lambda DNA-*BstE* II arker

IV.6.3. Cloning of each gene into pGEM®-T easy vector

Four genes were amplified from the *M. tuberculosis* genome with their respective primers and were subjected to agarose gel electrophoresis (Figures 14 & 15). Primers were designed with restriction sites for *Kpn*I and *Hind*III. Amplified genes were gel eluted. Each gene was ligated into pGEM®-T Easy Vector using the ligase enzyme. The ligated pGEM®-T Easy Vector-gene construct (recombinant plasmid) was used in transforming *E. coli* JM109 competent cells. Selection for transformants was performed on LB/ampicillin/ IPTG/X-Gal plates. The selection criteria were blue/white colour screening and ampicillin resistance. Colonies were checked for the presence of correct insert by performing a colony PCR with plasmid specific SP6 promoter and T7 terminator primers (Figure 16 &17). The colonies which were positive by this method were cultured in LB media overnight and recombinant plasmid was isolated in high yield, and they were confirmed for the presence of insert by standard PCR with plasmid specific SP6 promoter and T7 terminator primers. The size of the expected amplified product was calculated, by considering particular gene size and size of amplified region of original non-recombinant plasmid (Figure 18 &19). Recombinant plasmids were labelled for each gene construct and stored at -20°C. Positive cultures were also stored as glycerol stocks.

The expected PCR product size of each gene when present in different vectors and expected end-protein size is given in Table 2.

IV.6.4. Sequencing analysis

Sequencing of each pGEM®-T Easy Vector -gene construct was performed on ABI 310 Automated sequencer. Sequencing was based on Sanger's dideoxy chain termination method. Sequencing confirmed the correct reading frame. Chances of mutation incorporated during amplification, were ruled out.

Table 2: Expected PCR product size of genes in pGEMT-easy vector and in pET-32a and expected protein size

Genes	Gene size	Expected PCR (SP6 promoter & T7 terminator) product size	Expected PCR (T7 promoter& T7 terminator) product size	Expected protein size (in pET-32a)
esat-6 Rv3875	288bp	487bp	636bp	14.89 KDa
hspX Rv2031c	435bp	634bp	783bp	20.56 KDa
tb8.4 Rv1174c	333bp	532bp	681bp	16.56 KDa
plcA Rv2351c	1539bp	1738bp	887bp	61.22 KDa

Figure 14

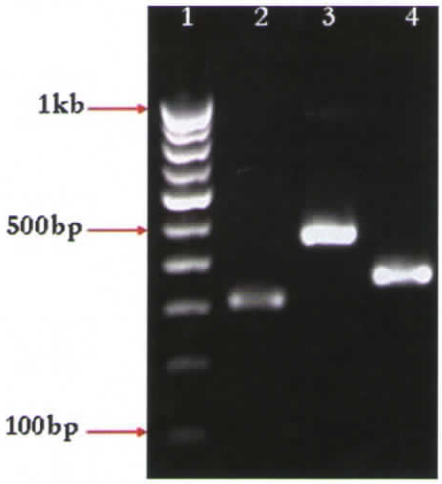


Figure 15

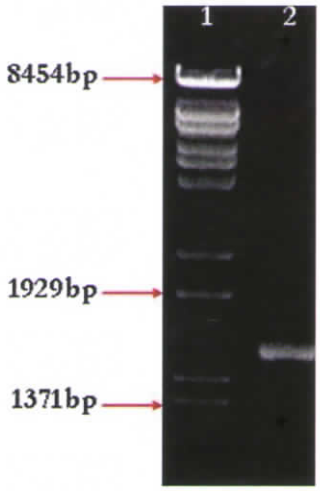


Figure 14: Amplification of *esat-6*, *hspX* and *tb8.4* (with *Kpn* I and *Hind* III sites) from *M. tuberculosis* H_{37}Rv: lane 1:100bp ladder, lane 2: amplified *esat-6,* lane 3: amplified *hspX*, lane 4: amplified *tb8.4*

Figure 15: Amplification of *plcA* (with *Kpn* I and *Hind* III sites) from *M. tuberculosis* H_{37}Rv: *lane1:* lambda DNA-*BstE* II marker, *lane 2:* amplifed *plcA*

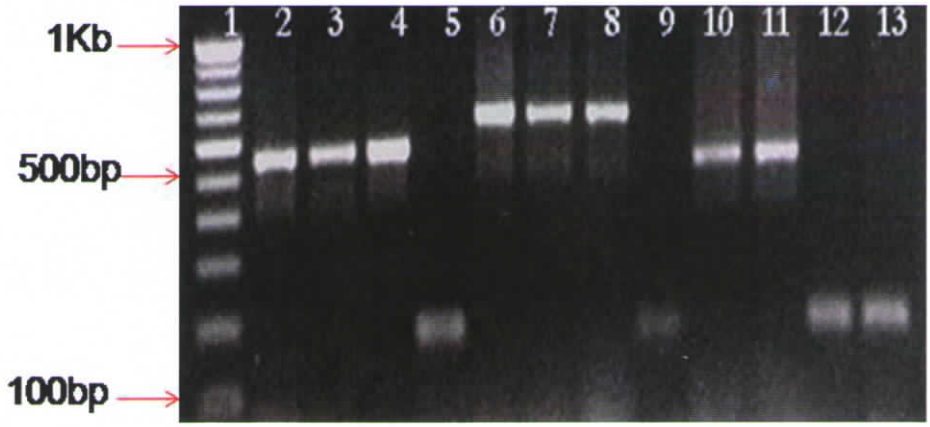


Figure 16: Colony PCR for selection of gene- *pGEMT easy* recombinant colonies: lane 1: 100bp ladder, lanes 2-5: *tb8.4* in pGEMT-easy, lanes 6- 9: *hspX* in pGEMT-easy, lanes 10-13: *esat-6* in pGEMT-easy

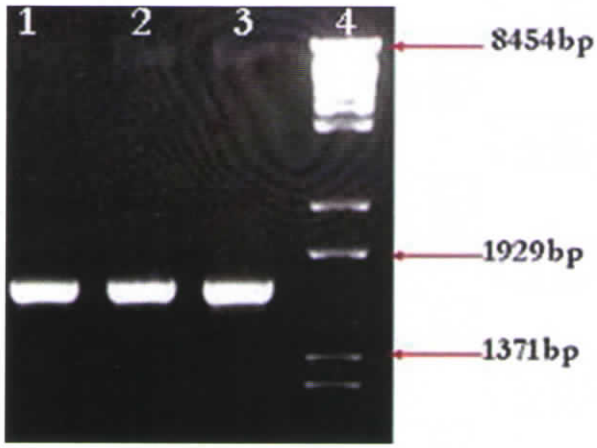


Figure 17: Colony PCR for selection of *plcA-pGEMT easy* transformed colonies: lanes 1-3: *plcA* in pGEMT-easy, lane 4: lambda DNA-*BstE* II marker

Figure 18

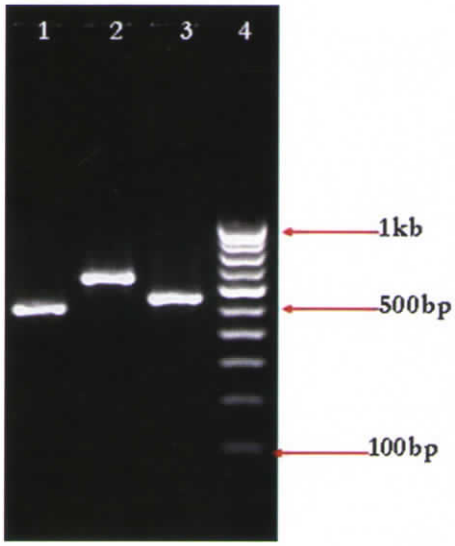


Figure 19

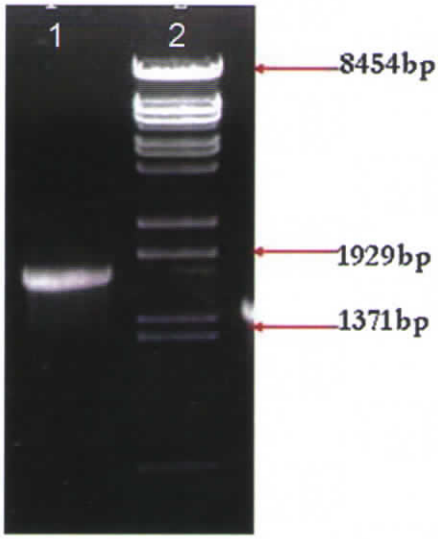


Figure 18: Amplification of gene-pGEM-T easy recombinant plasmid by SP6, T7 terminator primers: lane 1: *esat-6* in pGEMT-easy, lane 2: *hspX* in pGEMT-easy, lane 3: *tb8.4* in pGEMT-easy, lane 4: 100bp ladder.

Figure 19: Amplification of *plcA*-pGEM-T easy recombinant plasmid by SP6, T7 terminator primers: lane 1: *plcA* in pGEMT-easy, lane 2: lambda DNA-*BstE* II marker

IV.6.5. Modification of pET-32a expression vector

To reduce the end-protein size a 'thioredoxin' (trx) tag from pET-32a was excised out using a single restriction reaction with *NdeI* enzyme, followed by self-ligation with ligase. This construct was inserted into *E. coli* JM109 competent cells, and plated in LB agar containing 60µg/ml ampicillin. Positive colonies were cultured overnight in LB broth containing ampicillin, and recombinant plasmids were isolated. Removal of 'trx' tag from pET-32a by *NdeI* restriction was checked by T7 promoter and T7 terminator specific primers (Figure 20). Modified plasmid was compared with un-digested pET-32a by PCR.

IV.6.6. Gene cloning into modified pET-32a expression vector

Each gene with its restriction sites were excised out of its particular pGEM®-T easy construct by digestion with *Kpn*I and *Hind*III enzymes (Figure 21). pET-32a was also digested with *Kpn*I and *Hind*III enzymes (Figure 22). Excised gene with its restriction sites were inserted into *Kpn*I and *Hind*III sites of prokaryotic expression vector pET-32a. *E. coli* JM109 competent cells were transformed with these gene-pET-32a ligation products and the transformation mixture was plated on LB agar containing 60µg/ml ampicillin. Positive colonies were checked by colony PCR with T7 promoter and T7 terminator specific primers (Figure 23 & 24).

A single positive colony positive for each recombinant plasmid was cultured overnight in LB broth containing ampicillin, and recombinant plasmids were isolated, confirmed again by a standard PCR with T7 promoter and T7 terminator specific primers (Figure 25 &26).

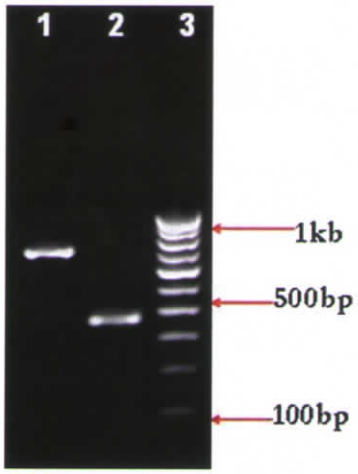


Figure 20: Removal of *trx* tag from pET-32a by *Nde* I restriction: lane 1: amplified pET-32a Trx +ve, lane 2: amplified pET-32a Trx -ve, lane 3: 100bp ladder

Figure 21

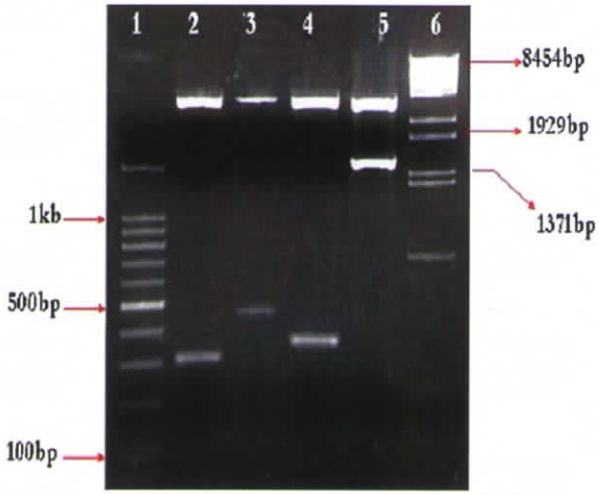


Figure 22

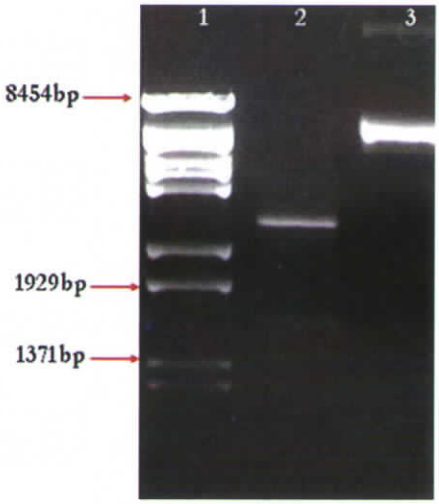


Figure 21: Release of genes from pGEMT easy recombinant vector by *Kpn*I and *HindIII* enzymes: lane 1: 100bp ladder, lane 2: *esat-6* release from pGEM-T easy, lane 3: *hspX* release from pGEM-T easy, lane 4: *tb8.4* release from pGEM-T easy, lane 5: *plcA* release from pGEM-T easy, lane 6: lambda DNA-*BstE* II marker.

Figure 22: Digestion of pET-32a vector by *Kpn*I and *Hind*III enzymes: lane 1: lambda DNA-*Bst*E II marker, lane 2: pET-32a (digested), lane 3: pET-32a (undigested)

Figure 23

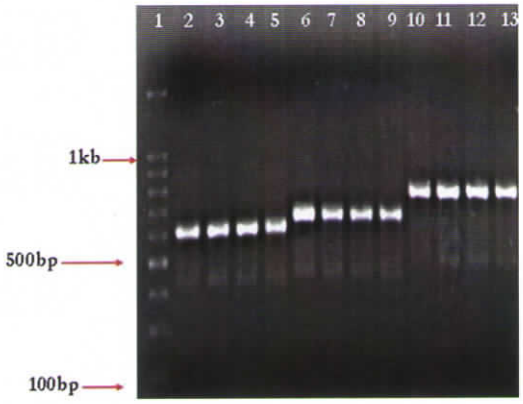


Figure 24

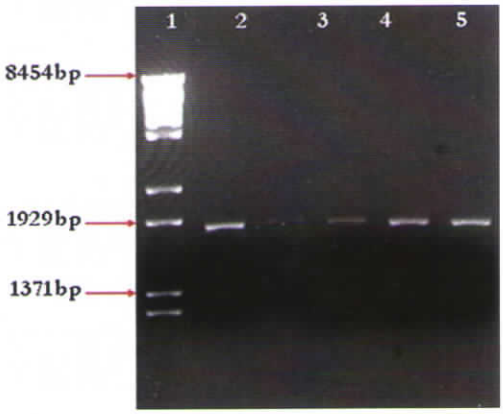


Figure 23: Colony PCR for selection of recombinant pET-32a transformed colonies: lane 1: 100bp ladder, lanes 2-5: *esat-6* in pET-32a, lanes 6- 9: *tb8.4* in pET-32a, lanes 10-13: *hspX* in pET-32a

Figure 24: Colony PCR for selection of *plcA*-pET-32a transformed colonies: lane 1: lambda DNA-*BstE* II marker, lanes 2-5: *plcA* in pET-32a

Figure 25

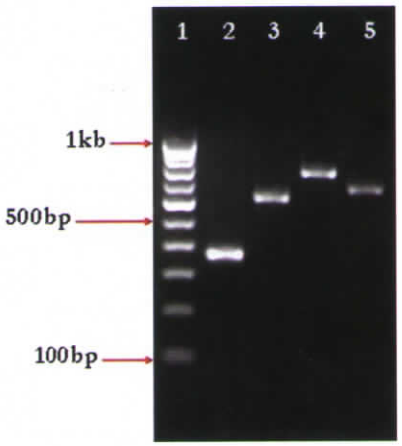


Figure 26

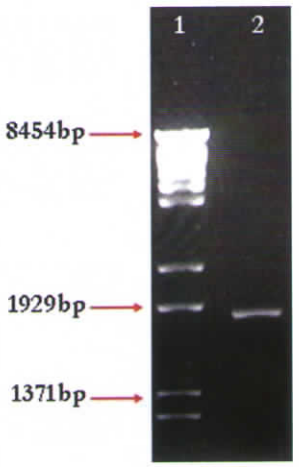


Figure 25: Amplification of gene-pET-32a plasmid by T7 promoter, T7 terminator primers: lane 1: 100bp ladder, lane 2: pET32a without insert, lane 3: *esat-6* in pET 32a, lane 4: *hspX* in pET 32a, lane 5: *tb8.4* in pET 32a

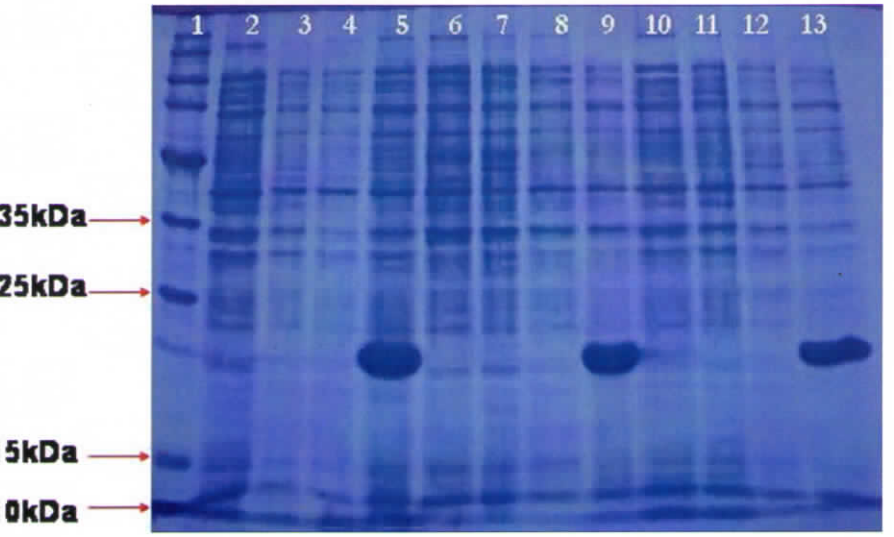
Figure 26: Amplification of *plcA*-pET-32a plasmid: lane 1: lambda DNA-*Bst*E II marker, lane 2: *plc A* in pET 32 a

IV.7. PROTEIN EXPRESSION

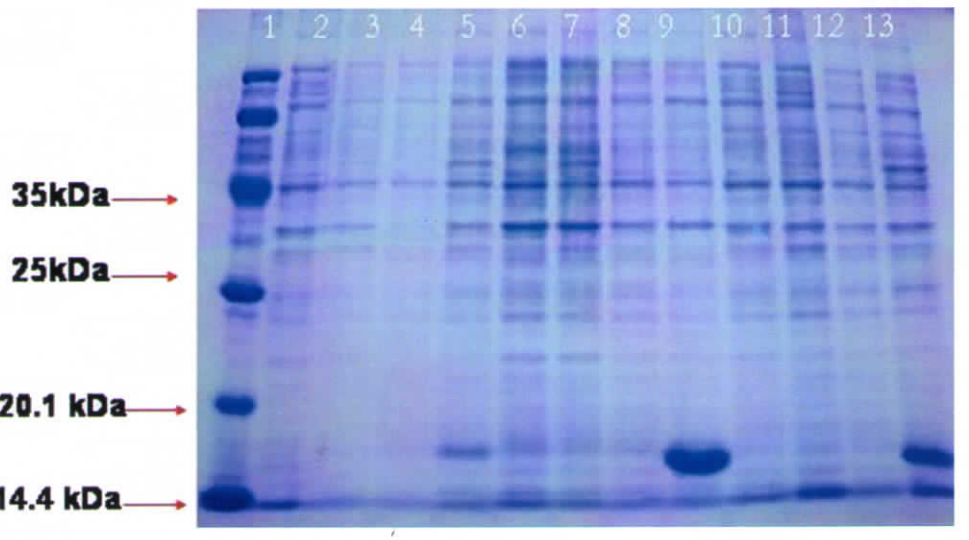
E. coli strains BL21 (DE3), BL21 (DE3) pLysS, JM 109(DE3*)* were utilized for protein over-expression. Gene-pET-32a recombinant plasmid was used for transformation. Selection was based on ampicillin resistance. Presence of inserts was confirmed by a colony PCR. The cultures were induced at 28ºC with 1mM IPTG for 4hrs. Protein expression was checked by SDS-PAGE followed by Coomassie blue gel staining.

Protein expressed was assessed by comparing the size with known protein markers. This migration was slightly higher than the molecular mass of the native molecule, which was expected because of the addition of the Histidine tag sequence and a thrombin site added to the recombinant protein to facilitate purification. HspX (Figure 27) was found over-expressed in all the three expression strains. Tb8.4 was found expressed in all three expression strains, but over-expression was present more in *E. coli* BL21 (DE3) pLysS (Figure 28). PlcA was over-expressed only in *E. coli* BL21 (DE3) pLysS (Figure 29).

ESAT-6 was not over-expressed (Figure 30) in any of the three strains while slight expression was present. Different induction temperatures and induction times were given, but an over-expression of ESAT-6 was not achieved. Hence another expression vector pET-28a was used for ESAT-6 expression.



gure 27: HspX protein expression on a 10% SDS-PAGE: lane 1: protein marker, ne 2: BL21(DE3), lane 3: pET-32a in BL21 (DE3), lane 4: uninduced, lane 5: duced, lane 6: BL21 (DE3) pLysS, lane 7: pET-32a in BL21 (DE3)pLysS, lane 8: induced, lane 9: induced, lane 10: JM 109(DE3) , lane 11: pET-32a in JM 109(DE3) ane 12: uninduced, lane 13: induced



gure 28: Tb8.4 protein expression on a 12% SDS-PAGE: lane 1: protein marker, ne 2: BL21(DE3), lane 3: pET-32a in BL21(DE3), lane 4: uninduced, lane 5: duced, lane 6: BL21(DE3)pLysS, lane 7: pET-32a in BL21(DE3)pLysS, lane 8: induced, lane 9: induced, lane 10: JM 109(DE3) , lane 11: pET-32a in JM 09(DE3) , lane 12: uninduced, lane 13: induced

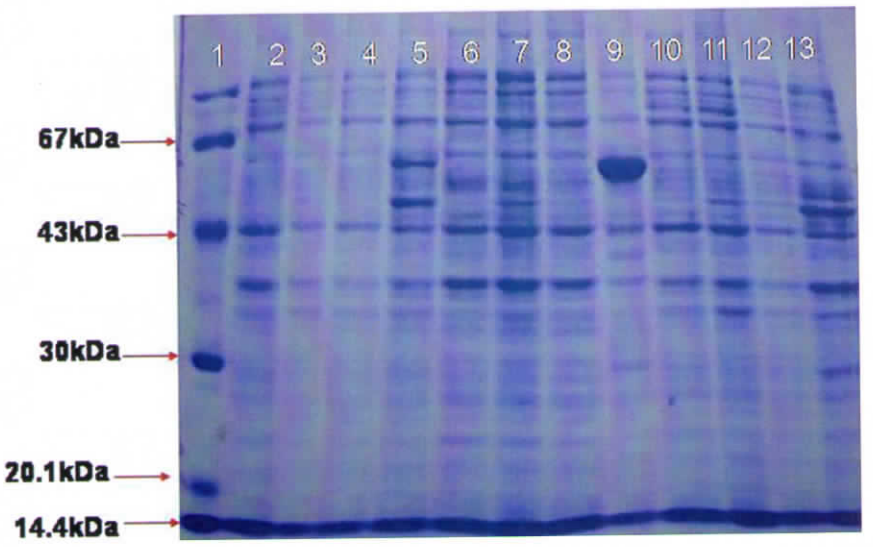


Figure 29: PlcA protein expression on a 10% SDS-PAGE: lane 1: protein marker, lane 2: BL21(DE3), lane 3: pET-32a in BL21(DE3), lane 4: uninduced, lane 5: induced, lane 6: BL21(DE3)pLysS, lane 7: pET-32a in BL21(DE3)pLysS, lane 8: uninduced, lane 9: induced, lane 10: JM 109(DE3) , lane 11: pET-32a in JM 109(DE3) , lane 12: uninduced, lane 13: induced

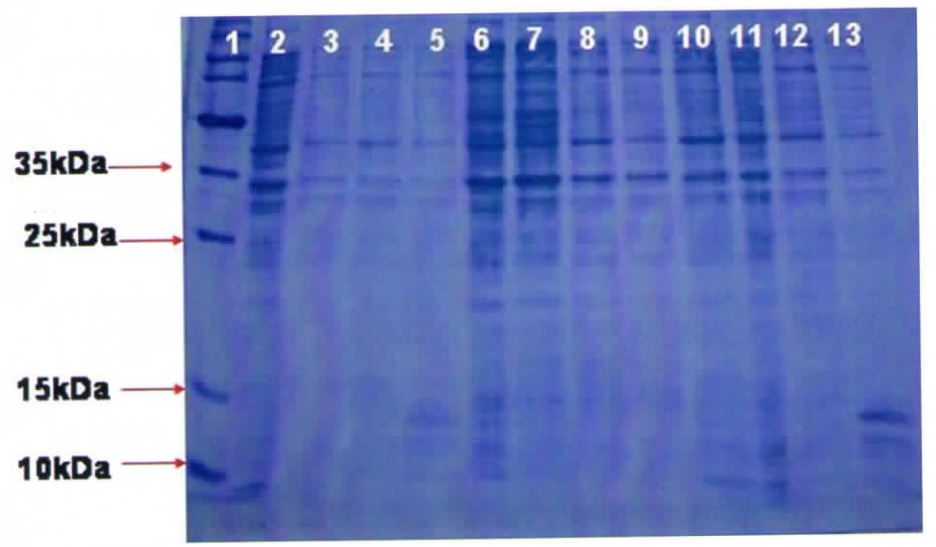


Figure 30: ESAT-6 protein expression on a 15% SDS-PAGE: lane 1: protein marker, lane 2: BL21(DE3), lane 3: pET-32a in BL21(DE3), lane 4: uninduced, lane 5: induced, lane 6: BL21(DE3)pLysS, lane 7: pET-32a in BL21(DE3)pLysS, lane 8: uninduced, lane 9: induced, lane 10: JM 109(DE3) , lane 11: pET-32a in JM 109(DE3) , lane 12: uninduced, lane 13: induced

IV.8. CLONING AND EXPRESSION OF *ESAT-6* IN pET-28A EXPRESSION VECTOR

The gene, *esat-6* (Rv3875) was amplified from *M. tuberculosis* H37Rv genome DNA. Primers were designed with restriction sites for *NdeI* and *Hind*III (Figure 31). PCR product was gel eluted and ligated into pGEM®-T Easy Vector and *E. coli* JM109 competent cells were transformed with this recombinant plasmid, and transformed colonies were checked by colony PCR (Figure 32). Plasmids were isolated in high yield from an overnight culture of the positive colony. Plasmid-gene construct was confirmed by a standard PCR using SP6 promoter and T7 terminator primers (Figure 33). Plasmid DNA sequencing was performed to check the open reading frame.

Gene *esat-6* with its restriction sites was restricted out of pGEM®-T easy recombinant vector by *Nde*I and *Hind*III enzymes (Figure 34) and inserted into *NdeI* and *Hind*III digested sites of prokaryotic expression vector pET-28a (Figure 35) by ligation. This recombinant plasmid was used in transforming *E. coli* JM109 competent cells. Selected was based on kanamycin resistance. Positive colonies were checked by colony PCR with T7 promoter and T7 terminator specific primers (Figure 36). Recombinant plasmids were isolated in high yields, again confirmed by standard PCR with T7 promoter and T7 terminator specific primers (Figure 37). This plasmid was transferred to *E. coli* expression host strains BL21 (DE3), BL21 (DE3) pLysS, JM 109(DE3). ESAT-6 was expressed in all the three strains. Protein expression was confirmed by a 15% SDS-PAGE analysis (Figure 38). The resultant band was slightly higher than the molecular mass of the native molecule, due the addition of the Histidine tag sequence and a thrombin site added to the protein to facilitate purification. The expected PCR product size of each gene when present in different vectors and expected end-protein size is given in Table 3.

Table 3: Expected PCR product size of *esat-6* in pGEMT-easy vector, and in pET-28a and expected protein size

Genes	Gene size	Expected PCR (SP6 promoter & T7 terminator) product size	Expected PCR (T7 promoter & T7 terminator) product size	Expected protein size (in pET-28a)
esat-6 Rv3875	288bp	487bp	548bp	12.65KDa

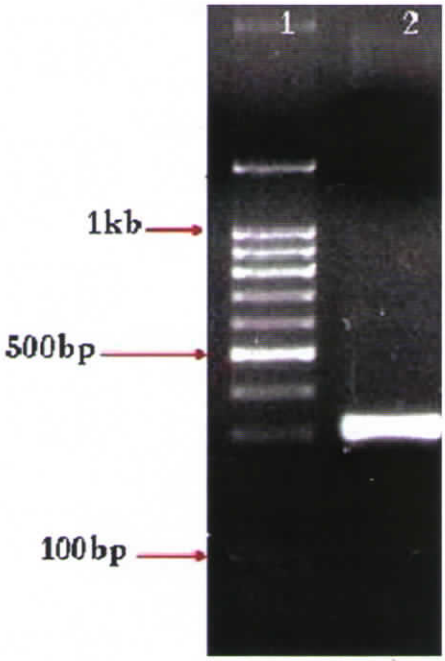


Figure 31: Gel picture showing *esat-6* (with *Nde*I and *Hind*III sites) amplification: lane 1: 100bp ladder, lane 2: amplified *esat-6*.

Figure 32

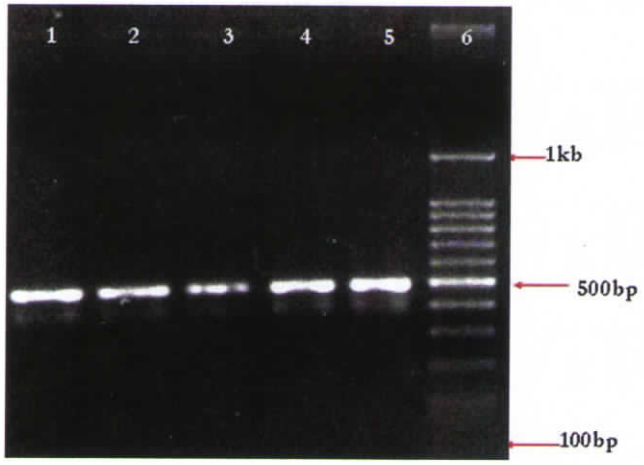


Figure 33

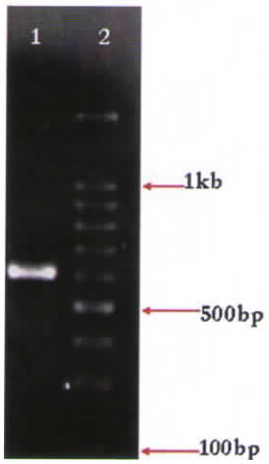


Figure 32: Colony PCR for selection of *esat-6*-pGEM-T *easy* transformed colonies: lanes 1-5: *esat-6* in pGEMT-easy, lane 6: 100bp ladder

Figure 33: Amplification of *esat-6*-pGEM-T easy plasmid by SP6, T7 terminator primers: lane 1: *esat-6* in pGEMT-easy, lane 2: 100bp ladder

Figure 34

Figure 35

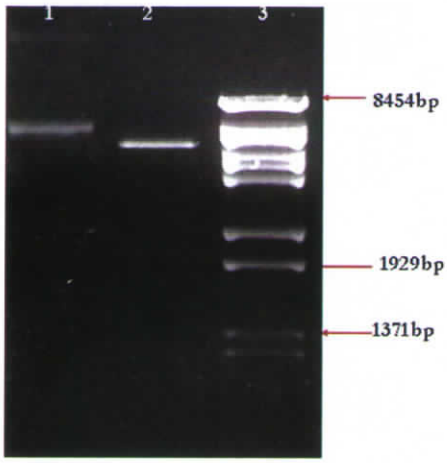


Figure 34: Release of *esat-6* from pGEM-T easy vector construct by *Nde*I and *Hind*III digestion: lane 1: 100bp ladder, lane 2: *esat-6* release from pGEM-T easy

Figure 35: Digestion of pET-28a vector by *Nde*I and *Hind*III enzymes: lane 1: pET-28a (undigested), lane 2: pET-28a (digested), lane 3: lambda DNA-*Bst*E II marker

Figure 36

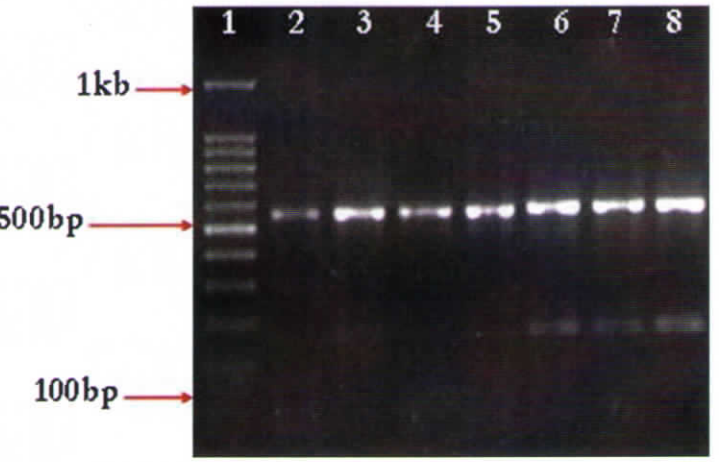
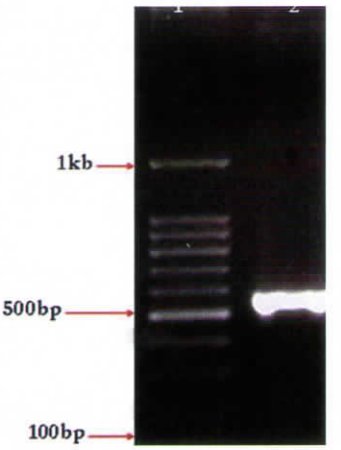
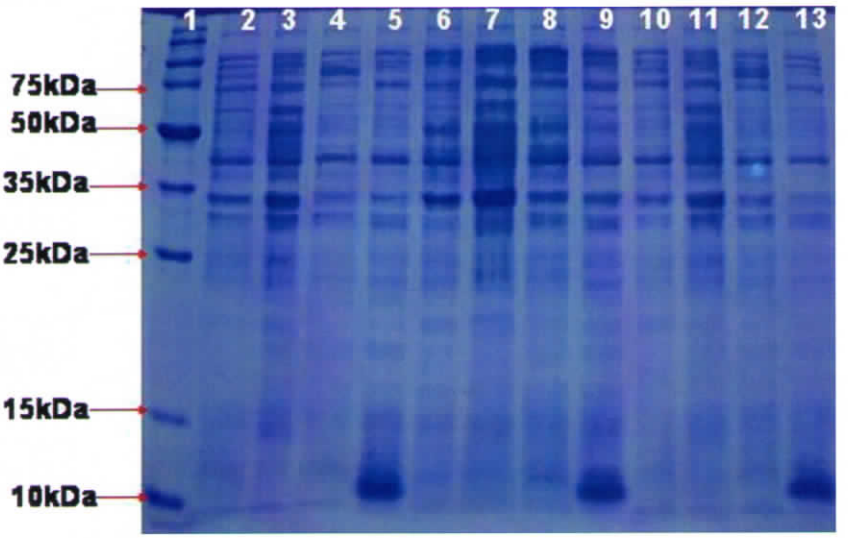


Figure 37



igure 36: Colony PCR for selection of *esat-6*-pET-28a transformed colonies: ne 1: 100bp ladder, lanes 2-8: *esat-6* in pET-28a

igure 37: Amplification of *esat-6*-pET-28a plasmid: lane 1: 100bp ladder, lane 2: *sat-6* in pET-28a



igure 38: ESAT-6 protein expression on 15% SDS-PAGE: lane 1: protein marker, ne 2: BL21(DE3), lane 3: pET-28a in BL21(DE3), lane 4: uninduced, lane 5: duced, lane 6: BL21(DE3)pLysS, lane 7: pET-28a in BL21(DE3)pLysS, lane 8: ninduced, lane 9: induced, lane 10: JM109(DE3) , lane 11: pET-28a in JM109(DE3), ne 12: uninduced, lane 13: induced

IV.9. PURIFICATION OF RECOMBINANT PROTEINS

E. coli BL21(DE3)pLysS expression strain was selected as all the four proteins were expressed in this strain. For obtaining high yield of recombinant proteins, induction experiments were performed in 100ml media, and following induction with IPTG, these cultures were incubated for 4 hours at 28°C. Cells were harvested and lysed. SDS-PAGE was performed to check the location of the expressed protein, i.e., whether they are present in the soluble or in the insoluble fraction. Proteins, PlcA, ESAT-6, and Tb8.4 were seen solely as inclusion bodies, while HspX was found in soluble fraction. Using Ni-NTA column chromatography, these proteins were purified to near-homogeneity. The proteins, which were present in as inclusion bodies, were purified using denaturing conditions, which involved the use of urea. HspX was at first purified under native conditions and eluted based on its competitive power with Imidazole to bind with Ni-NTA, but very low yield of purified HspX was obtained through native purification. Hence another fraction of crude proteins containing HspX was purified under denaturing conditions as well. Purification was checked by SDS-PAGE (Figures 39a, 40a, 41a and 42a) and His-tagged proteins were confirmed by probing blots containing these proteins using monoclonal anti- His antibody (Figures 39b, 40b, 41b and 42b).

IV.9.1. Desalting of denatured proteins

PD10 columns were used for desalting the protein solutions. Desalting through PD10 columns resulted in high dilutions of protein solution and hence dialysis in urea at decreasing concentration was used to desalt and renature the denatured proteins.

IV.10. PROTEIN QUANTIFICATION

Proteins were found to be expressed in following quantities:

PlcA- 69mg/L, ESAT-6- 370mg/L, HspX- 284mg/L, Tb8.4 - 100mg/L

Figure 39a

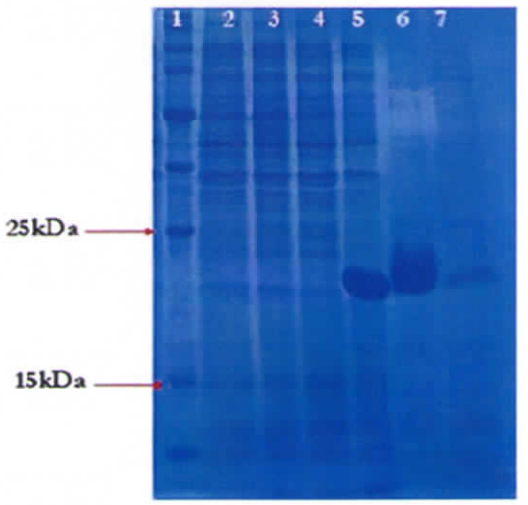


Figure 39b

Figure 39a: SDS-PAGE showing His-tagged HspX purification: Lane (1): protein marker, (2):*E. coli* BL21(DE3) pLysS, (3):pET-32a in BL21(DE3) pLysS, (4):uninduced, (5): induced, (6): HspX purified (denatured), (7) HspX purified (native).

Figure 39b: Immunoblot analysis of His-tagged HspX

Figure 40 a

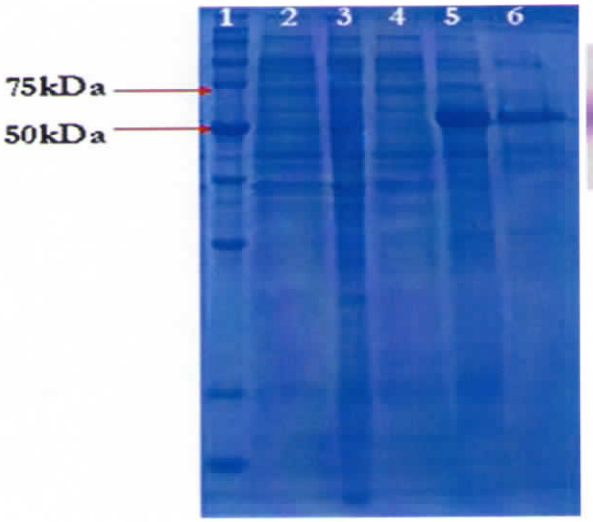


Figure 40 b

Figure 40a: SDS-PAGE showing His-tagged PlcA purification: Lane (1): protein marker, (2): *E. coli* BL21(DE3)pLysS, (3):pET-32a in BL21(DE3) pLysS, (4):uninduced, (5): induced, (6): purified PlcA

Figure 40b: Immunoblot analysis of His-tagged PlcA

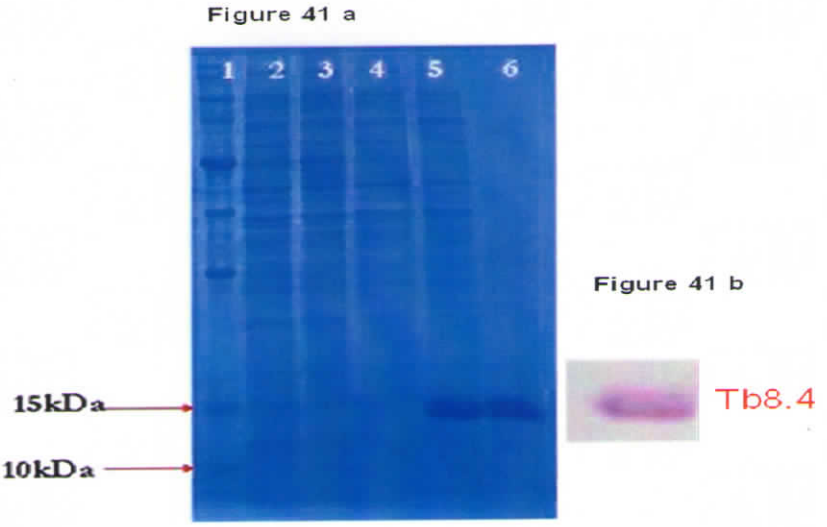


Figure 41a: SDS-PAGE showing His-tagged Tb8.4 purification: Lane (1): protein marker, (2): *E. coli* BL21(DE3)pLysS, (3):pET-32a in BL21(DE3) pLysS, (4):uninduced, (5): induced, (6): purified Tb8.4

Figure 41b: Immunoblot analysis of His-tagged Tb8.4

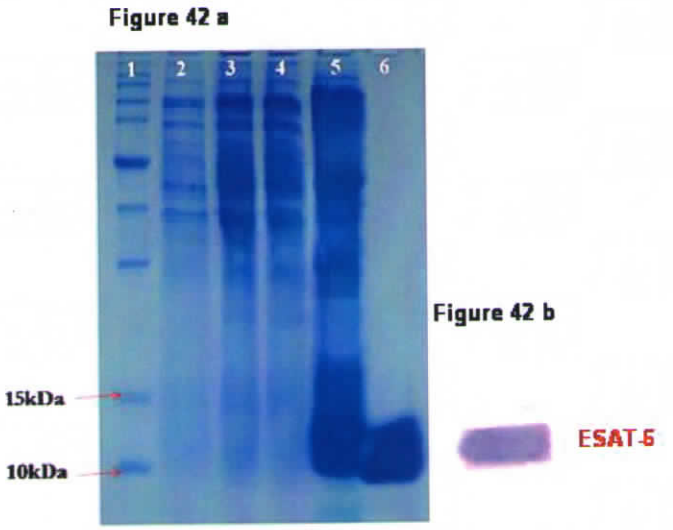


Figure 42a: SDS-PAGE showing His-tagged ESAT-6 purification: Lane (1): protein marker, (2):*E. coli* BL21(DE3) pLysS, (3):pET-32a in BL21(DE3) pLysS, (4): uninduced, (5): induced, (6): purified ESAT-6.

Figure 42b: Immunoblot analysis of His-tagged ESAT-6

IV.11. HUMORAL IMMUNE RESPONSES AGAINST PURIFIED ANTIGENS IN CLINICAL SPECIMENS

IV.11.1. Detection of antibody to mycobacterial PlcA, HspX, Tb8.4, ESAT-6 antigens and multiantigen cocktail in sera samples

Standardization of ELISA with PlcA, HspX, Tb8.4 and ESAT-6 antigens showed that 500pg/ml antigen concentration gave the 'best' discrimination between controls and tuberculosis patient at 1:5000 dilution of sera samples. Hence these concentrations were used in this study.

As a result of diverse antibody response in TB patients to individual antigens, it became necessary to combine several immunoreactive mycobacterial antigens in this ELISA to enhance sensitivity and specificity. Hence an ELISA was performed with all four recombinant antigens were mixed in equal concentration (200pg/ml).

In tuberculosis patients, the mean absorbance value of IgG was found to be significantly elevated ($P<0.001$) than healthy control and diseased control sera. Diseased control group had a higher mean antibody titer than healthy control but the difference was not statistically significant ($P>0.05$).

The mean absorbance values of anti- PlcA, HspX, Tb8.4, ESAT-6 and multiantigen antibody titre in each group are summarized in Table 4. The 'cut-off' value separating the tuberculosis from the diseased control group by ELISA was determined using the following formula: mean absorbance of disease controls + 2 SDs. A test was found positive when the absorbance is greater than 0.637, 0.811, 0.797, 0.882 and 0.876 for PlcA, HspX, Tb8.4, ESAT-6 and multiantigen complex respectively.

Based on this criterion, the sensitivity of PlcA, HspX, Tb8.4, ESAT-6 and multiantigen cocktail were 41.7%, 63.3%, 45%, 53.3% and 76.7%. A higher sensitivity in ELISA was obtained when a multiantigen cocktail consisting of all the four antigens. Not a single false positive results were obtained in BCG vaccinated healthy individuals. Few positive results were obtained in non-TB pulmonary disease control group. The specificity of PlcA, HspX, Tb8.4, ESAT-6 and multiantigen cocktail in non-tuberculous pulmonary disease control patients were 95.7%, 93.5%, 94.6%, 93.5% and 94.6%.

Table 4: Mean IgG titre in tuberculous and control sera specimens

Antigens	Tuberculosis (mean±SD) (n=60)	Diseased control (mean±SD) (n=92)	Healthy control (mean±SD) (n=42)
PlcA	0.722±0.38	0.431±0.103	0.284±0.099
HspX	0.883±0.461	0.399±0.206	0.278±0.106
Tb8.4	0.811±0.392	0.463±0.167	0.279±0.112
ESAT-6	0.868±0.291	0.488±0.197	0.361±0.176
Multiantigen	0.915±0.52	0.522±0.177	0.357±0.098

IV.11.2. Detection of antibody to mycobacterial PlcA, HspX, Tb8.4, ESAT-6 antigens and multiantigen cocktail in pleural fluid samples

The mean absorbance and standard deviation in tuberculous and malignant pleural effusion with individual recombinant mycobacterial antigens and their multiantigen cocktail is given in Table 5. The 'cut-off' value separating the PTB from the diseased control group by ELISA was determined using the following formula: mean absorbance of disease controls $\pm$ 2 SDs. A test was found positive when the absorbance is greater than 0.513, 0.604, 0.506, 0.521 and 0.649 for PlcA, HspX, Tb8.4, ESAT-6 and multiantigen complex respectively. Based on this criterion, IgG positivity for PlcA, HspX, Tb8.4, ESAT-6 antigens and multiantigen complex were 49.3%, 60.8%, 49.3%, 53.6% and 75.4% respectively. Antibody titre in culture positive plural fluids was significantly higher than culture negative patients with PTB ($P < 0.05$). Sensitivity of the assay using multi-antigen complex was higher than individual antigens. The differences between the multi-antigen complex and PlcA, Tb8.4, HspX and ESAT-6 were significant ($P < 0.01$). All the four antigens and multiantigen gave positive results in three pleural fluids with malignant pleural effusion.

Table 5: Mean IgG titre in tuberculous and malignant pleural effusions

Antigens	Control group (n=71)	Tuberculosis test group (n=69)
PlcA	0.247±0.133	0.598±0.145
HspX	0.36±0.122	0.615±0.187
Tb8.4	0.38±0.098	0.567±0.089
ESAT-6	0.281±0.12	0.653±0.143
Multi-antigen cocktail	0.289±0.18	0.798±0.173

IV.11.3. Immunohistochemical demonstration of specific mycobacterial antigens

PlcA, HspX, Tb8.4, ESAT-6, TBGL and CFA were used in the production of antibodies in rabbits. These antibodies were used in immunodetection of mycobacterial antigens in infected lymph nodes.

Immunostaining using the six antibodies gave positive results in all the 'confirmed' TBL cases. Mycobacterial antigens were demonstrated within the cytoplasm of several macrophages and Langhans' giant cells in the tuberculous lesion. They appeared as granular brownish material. Negative immunostaining was obtained when an IHC with normal rabbit serum was performed on sections of lymph nodes from 'confirmed' tuberculosis (Figure 43 & 44).

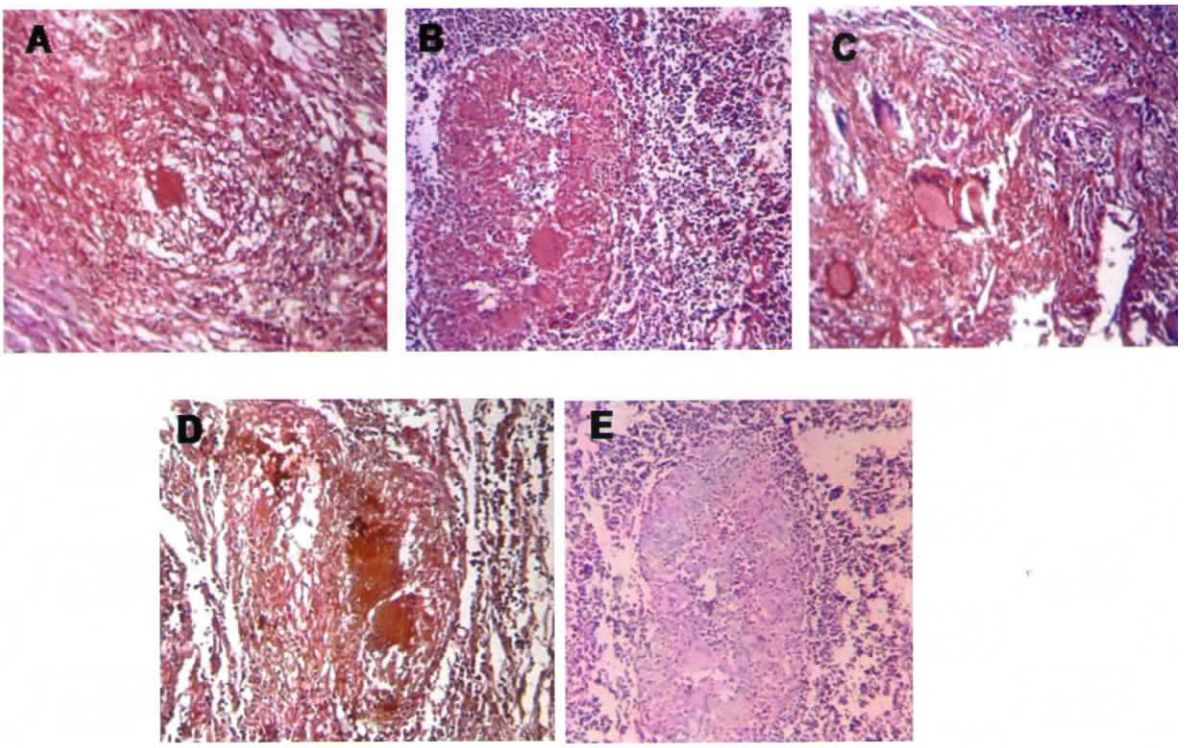
A polyclonal antibody to ESAT-6 was found to be highly reactive when compared to other antibodies. Anti-ESAT-6 antibody detected 27 out of 31 'probable' TBL cases. Anti- HspX, anti-Tb8.4, anti-TBGL and anti-CFA antibodies gave positive results with IHC in disease control group. False positive results were not obtained

with anti-ESAT-6 and anti-PlcA antibodies. The results of the study in terms of sensitivity and specificity are given in Table 6.

Table 6: Results of IHC using antibodies against six mycobacterial antigens

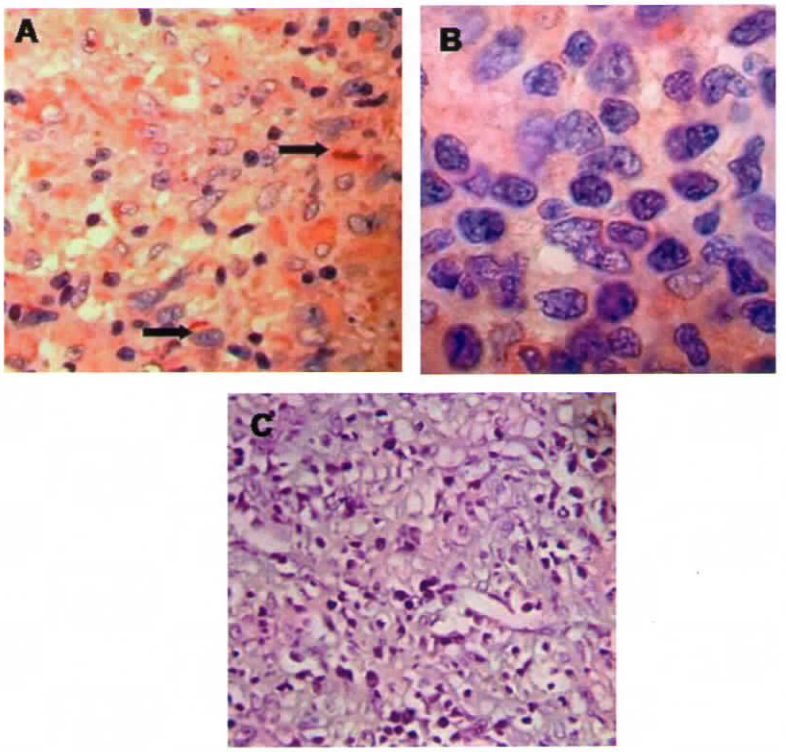
Antibodies	Sensitivity (%)			Specificity (%)
	Confirmed TBL (n=4)	Probable TBL (n=31)	Total TBL (n=35)	Disease control (n=36)
Anti-ESAT-6	100	87.1	88.6	100
Anti-PlcA	100	64.5	68.6	100
Anti-HspX	100	74.2	77.1	91.7
Anti-Tb8.4	100	67.7	71.4	94.4
Anti-TBGL	100	61.3	65.7	86.1
Anti-CFA	100	54.8	60	86.1

Figure 43: Photomicrographs showing immunohistochemical staining in formalin-fixed lymph node sections of TBL with anti-ESAT-6, anti-HspX, anti-Tb8.4 and anti-PlcA antibodies.



Photomicrographs showing Immunohistochemical staining with (A) anti- ESAT-6, (B) anti-HspX, (C) anti-Tb8.4, (D) anti-PlcA antibodies: The cytoplasm of macrophages and Langhan's giant cells are positive for mycobacterial antigens in a tuberculous granuloma (ABC X200). **(E) Photomicrograph showing IHC using normal rabbit serum:** The cytoplasm of macrophages and Langhan's giant cells are devoid of immunostaining for mycobacterial antigens in a tuberculous granuloma (ABC X200).

gure 44: Photomicrographs showing immunohistochemical staining in rmalin-fixed lymph node sections of TBL with anti-TBGL and anti-CFA ntibodies



notomicrograph showing IHC with (A) anti-TBGL antibody: Immunostaining owing intra-cytoplasmic rod-like structure in a tuberculous granuloma (ABC X200), **anti-CFA antibody:** The cytoplasm of macrophages showing light mmunostaining for mycobacterial antigens in a tuberculous granuloma (ABC X300),) **normal rabbit serum:** The cytoplasm of macrophages devoid of immunostaining r mycobacterial antigens in a tuberculous granuloma (ABC X200).

IV.12. CELL-MEDIATED IMMUNE RESPONSES AGAINST PURIFIED ANTIGENS

IV.12.1. Human PBMC culture and protein stimulation

PBMCs from patients with active pulmonary tuberculosis, house-hold contacts and BCG vaccinated individuals were isolated, cultured in RPMI cell culture medium. These PBMCs were allowed to adhere to wells of tissue culture plates for three hours at 37°C at 5% CO_2 (Figure 45 & 46).

To assess cytokine release, PBMCs from above groups of individuals were incubated for 96 hours with PlcA, HspX, Tb8.4 and ESAT-6 proteins and commercial PPD. Bases on dose-response standardisation, 5µg/ml was chosen as working concentration for recombinant antigens. PPD was used at 3µg/ml final concentration. For positive controls, PBMC were cultured in the presence of Concanavalin A (10µg/ml). Cultures incubated with medium alone served as negative controls.

IV.12.2. MTT assay

Results were analysed in stimulation index units. Stimulation index was the ratio of absorbance at 570nm of culture incubated in the presence of antigen to absorbance at 570 nm of culture incubated in the absence of antigen. Distinction based on MTT assay was more connected with status of pre-sensitization. TST positive cases irrespective of categories had an increased proliferation. Healthy house-old contacts of TB showed maximum proliferation against mycobacterial antigens, than BCG vaccinated healthy individuals. Proliferation responses of the PBMCs, to *M. tuberculosis* PPD and individual recombinant mycobacterial antigens are given in Figures 47.

Figure 45: Photomicrograph showing PBMCs after 2 hours (10X objective)

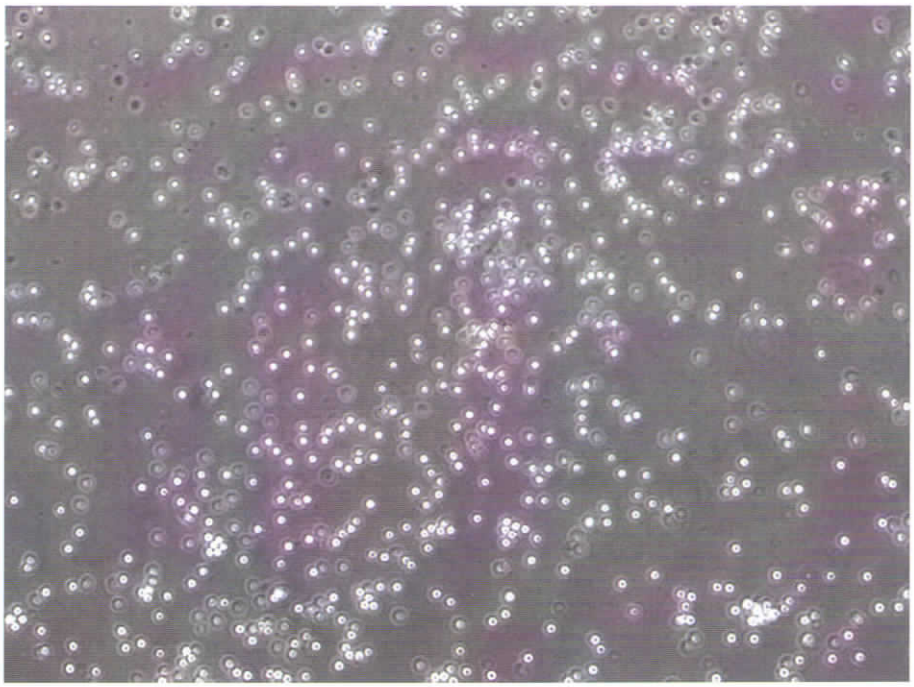


Figure 46: Photomicrograph showing PBMCs after 3 days (10X objective)

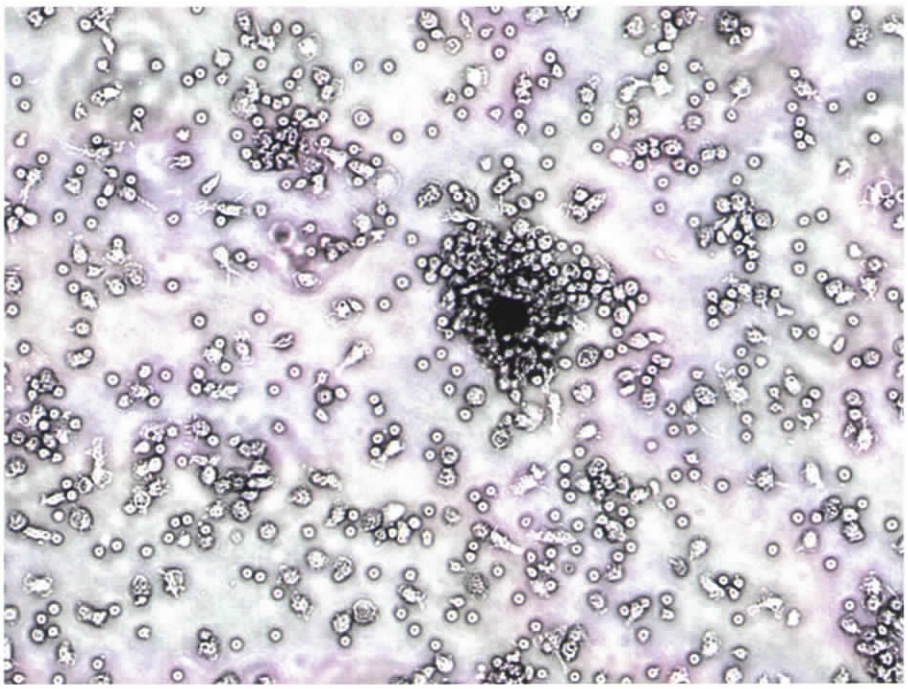
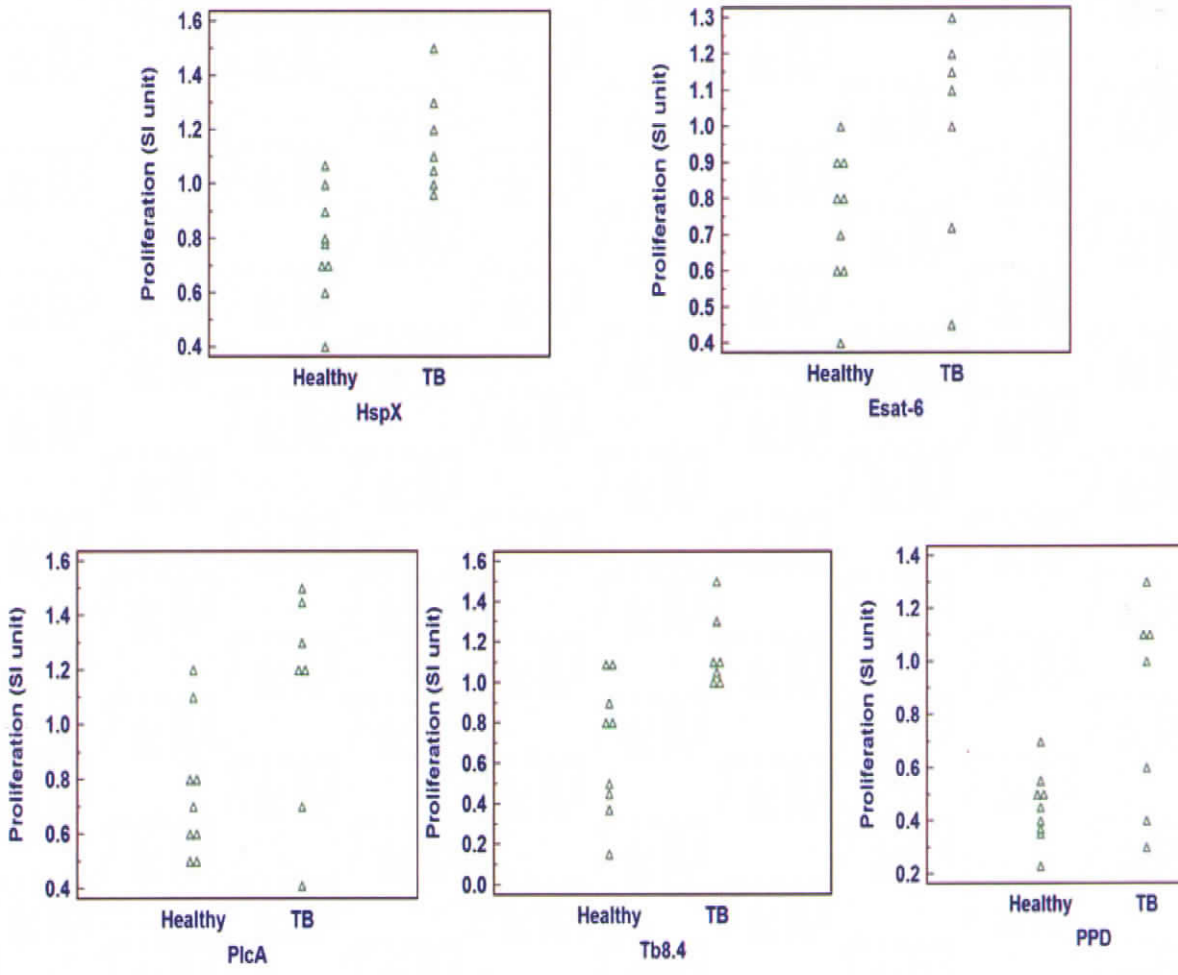


Figure 47: Dot-plot showing proliferative responses of human peripheral blood mononuclear cells (PBMC) in response to commercial PPD, PlcA, HspX, Tb8.4 and ESAT-6 antigens.



IV.12.3. Cytokine release assay

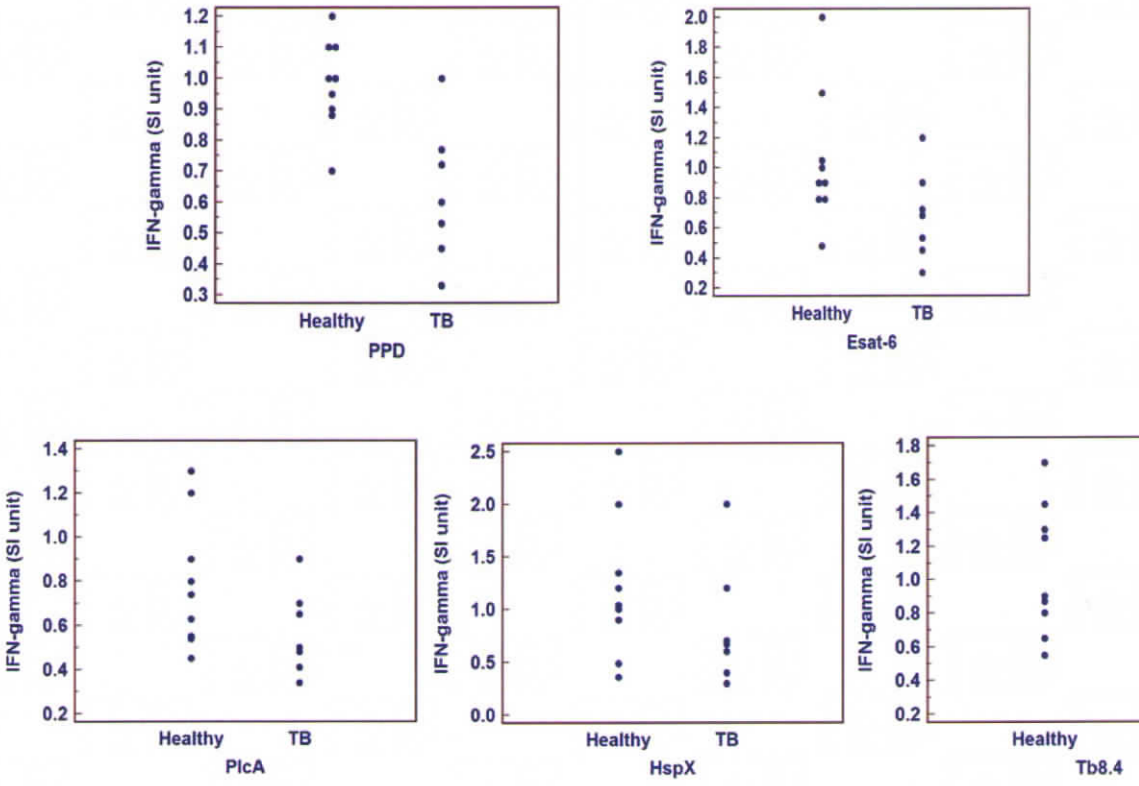
Levels of IFN-γ, TNF, IL-4 and IL-10 in supernatants from stimulated and un-stimulated cultures were determined by sandwich ELISA with commercial kits (BD Biosciences). Samples were assayed in triplicates and results were expressed as the mean value of the three readings in an ELISA reader at 450 nm. Cytokines were quantified with reference to standard curves generated using human recombinant cytokines (issued by manufacturer). The sensitivity of the assays was 1 pg/ml for IFN-γ, while 2pg/ml for TNF- α, IL-4 and IL-10.

Cytokines were expressed in picograms/ml. Cytokine values were highly variable in same individuals of the same study group. Also cytokines released in negative controls varied in individuals. For a proper comparison, cytokine analysis was performed in stimulation index (SI) units. Stimulation index was the ratio of cytokine released by culture incubated in the presence of protein to cytokine released in culture incubated in the absence of protein.

IV. 12.3.1. IFN- γ release assay

PBMC from all subjects elicited IFN-γ in response to all the four recombinant mycobacterial antigens and commercial PPD. IFN-γ released in response to recombinant mycobacterial antigens was found reduced in active TB individuals than in healthy donors. TB patients undergoing treatment showed an elevated IFN-γ response. Healthy house-hold contacts produced more IFN-γ than BCG vaccinated healthy donors. Analysis of cytokine released in response to different antigens in different study subjects was done using SI units (Figures 48). PPD based IFN-γ release was significantly more in TST positive individuals than TST negative individuals ($P < 0.05$).

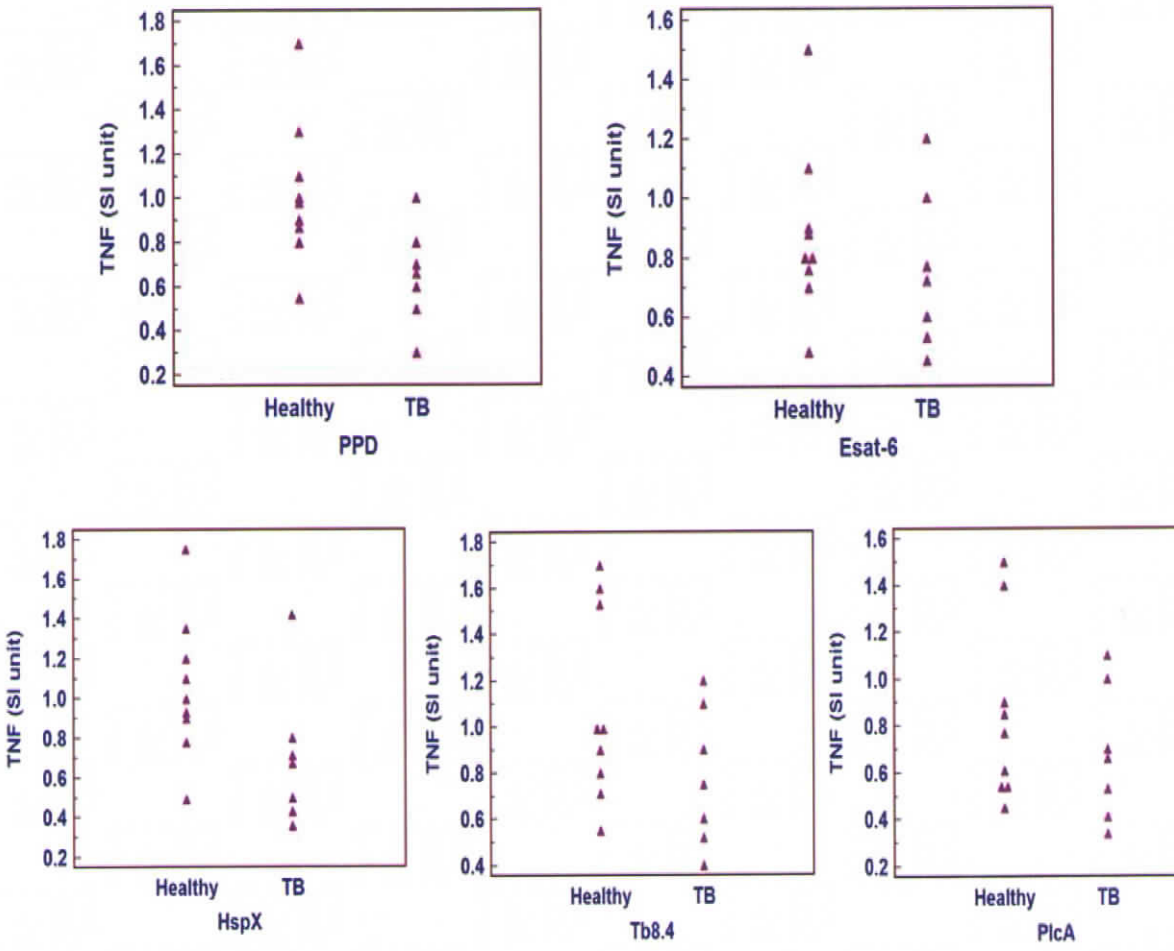
Figure 48: Dot-plot showing gamma interferon release from PBMC in response to commercial PPD, PlcA, HspX, Tb8.4 and ESAT-6 antigens.



IV. 12.3.2. TNF release assay

PBMC from all subjects elicited TNF in response to all the four recombinant mycobacterial antigens and commercial PPD. TNF released in response to recombinant mycobacterial antigens was found reduced in active TB individuals than in healthy donors. TB patients undergoing treatment showed an elevated TNF response. Healthy house-hold contacts produced more TNF than BCG vaccinated healthy donors. Analysis of cytokine released in response to different antigens in different study subjects was done using SI units (Figures 49). PPD based TNF release was more in TST positive individuals than TST negative individuals. The difference was insignificant statistically ($P > 0.05$). Difference between TNF release in response to PPD, in healthy and house-hold contacts was also not significant ($P > 0.05$).

Figure 49: Dot-plot showing Tumor necrosis factor release from PBMC in response to commercial PPD, PlcA, HspX, Tb8.4 and ESAT-6 antigens.



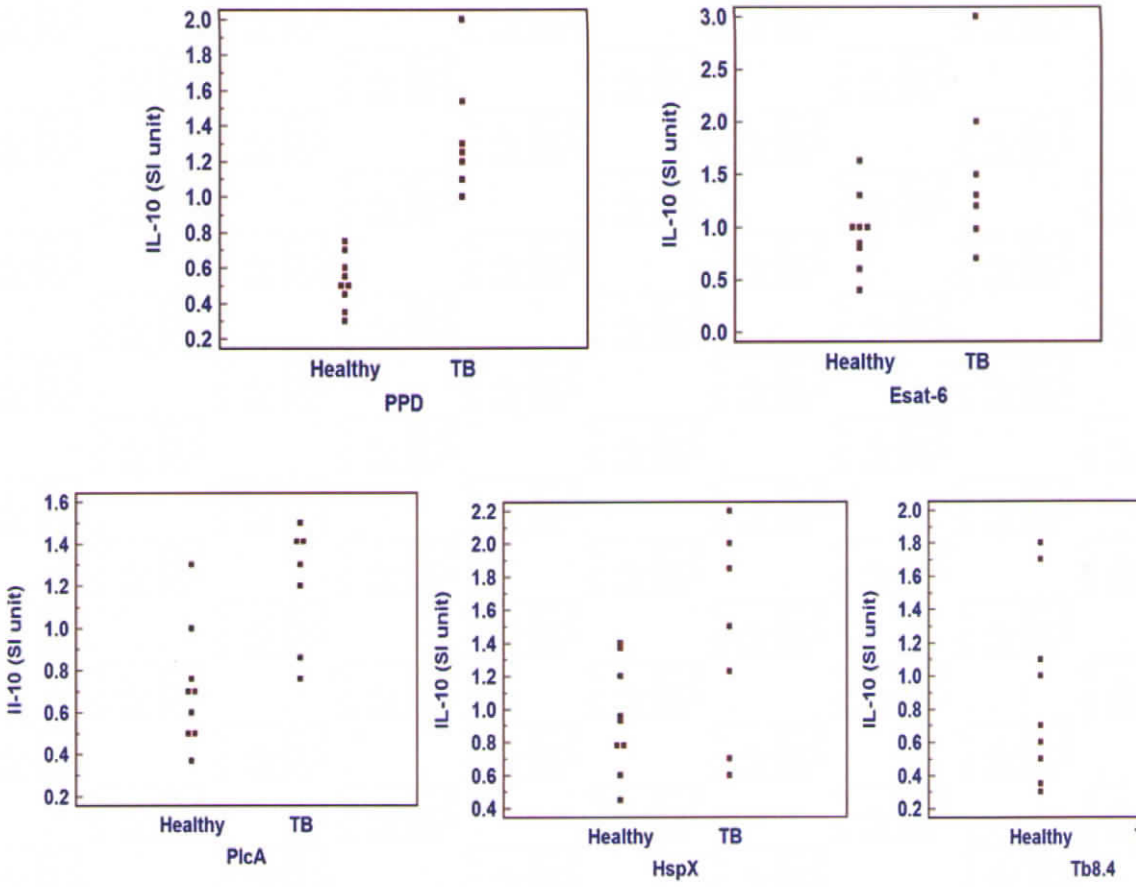
IV. 12.3.3. IL-10 release assay

IL-10 was elicited in response to all the four antigens and PPD. IL-10 was found increased in active TB individuals, TST positive patients in particular. Tuberculosis patients undergoing treatment showed a depression in IL-10 response unlike IFN- γ and TNF. On stimulation with mycobacterial antigens, house-hold contacts of tuberculosis patients showed an increased production of IL-10 when compared to BCG vaccinated healthy controls. Analysis of cytokine in different categories was done using SI units (Figures 50). PBMC from active TB patients produced significantly more IL-10 after stimulation with PPD, compared with healthy individuals ($P < 0{\cdot}05$)

IV. 12.3.4. IL-4 release assay

IL-4 was below the detectable range of the assay, and was hence considered to be not elicited against these antigens by PBMC from any of the study subject.

Figure 50: Dot-plot showing interleukin-10 release from PBMC in response to commercial PPD, PlcA, HspX, Tb8.4 and ESAT-6 antigens.



V.DISCUSSION

V.1. DIAGNOSIS OF PAUCIBACILLARY TUBERCULOSIS

V.1.1. Diagnosis of tuberculous lymphadenitis and pleural tuberculosis

Pleural tuberculosis (PTB) and tuberculous lymphadenitis (TBL) are frequent clinical manifestations of extra-pulmonary tuberculosis. Both PTB and TBL are potentially curable, provided an early diagnosis can be made and appropriate anti-tuberculous chemotherapy (ATT) is instituted. One of the common manifestations in PTB patients is the occurrence of pleural effusion. Pleural effusion can also occur in non-tuberculous pulmonary diseases such as bronchial carcinoma or pleural metastasis. The radiological appearances in thorax of patients with tuberculous and malignant pleural effusion bear close resemblance to each other and often create diagnostic enigma at the bed-side diagnosis and management. The distinction becomes extremely significant as treatment modalities in tuberculous and malignant pleural effusions differ vastly. Cytological and bacteriological investigation of pleural fluid is essential in distinguishing these pleural effusions.

Laboratory diagnosis of TBL is usually made by fine needle aspiration cytology (FNAC) and histopathological examination in lymph node biopsies. Both fine needle aspirates and lymph node biopsies in TBL demonstrate granulomatous lymphadenitis composed of aggregates of epithelioid cells, macrophages and Langhan's giant cells in and around zones of caseous necrosis. Granulomatous lymphadenitis has extensive differential diagnosis and is known to occur in sarcoidosis, sarcoid-like granulomatous reaction in the draining lymph nodes of malignancies of epithelial origin, fungal and parasitic infections (filariasis) and lymphogranuloma venerium. Demonstration of the causative agent - acid-fast bacilli (AFB), *Mycobacterium tuberculosis* either by Ziehl-Neelsen (ZN) staining or by

bacteriological culture of biopsies and hence considered as the 'gold standard' for the diagnosis of TBL.

V.1.2. Bacteriological diagnostic methods in PTB and TBL

In this study, AFB in cytospin smears of pleural fluids were demonstrated only in 4.3% patients with tuberculous pleural effusion (TPE). In paraffin sections of 35 TBL cases, AFB was demonstrated in four patients (11.4% sensitivity). *M. tuberculosis* was isolated by culture in 6/69 tuberculous pleural fluids and 4/35 TBL cases. Bacteriological methods were less sensitive and yielded many false negative results in patients with PTB and TBL. These results of bacteriology were in concordance with earlier published reports (120-123).

Several factors may account for the low sensitivity of bacteriological methods in diagnosis of PTB. Accumulation of fluid within pleural space in PTB patients usually occurs as a result of the discharge of mycobacterial antigens from sub-pleural tuberculous lesions (124). Occasionally, tubercle bacilli are also released into pleural space from sub-pleural caseous lesions. In several occasions, tubercle bacilli get entangled in the network of fibrinous exudates in the basal regions. During thoracocentesis, pleural fluid in PTB patients is often aspirated from the most accessible locations rather than basal fibrinous regions. Hence TPE specimens are either devoid of bacilli or present in sub-optimal number.

In lymph nodes from TBL patients, there is a florid granulomatous inflammation. Macrophages and epithelioid cells in this granulomatous lesion phagocytose the tubercle bacilli and consequently bacilli become non-viable and their morphology gets distorted (125). This may account for the low detectability of ZN staining. Alternate diagnostic approaches have thus become essential in the successful bed-side management of PTB and TBL patients.

V.1.3. Evaluation of standard and nested PCR methods in paucibacillary tuberculosis

PCR has gained wide-spread importance in the clinical diagnosis of *M. tuberculosis,* and is currently regarded as a valuable support in the clinical diagnosis of TB in developed countries. Despite substantial demands for technical skills and equipment, they are being exploited productively in referral laboratories in developing countries. PCR for *M. tuberculosis* is less time-consuming than bacterial methods, and more sensitive than ZN staining method.

A standard PCR assay based on the amplification of a 245 base pair region from the right arm of the insertion sequence, *IS6110* of *M. tuberculosis* complex was undertaken in PTB and TBL cases. *IS6110* is specific for the members of *M. tuberculosis* complex and present in 15-20 copy numbers (59). The results of PCR were correlated with ZN staining. *IS6110* PCR was found to be more sensitive than ZN method.

IS6110 PCR in specimens from tuberculous and malignant pleural effusion yielded 84% sensitivity and 84.5% specificity in this study. Some earlier studies (126, 121) reported a sensitivity ranging from 70% to 74% in tuberculous pleural effusion. The enhanced sensitivity of PCR in this study might be due to the inclusion of a CTAB–NaCl incubation step during the DNA extraction, which reduced the inhibitors of PCR (127). Positive results were obtained in eleven patients with malignant pleural effusion. Some of these positive results in malignant pleural effusion may be due to the reactivation of old tuberculous lesions, a phenomenon well-known to crop up in patients with malignancies. Yet all the eleven cases could not be considered as reactivation of tuberculosis lesions, indicating towards non-specificity of this target.

Possibility of amplicon cross-contamination was also present even after stringent laboratory practices.

An in-house I*S6110* PCR in formalin-fixed paraffin embedded lymph nodes from 35 TBL and 36 disease control group was carried out. Initially PCR was attempted in FNA of lymph nodes. But FNA was seldom adequate for various methods studied and more often non-representative of the lesion. *IS6110* PCR in lymph node biopsies gave an overall 74.3% sensitivity and 83.3 % specificity. These results were more or less comparable to several earlier published reports (128, 129). Positive results in lymph nodes from disease control group were encountered in one patient with fungal granuloma, four patients with reactive follicular hyperplasia and in one patient with non-specific lymphadenitis of unknown aetiology.

A study by Kent *et al* has reported a homology between *IS6110* of *M. tuberculosis* and DNAs of other *Mycobacterium* species (76). Some isolates of *M. tuberculosis* from South India have been reported to lack *IS6110* sequences leading to false negative results (77). The large number of false positive results of *IS6110* PCR in this study prompted us to the selection of a more specific target than *IS6110* for PCR-based diagnosis of PTB and TBL patients.

A single copy gene, *mtp40*, present in *M. tuberculosis* was reported to be absent from *M. bovis* and other members of *M. tuberculosis* complex by Portillo *et al* (130). This target was employed in detecting *M. tuberculosis* in different clinical samples, such as sputum, urine, and cerebrospinal fluid and was reported to be specific for the diagnosis of infection with *M. tuberculosis*. Herrera *et al* (69) reported that *mtp40* PCR was more specific than *IS6110* amplification in differentiating *M. tuberculosis* from other related mycobacteria.

To minimize the occurrence of false positive results obtained with *IS6110* PCR, in this study, a standard PCR targeting *mtp40* gene was performed. Standard *mtp40* PCR gave an overall sensitivity of 43.5% in TPE specimens. Positive results were detected in six patients with malignant pleural effusion enhancing the specificity to 91.5%. Standard *mtp40* PCR gave two false positive results in reactive follicular hyperplasia group and one in fungal granuloma. The specificity of standard *mtp40* PCR was found to be 91.6%.

Though the specificity of PCR diagnosis was increased with *mtp40* specific primers, sensitivity was less than 50%. The high specificity and low sensitivity of standard PCR using *mtp40* genomic sequence can be described in terms of its low detectability and hence a nested PCR targeting this gene became essential to achieve improved outcome.

A nested PCR using *mtp40* was found to be more sensitive and specific than *IS6110* for the diagnosis of PTB and TBL cases. Nested PCR gave 91.3% sensitivity and 90.1% specificity in tuberculous and malignant pleural effusions. Nested PCR gave an overall sensitivity of 88.6% in patients with TBL. Two false positive results were encountered in reactive follicular hyperplasia group and fungal granuloma. The specificity of nested PCR was found to be 91.6%.

A nested PCR based on *mtp40* gene will be particularly useful for the diagnosis of tuberculous pleural effusion and tuberculous lymphadenopathy in patients where conventional diagnosis fails and where the provisional diagnosis of TB is made on the basis of clinical presentation and histology/cytology examination without evidence of AFB. It is also more specific than the *IS6110* target and is

especially useful for detecting those *M. tuberculosis* strains which contain no copy of the IS*6110*.

However, standard and nested PCR methods could not distinguish between active, treated, and latent cases of TB. The stringent conditions needed for reducing cross-contamination would be very difficult to achieve in labs of developing countries.

V.1.4. Evaluation of *in situ* PCR in formalin-fixed lymph node specimens

In situ PCR was evaluated in formalin-fixed paraffin sections of patients with TBL. This method appeared very appealing theoretically as no DNA isolation was involved, reducing chances of cross-contamination leading to false positive results. *In situ* PCR also facilitates the demonstration of amplified mycobacterial DNA in true cytological context. Hernandez Pando *et al* employed an *in situ* PCR to reveal mycobacterial DNA in macroscopically normal lung tissues indicating that this method can be used in the detection of latent tuberculosis cases (78).

In this study, *in situ* PCR could be applied to few numbers of samples. In such TBL specimens, *in situ* PCR demonstrated the presence of mycobacterial DNA in the form of bluish-black bodies in and around granulomas. The adhesion of tissue sections on to the slide varied greatly depending on the histopathological variations of tuberculous lesion. Different slides were used for the study, and different slide-coating methods were implemented, but the results were inconsistent and hence *in situ* PCR was not undertaken any further.

None of the above methods either evaluated or modified was found to be adequate for meeting the objective of the study. An alternative diagnostic method which is sensitive and specific like PCR and devoid of cross contamination issues evoked by PCR was hence to be attempted. Serodiagnosis of tuberculosis has been

traditionally regarded as a non-reliable practice, mainly because of the low sensitivity and specificity of these tests. Apart from the intrinsic complexity of the immunological characteristics of tuberculosis (chronic disease that induces predominantly cellular immunity and weak humoral immunity), one possible explanation for the general lack of success of most serological tests is the complex mixture of crude antigens that has been used in these tests (58). More recently, a series of highly purified *M. tuberculosis* recombinant antigens have been described and evaluated as serological markers of the disease. Some of these antigens have been more extensively evaluated and shown to have high sensitivity and specificity for the identification of pulmonary TB patients (109, 131 - 136). These findings have revived the concept that serological tests for the diagnosis of tuberculosis, as for most infectious diseases, are definitely feasible.

Taking this lead, four genes of *M. tuberculosis* were cloned in *E. coli*; their respective proteins were over-expressed and purified in high yield. The potential application of these antigens in the diagnosis of pulmonary and extra-pulmonary tuberculosis was evaluated.

V.2. CLONING, EXPRESSION AND PURIFICATION OF FOUR MYCOBACTERIAL ANTIGENS

In this study, *M. tuberculosis* genes coding for four mycobacterial antigens PlcA, HspX, Tb8.4 and ESAT-6 were cloned, expressed and purified them in high yield. PlcA (Rv2351c) is probable membrane associated phospholipase C1 of *M. tuberculosis*, which is also referred to as Mtp40 antigen (37) whose activities are restricted to pathogenic *Mycobacterium* subsp. ESAT-6 is the early-secreted antigenic target 6-kDa protein, specific for *M. tuberculosis* complex, but is reported to be absent from *Mycobacterium bovis* BCG (106). ESAT-6 (Rv3875) has been

reported to elicit strong antibody responses and delayed type hypersensitivity skin reactions in guinea pigs (109). HspX (Rv2031c/ *acr* /16-kDa antigen / alpha-crystallin) was reported to be more sensitive than ESAT-6, CFP10 and antigen 85 in terms of sensitivity in serodiagnosis for the diagnosis of pulmonary tuberculosis (137). Tb8.4 (Rv1174c) is an immunodominant T-cell antigen of *M. tuberc*ulosis and this antigen predominantly elicit cell-mediated immunological responses in human beings and animal models (38).

Genes were amplified from *M. tuberculosis* genome and cloned into pGEM®-T Easy vector. Taq polymerase often adds a single deoxyadenosine, in a template-independent fashion, to the 3′-ends of the amplified fragments (138). pGEM®-T Easy vector with its single 3′-T overhangs at the insertion site greatly improved the efficiency of ligation of a PCR product into the plasmids by preventing recircularization of the vector and providing a compatible overhang for PCR products generated by Taq polymerase. These high copy number vectors contain T7 and SP6 RNA polymerase promoters flanking a multiple cloning region within the α-peptide coding region of the enzyme β-galactosidase. Transformed clones were selected based on antibiotic resistance and blue/white screening as insertional inactivation of the α-peptide allows recombinant clones to be directly identified by color screening on indicator plates.

Cloning into pGEM®-T Easy vector increased the cloning efficiency, and this construct was used in DNA sequencing. Successful cloning of open reading frames was checked and chance of mutation due to initial amplification was eliminated. Genes were excised out of pGEM®-T Easy construct and were separately cloned into the *Kpn*I - *Hind* III site of pET-32a.

In pET system, both the T7 promoter and the lac operator are located 5' to the genes of interest. When T7 RNA polymerase is present, the lac operator is not repressed and the transcription of gene of interest proceeds rapidly. T7 promoters being a viral promoter transcribe rapidly and profusely as long as the T7 RNA polymerase is present. The expression of gene of interest increases rapidly as the amount of mRNA transcribed from gene of interest increases. Within a few hours the inserted gene becomes one of the most prevalent components of the cell.

Gene of interest is not transcribed unless the T7 RNA polymerase is present. Prokaryotic cells do not produce this type of RNA and therefore for protein production, a recombinant plasmid is transferred to host *E. coli* strains containing a chromosomal copy of the gene for T7 RNA polymerase. These hosts are lysogens of bacteriophage DE3, a lambda derivative that has the immunity region of phage *21* and carries a DNA fragment containing the *lacI* gene, the *lacUV5* promoter, and the gene for T7 RNA polymerase (139). This fragment is inserted into the *int* gene, preventing DE3 from integrating into or excising from the chromosome without a helper phage. Once a DE3 lysogen is formed, the only promoter known to direct transcription of the T7 RNA polymerase gene is the *lacUV5* promoter, which is inducible by isopropyl-b-D-thiogalactopyranoside (IPTG). Addition of IPTG to the growing culture of the lysogen induced T7 RNA polymerase, which in turn transcribed the target DNA in the plasmid.

Three proteins, HspX, PlcA and Tb8.4 were over-expressed in E.coli BL21 (DE3) pLysS. But ESAT-6 was not over-expressed with this vector, and hence the gene *esat-6* was cloned into pET-28a, which gave an over-expression of ESAT-6.

Expressed proteins were Histidine (His) tagged using pET system for easiness of purification. His tagged proteins were purified using HisLink protein purification resin which is a macroporous silica resin modified to contain a high level of tetradentate-chelated nickel) for efficient capture and purification of bacterially

expressed polyhistidine proteins. Three proteins, ESAT-6, PlcA and Tb8.4 were present as inclusion bodies, while HspX was seen in soluble fraction. Inclusion bodies were purified under denaturing conditions and HspX protein was purified under native conditions. To increase the yield of purification of HspX, later this protein was also purified under denaturing conditions. The denaturing agents present in the final protein solution was dialyzed out by dialysis and used in further immunological evaluation. HspX, purified under both native and denatured conditions were compared in immunoassays and their activities were identical. The slow growing nature of *M. tuberculosis* hinders large-scale purification of specific antigens. Recombinant DNA technology described above was able to produce mycobacterial antigens in its pure version and in very high yield.

V.3. HUMORAL IMMUNE RESPONSES AGAINST PURIFIED ANTIGENS IN CLINICAL SPECIMENS

V.3.1. Detection of serum antibody response to mycobacterial PlcA, HspX, Tb8.4, ESAT-6 antigens and multiantigen cocktail

Mycobacterial antigens in immunocompetant individual essentially elicit cell mediated immune response. These activated T-lymphocytes produce cytokines and chemokines and play an important role determining the course of the infection. B-lymphocytes on the other hand produce antibodies in response to mycobacterial antigens. Most of the antibody to *M. tuberculosis* belongs to IgG clan while IgM and IgA antibodies can also be produced in response to mycobacterial antigens. Immunoassays aiming at detection of antibodies hence can be used in the diagnosis of tuberculosis. Immunoassay to detect antibodies in the sera and other body fluids represent a reliable approach for the diagnosis of TB, provided specific antigens are

being used. Several antigens need to be identified and isolated in large-scale. The choice of particular mycobacterial antigen to be used in immunoassay is complicated due to the heterogenous antibody response against different antigens in patients with TB. Four mycobacterial antigens purified in this study were evaluated for antibody response against them in sera specimens from pulmonary tuberculosis patients.

All the four mycobacterial antigens elicited antibody response in patients with pulmonary TB. As the sensitivity of the individual antigens in ELISA was not promising, a cocktail of all these antigens were used in ELISA. Multiantigen cocktail provided higher sensitivity than individual antigens, while among individual antigens, HspX was found to more sensitive. Earlier studies reported that augmented immune responses to HspX are observed in latent TB (140). But the results of this study confirmed that antibodies to HspX are predominantly found in patients with active TB as reported (141).

Multiantigen did not give any false positive results in healthy controls. Few positive results were obtained in non-TB pulmonary disease control, especially bronchial carcinoma patients. Reactivation of tuberculous lesions is well known to occur in patients with bronchial carcinoma (142). Therefore it is likely that IgG antibody to antigens detected by ELISA in these patients with bronchial carcinoma could be due to the reactivation of tuberculous lesions. Hence presence of IgG antibodies to *M. tuberculosis* in patients with bronchial carcinoma could not be entirely ruled out as false positive results.

V.3.2. Detection of antibody to mycobacterial PlcA, HspX, Tb8.4, ESAT-6 antigens and multiantigen cocktail in pleural fluid specimens

Immunoassays to detect the presence of mycobacterial antigen as well as antibody response to *M. tuberculosis* have been developed as reliable markers for the

diagnosis of tuberculous pleural effusion (TPE) (124, 143). Exudates in pleural effusion in PTB patients are usually rich in lymphocytes. These lymphocytes are composed of both T and B lymphocytes. T lymphocytes are more predominant than B lymphocytes in tuberculosis. Several studies have highlighted to T lymphocyte responses to mycobacterial antigens in patients with tuberculous pleural effusion. These include the release of cytokines like IFN-γ (144). On the other hand B lymphocytes produce antibodies against mycobacterial antigens. Immunoassays to detect the presence of antibodies against purified protein derivative (145), tuberculosis glycolipid antigen (146) and lipoarabinomannan (147) in patients with TPE have been documented. Recently, Kaisermann et al detected IgA response against MPT-64 and MT-10.3 recombinant mycobacterial antigens in pleural fluids (148). Though these assays were sensitive, false positive results were encountered in patients with non- tuberculous pleural effusion.

The role of PlcA, HspX, Tb8.4 and ESAT-6 in accurate PTB diagnosis was evaluated. The sensitivity of ELISA using individual antigens were found to be low and ranged between 40-60%. But an ELISA using the multiantigen cocktail antigen gave a sensitivity of 75.4%. HspX was highly sensitive than other three when assayed individually in active tuberculosis cases. Even though not highly sensitive individually, PlcA, Tb8.4 and ESAT-6 antigens contributed in increasing sensitivity in the multiantigen complex, without any loss of specificity.

False positive results were encountered in three pleural fluid specimens from patients with bronchial carcinoma. These three patients subsequently underwent surgery and the pneumoectomy specimens were subjected to histopathology. In all the three patients the histological factors were suggestive of squamous cell carcinoma. In addition, non-caseating granuloma suggestive of tuberculosis was also

observed in the histological sections of lungs. Therefore it is likely that IgG antibody to recombinant antigens detected by ELISA in these three patients with bronchial carcinoma could be due to the reactivation of tuberculous lesions.

Results of ELISA in sera and pleural fluid specimens indicates that a multiantigen complex composed of PlcA, HspX, Tb8.4 and ESAT-6 is a specific and sensitive marker in making a diagnosis of TB. The assay is reliable and reproducible. Unlike cell- mediated assays, IgG detection is a rapid and cost-effective method.

V.3.3. Immunohistochemical method for the demonstration of mycobacterial antigens in FFPE lymph nodes

Demonstration of *M. tuberculosis* bacilli by bacteriological methods in lymph node biopsies is considered as the 'gold-standard' for making a diagnosis in TBL. In our study AFB was demonstrated in a small number of TBL cases.

During the past decades IHC assumed greater significance in the diagnosis of several infectious diseases including tuberculosis. Role of IHC in the diagnosis of tuberculosis in formalin-fixed paraffin embedded lymph node biopsies have been reported in several published studies. (149, 84) Mukherjee et al used a commercial anti-BCG antibody in their study and recorded 74% sensitivity, while Purohit et al applied anti MPB-64 antibody with 80% positivity in patients with TBL. In another study, Goel et al (125) developed an immunocytochemical method using anti-38kDa antibody in the FNA of TBL specimens recording a sensitivity of 96.7%. It becomes relevant to explain that the sensitivity of IHC for TBL diagnosis depends on (a) distribution of mycobacterial antigen in granuloma (b) clinical stage of disease (c) duration of ATT received prior to biopsy (d) specificity of primary antibody used in IHC.

ESAT-6, PlcA, HspX, and Tb8.4 were used to produce respective polyclonal antibodies in albino rabbits. These antibodies together with anti-TBGL and anti- CFA was used in IHC. The results of IHC demonstrate the high sensitivity of anti-ESAT-6 antibody. Anti-HspX antibody gave positive results in two cases of metastatic carcinoma cases and in one reactive hyperplasia patient. Tb8.4 antibody also gave positive immunostaining in two patients with metastatic carcinoma. Anti-TBGL and anti-CFA gave false positive results in four metastatic cases. These results were regarded as false positive. The other two antibodies, anti-ESAT-6, and anti-PlcA did not give any false positive reaction in disease control group. In this IHC study, anti-ESAT-6 antibody was more sensitive than anti-PlcA. Anti-TBGL antibody demonstrated 'rod-like' structures within the cytoplasm of Langhans' giant cells and epithelioid cells in the granulomatous lesions were observed, which closely resembled AFB morphological characteristics and appeared more prominent than AFB as seen in ZN stained sections. This may be due to the fact that the anti-TBGL antibody captured the entire glycolipid component of bacilli.

Application of IHC assumes greater significance in situations such as 'sarcoid' like, non- caseating granulomatous lesions. The histopathological features in such lesions resemble TBL and become indistinguishable on histopathological grounds. IHC using anti-ESAT-6 holds promise for the specific detection of *M. tuberculosis* in tuberculosis involvement of lymph nodes. IHC allows retrospective analysis of paraffin section without alteration in the intensity of immunostaining. IHC using anti-ESAT-6 antibody is a better choice for the diagnosis of TBL, in patients in whom conventional diagnostic methods did not confirm the disease.

V.4. CELL-MEDIATED IMMUNE RESPONSES TO MYCOBACTERIAL ANTIGENS

Under the direct influence of mycobacterial antigens host T lymphocytes undergo activation during the active stages of tuberculosis. This activated cells produces pro-inflammatory cytokines like IFN-γ and TNF. At the same time T-lymphocytes also produces anti-inflammatory cytokines such as IL-10 (150). In healthy individuals a balance between pro-inflammatory and anti-inflammatory cytokines does exist. Thus the cell mediated immune responses against mycobacterial antigens result in the elimination of tubercle bacilli or may sometimes result in a latent phase of disease. An imbalance between pro-inflammatory and anti-inflammatory cytokines, occur during active stages of tuberculosis (151). Anti-inflammatory cytokines predominate over pro-inflammatory cytokines in such stages of disease. In this study, four cytokines (IFN-γ, TNF, IL-10 and IL-4) released from human peripheral blood mononuclear cells (PBMC) when exposed to specific mycobacterial antigens were evaluated.

In this study, pro-inflammatory cytokines like IFN-γ and TNF were found to be down-regulated in active TB patients. Anti-inflammatory cytokine, IL-10 was present in increased quantities in active TB cases. Stimulation of PBMC with mycobacterial antigens did not produce IL-4. All the four recombinant mycobacterial antigens could distinguish the house-hold contact individuals of TB patients from BCG sensitized healthy voluntary donors based on IFN-γ and TNF assays. IL-10 release in active TB patients was seen up-regulated. House-hold contacts released more IL-10 than BCG vaccinated healthy donors. Following ATT treatment the cytokine responses in TB patients have undergone a distinct reversal.

The recombinant mycobacterial proteins in this study were found to elicit strong TH1 response, i.e. elevated IFN- γ expression associated with lower expression of IL-4. IL-10, another TH2 cytokine, was augmented which is in correlation with previous

studies on prominent mycobacterial antigens. It has been reported that IL-10 inhibits the synthesis of cytokines by TH1 cells (152). This may attribute, at least partially, to the decreased amounts of protective cytokines, IFN-γ and TNF.

A commercial PPD was used along with the four antigens in this study. PPD was able to distinguish between active TB and healthy donors. IFN- γ and TNF was found to be elevated in healthy donors compared to active tuberculosis patients. IL-10 was found increased in active TB patients. Like other antigens, PPD was also not able to induce IL-4 release from any of the subject groups. With PPD induction, difference in cytokine production between BCG vaccinated donors and house-hold contacts were not statistically significant. Differences in cytokine production before and during treatment were also statistically insignificant.

ESAT-6 has been reported to induce IFN- γ in pulmonary TB patients (153). But all the individuals irrespective of categorization generated cytokines when induced with ESAT-6. ESAT-6 as reported by earlier studies is not recognized by TST negative BCG vaccinated subjects (154). This contradictory performance can be due to the endemic nature of *M. tuberculosis* infection in our population. The results of this study can be correlated to some earlier published works where T-cell dominant antigens like ESAT-6 was shown to down-regulate pro-inflammatory cytokines like IFN- γ (155, 156). A recent study has proved that ESAT-6 inhibited production of IFN-γ by *Mycobacterium tuberculosis*-responsive human T Cells (157).Other three antigens, PlcA, HspX and Tb8.4 also produced similar results as ESAT-6, in this study.

The difference between cytokines released by BCG vaccinated healthy donors and house-hold contacts of TB patients were more significant with HspX and Tb8.4. These antigens gave significantly higher IFN- γ and TNF in house-hold contact individuals compared to ESAT-6 and PlcA. Large population study with these

antigens will help in ascertaining these results thereby its evaluation in latent TB detection.

MTT assay was reported in several studies to delineate proliferation profiles against mycobacterial antigens in different groups of patients (158). MTT assay in this study indicated that PBMC from most of the individuals were responding to the four antigens and PPD. But great deal of information was not obtained by means of this test, except that TST-positive individuals were responding more with stimulation index greater than one, when compared to TST negative subjects. The disease condition and treatment stages did not produce any significant difference in MTT assay.

PBMC were isolated from non-tuberculous pulmonary disease controls like bronchial carcinoma and bacterial pneumonia patients. Cytokine release assays in them were similar to healthy BCG vaccinated individuals. The results were not used for further evaluation as sample number was too low to make any inference and also cytokine release in such patients will be affected by their disease conditions.

Results of the study shows that TNF and IL-10 assays can be made useful as adjuncts in IFN- γ release assays, and a panel of specific mycobacterial antigens can be included in assays instead of ESAT-6, to increase the overall sensitivity and specificity of the diagnosis.

V.5. APPLICATION OF RECOMBINANT MYCOBACTERIAL ANTIGENS IN DETECTION OF LATENT TUBERCULOSIS

A significant proportion of individuals in our population are exposed to *M. tuberculosis* infection. By virtue of an intact immunosurveillance, these individuals are able to limit the growth of mycobacteria and become latently infected. Individuals with latent tuberculosis are at greater risk of developing active disease when their immunological status gets altered as in cases of HIV infection, severe malnutrition etc

(159). The standard method for detecting individuals with latent tuberculosis is the use of intradermal tuberculin test using PPD antigen of *M. tuberculosis*. The TST exploits the fact that latent TB induces a strong cell-mediated immune response by measuring the DTH response to intradermal inoculation of PPD, a crude mixture of more than 200 *M tuberculosis* proteins. DTH-induced cutaneous induration by PPD is not a sensitive marker of immune sensitization in immunosuppressed individuals with latent TB, and DTH response to PPD is not specific for latent TB because of the antigenic cross-reactivity of PPD with BCG vaccination and environmental mycobacteria (160). This situation calls for the development of newer tests with specific antigens of *M. tuberculosis.*

The antigens cloned and purified in this study, has been evaluated for its cell mediated immune response in terms of its potential for cytokine release. The results of this preliminary study indicates that, such specific antigens of *M. tuberculosis* have greater potential compared to crude PPD antigen in cell mediated diagnosis of tuberculosis cases and also in the detection of latent tuberculosis cases.

VI. SUMMARY AND CONCLUSIONS

VI.I. SUMMARY AND CONCLUSIONS

Major observations in this study are as follows:

- Nucleic acid amplification assays were more sensitive than conventional bacteriological methods for the detection of *M. tuberculosis* in lymph nodes and pleural fluids from patients with extra-pulmonary tuberculosis.
- Nested PCR using primers specific for *mtp40* genomic sequence of *M. tuberculosis* was more sensitive than conventional PCR with *IS6110* and hence nested PCR is recommended as a diagnostic approach in clinical specimens from patients with extra-pulmonary tuberculosis.
- PCR methods, though sensitive was found to be yielding false positive results due to cross-contamination.
- During the course of study, genes coding for four mycobacterial antigens PlcA, HspX, Tb8.4 and ESAT-6 were cloned and expressed in *E. coli*. These proteins were purified to near-homogeneity and in large quantities.
- Antibody detection assays using multiantigen cocktail formulated by recombinant mycobacterial antigens, PlcA, HspX, Tb8.4 and ESAT-6 was found to be highly specific in detecting IgG antibodies in sera samples from pulmonary TB patients and in pleural fluid specimens from pleural TB patients. Based on high specificity and operational advantages, the assay has potential for application in routine diagnosis of pulmonary and extra-pulmonary tuberculosis. This assay is also suited to laboratories in developing countries.
- Using a panel of antibodies against *M. tuberculosis* antigens, an immunohistochemical method was successfully developed to demonstrate specific mycobacterial antigens in the formalin-fixed sections of lymph nodes of patients with TBL. Among these, anti-ESAT-6 antibody was highly specific

and evaluated as an 'ideal' candidate as a primary antibody in IHC for TBL diagnosis.

- *In vitro* studies suggested that there is a down-regulation of pro-inflammatory cytokines - IFN-γ and TNF, and an up-regulation of anti-inflammatory cytokine, ie, IL-10 when PBMC of patients with active pulmonary tuberculosis were stimulated with the four mycobacterial antigens. Following optimal ATT treatment there was a reversal in the ratio of pro-inflammatory and anti-inflammatory cytokine profiles in these patients.
- In this preliminary study, it was observed that the four mycobacterial antigens could distinguish the house-hold contact individuals of TB patients from BCG sensitized healthy donors. The results of this study revealed an enhanced production of IFN-γ, TNF and IL-10 cytokines in house-hold contacts than in BCG vaccinated healthy donors. Such a distinction was not possible when cytokine release assay was performed with PPD antigen. This observation could be used in distinguishing individuals with latent TB infection from BCG vaccinated healthy individuals.

VI.2. FUTURE DIRECTIONS

- It is being proposed that immunohistochemistry with anti-ESAT-6 antibody can be used for the demonstration of mycobacterial antigens in tuberculosis-infected tissues other than lymph nodes. Based on the same principle, an 'immunocytochemistry' can be applied in visualizing mycobacterial antigen in body fluid smears as in cerebrospinal fluid, pericardial fluid, synovial fluid, pleural fluids and bronchoalveolar lavages.

- Serum IgA and IgM profiles against these recombinant mycobacterial antigens should be undertaken.
- A multicentre trial to evaluate the potential application of the four mycobacterial antigens in diagnosis of pulmonary tuberculosis is essential.
- Cytokine release assays based on *M. tuberculosis* antigens PlcA, HspX, Tb8.4 and ESAT-6 in large population may lead to the development of a diagnostic parameter for latent tuberculosis. These specific antigens may be evaluated for their efficiency in intradermal tests.
- The ability of these specific antigens in eliciting pro-inflammatory (protective) cytokines in healthy individuals can be made useful in potential vaccine development.

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VIII.PUBLICATIONS

Anie Y, Sumi S, Varghese P, Madhavi LG, Sathish M, Radhakrishnan VV. Diagnostic approaches in patients with tuberculous pleural effusion. Diagnostic Microbiology and Infectious Diseases 2007;59(4):389-94.

Sumi S and Radhakrishnan VV. Evaluation of immunohistochemistry with a panel of antibodies against recombinant mycobacterial antigens for the diagnosis of tuberculous lymphadenitis. Accepted in International Journal of Medicine and Medical Sciences.

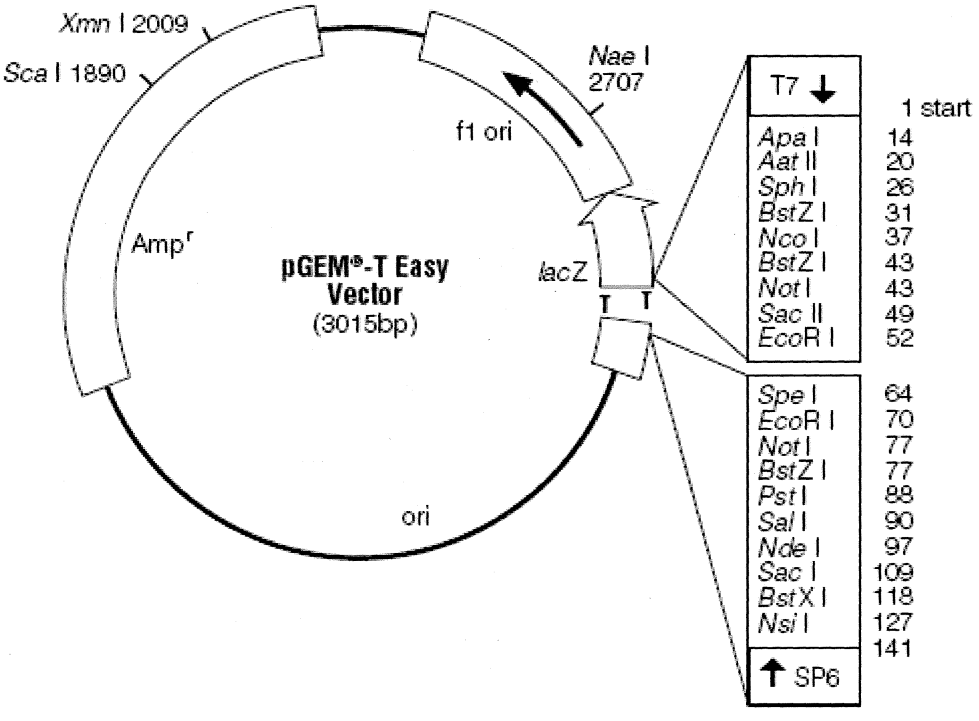
Sumi S and Radhakrishnan VV. Diagnostic significance of humoral immune responses to recombinant antigens of Mycobacterium tuberculosis in patients with pleural tuberculosis. Communicated to Diagnostic Microbiology and Infectious Diseases

Sumi S, Madhavilatha GK, Sathish Mundayoor and Radhakrishnan VV. Assessment of four recombinant mycobacterial antigens as serodiagnostic markers for pulmonary tuberculosis. Communicated to The International Journal of Tuberculosis and Lung Disease.

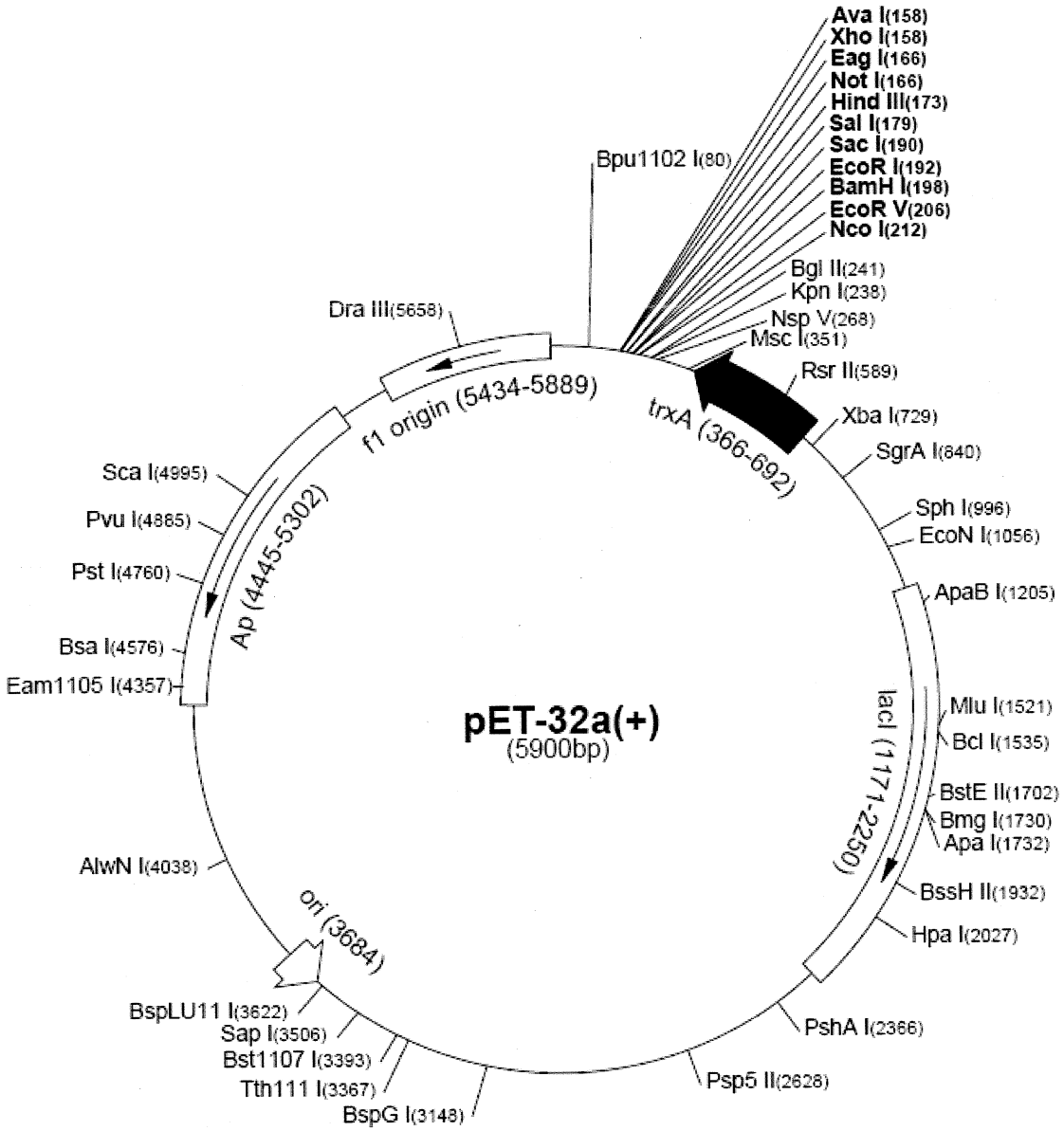
Sumi S, Madhavilatha GK, Sathish Mundayoor and Radhakrishnan VV. Molecular cloning, over-expression, purification, and immunological characterization of phospholipase C 1 (PlcA) of *Mycobacterium tuberculosis.* . Communicated to FEMS Immunology and Medical Microbiology.

IX. ANNEXURES

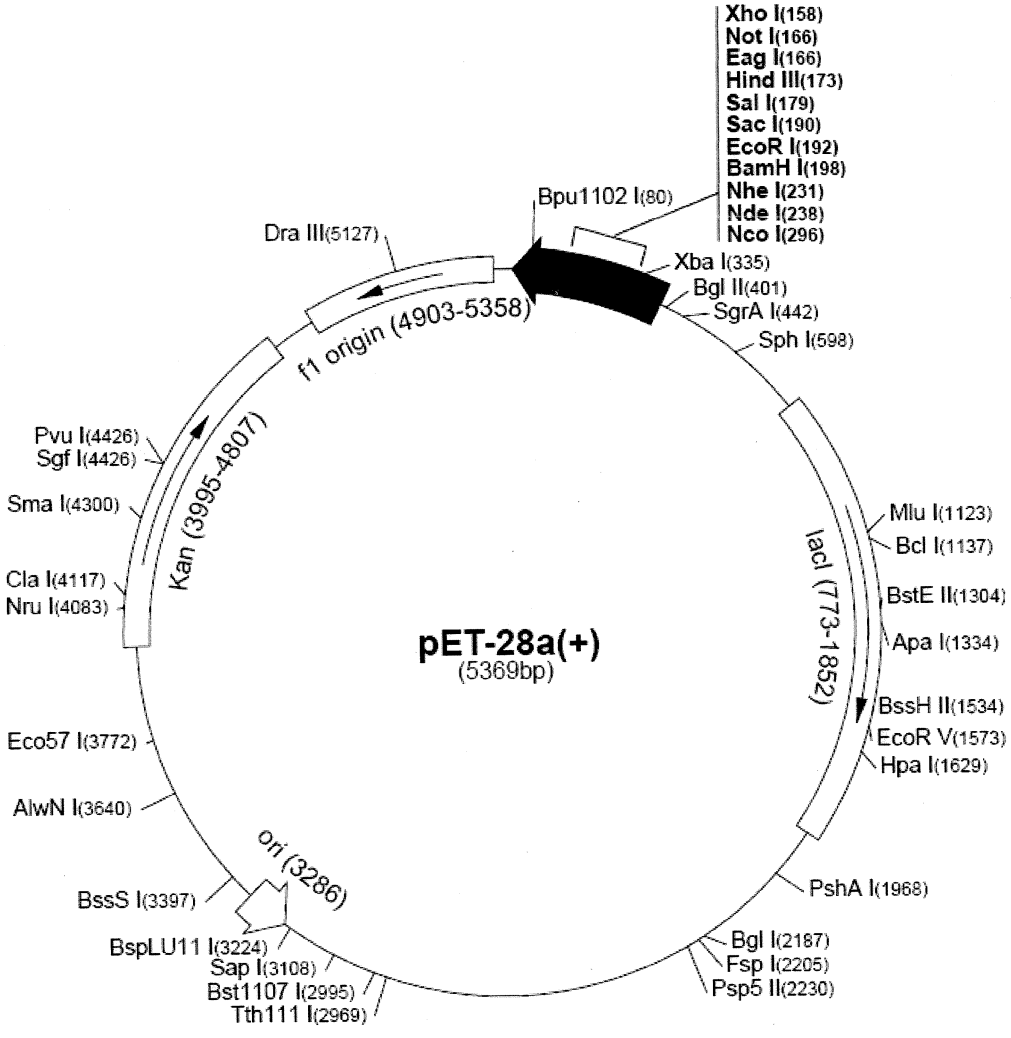
IX.1. pGEMT EASY VECTOR MAP (PROMEGA)



IX.2. pET-32a VECTOR MAP (NOVAGEN)



IX.3. PET-28a VECTOR MAP (NOVAGEN)



IX.4. INFORMED CONSENT FORM FOR THE USE IN COLLECTION OF CLINICAL SAMPLES TO BE USED IN TUBERCULOSIS RESEARCH

1.Project title : Molecular and Immunological approaches in the diagnosis of human tuberculosis

2.Project details: For the effective management of tuberculosis, novel vaccines and specific diagnostic methods are being developed. The study involves the efficient utilization of four isolated recombinant mycobacterial antigens in the early diagnosis of tuberculosis.

3.Name, address, of the Institute : Sree Chitra Tirunal Institute For Medical Sciences And Technology, Thiruvananthapuram-695011

4.Name, address and telephone number of the principal investigator : Dr.V.V.Radhakrishnan
Professor and Head
Department of Pathology
Sree Chitra Tirunal Institute For
Medical Sciences and Technology,

Thiruvananthapuram-695011

Phone:2524508

5.I have been explained the purpose of the research being undertaken and I have understood them : Yes/No

6.I have had the opportunity to ask questions and am satisfied with the answers provided to me : Yes/No

7.I have been informed of the steps to be implemented for protecting my privacy and confidentiality and I am satisfied with them : Yes/No

8.I have been informed that certain screening test may be performed on the samples donating by me and that I will not be provided with the results of these tests : Yes/No

9.I have been informed that I will not

derive any direct benefits from

the study : Yes/No

10.I have been informed that I can

withdraw my consent for the study

at any time without facing any penalty : Yes/No

I am willingly donating my sample for the purpose of this research study and I confirm that I have not been coerced, directly or indirectly, to donate my sample

Signature of the Donor Date

Signature of the Witness Date

NOTE: This study was duly approved by Institute Ethics Committee and Animal Experimentation Ethics Committee.