UNIVERSITE CLAUDE BERNARD – LYON 1

FACULTE DE PHARMACIE

INSTITUT DES SCIENCES PHARMACEUTIQUES ET BIOLOGIQUES

2013 **THESE n° 114**

THESE

Pour le DIPLOME D'ETAT DE DOCTEUR EN PHARMACIE

Présenté et soutenu publiquement le 23 septembre 2013

Par

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né le 12 décembre 1955

à Flers

*****

Clinical Evaluation of a tuberculosis diagnostic tool box in an endemic country: India

*****

Jury

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A notre Jury de THESE

A Monsieur le Professeur Jean FRENEY, Professeur des Universités-Praticien Hospitalier,
Votre aide très précieuse a été essentielle à la réalisation de cette thèse. C'est un très grand honneur pour moi que vous ayez accepté d'être mon Président de jury, vous qui êtes l'auteur du livre référent de la microbiologie présent dans tous les laboratoires. Ce fut aussi un très grand plaisir de m'avoir fait partager votre passion sur l'histoire de la microbiologie.

A Monsieur le Professeur Gérard LINA, Professeur des Universités-Praticien Hospitalier,
Je garderai toujours en moi le souvenir du travail effectué ensemble pendant plusieurs années sur la toxine Leucocidine de Panton Valentine de *staphylococcus aureus.* Je suis très honoré que vous participiez au jury de ma thèse, vous qui êtes en charge du diagnostic de la tuberculose pour l'ensemble des Hospices Civils de Lyon.

A Monsieur le Docteur Christophe LONGUET, Docteur en Médecine,
Nous avons parcouru ensemble l'Inde pour suivre ce projet d'évaluation clinique d'outils de diagnostic de la tuberculose ; grâce à votre formation de médecin clinicien, vous avez joué un rôle très important pour que cette étude reste proche des besoins médicaux. Je vous remercie infiniment d'avoir accepté de participer au jury de cette thèse.

A Madame Geneviève INCHAUSPE, Docteur-ès Sciences,
Notre proximité professionnelle nous a fait échanger si souvent sur la tuberculose et d'autres thèmes scientifiques que je suis très heureux et honoré que vous participiez au jury de ma thèse.

A Monsieur Dominique VAUR, Docteur en Pharmacie,
Après de très nombreuses années d'amitiés et nos nombreuses discussions sur la biologie médicale, je suis très ému que vous participiez au jury de cette thèse, thèse que nous attendions depuis longtemps… Merci infiniment d'avoir accepté de traverser la France pour être parmi nous.

Mes remerciements vont également à :

A Satheesh KUMAR qui a réalisé un énorme travail pour le suivi de l'étude clinique. Grâce à sa très forte implication et son professionnalisme, un travail de qualité homogène et fidèle au protocole a été réalisé sur l'ensemble des sites impliqués dans cette étude clinique.

Au Professeur Philippe Henri LAGRANGE qui a su, grâce à son énergie, enthousiasme et expertise reconnue internationalement, fédérer l'ensemble des partenaires indiens autour de cette étude clinique.

Une pensée profonde va aussi à mon ami Youssouf ISSABRE avec lequel j'ai partagé une phase importante de ma carrière sur le développement de tests de diagnostic rapide pour les pays en voie de développement. Youssouf a su me transmettre les réels besoins du terrain pour les pays dits « du sud ».

A Jean Louis MACHURON, un des meilleurs experts des problèmes de santé publique en Afrique. Jean Louis avec son optimisme et dynamisme permanent est toujours à mes côtés pour me guider sur ma reconversion professionnelle.

A ma famille et particulièrement ma femme Isabelle et mes 3 enfants : Guillaume, Marie et Camille qui m'ont fortement soutenu pour cette thèse et la nouvelle orientation de ma carrière à l'issue d'une rupture difficile de mon activité professionnelle.

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Abbreviations

AFB	Acid fast bacilli
Ag	Antigen
AIDS	Acquired immunodeficiency syndrome
ART	Antiretroviral therapy
ARTI	Annual risk of tuberculosis infection
BCG	Bacille calmette et Guerin
CDC	Centre for disease control
CHC	Community Health Centre
DAT	Diacyl trehalose
DC	Dendritic cells
DOT	Direct observed treatment
DRS	Drug resistance surveillance
DST	Drug susceptibility testing
ELISA	Enzyme-linked immunosorbent assay
FRU	First referal unit
GDP	Gross Domestic Product
HIV	Human immunodeficiency virus
ICMR	Indian Medical Council Research
IFN-ϒ	Interferon gamma
IGRA	Interferon gamma release assay
IL	Interleukin
INH	Isoniazid
IPHS	Indian Public Health Standards
IRIS	Immune reconstitution inflammatory syndrome
LAM	Lipoarabinomannan
LAMP	Loop-mediated isothermal amplification
LED	Light-emitting diodes
LOS	Lipooligosaccharide
LJ	Loewenstein jensen
LTBI	Latent tuberculosis infection
MDG	Millennium Development Goals
MDR	Multi drug resistance
MCE	Multicenter clinical evaluation
MOTT	Mycobacteria other than tuberculosis
MTB	*Mycobacterium tuberculosis*
MTBC	*Mycobacterium tuberculosis* complex
NAA	Nucleic acid amplification
NAAT	Nucleic acid amplification test
NK	Natural killer
NMR	Nuclear magnetic resonance
NTI	National Tuberculosis Institute

NTP	National Tuberculosis Programme
PAS	Para aminosalicyclic acid
PCR	polymerase chain reaction
PGL	Phenol glycolipid
PHC	Primary Health Care
PLHIV	People living with HIV
PPD	Purified protein derivative
PPP	Purchasing power parity
QFT-GIT	QuantiFERON-TB Gold
RIF	Rifampicin
RNTCP	Revised National Tuberculosis Control Program
TB	Tuberculosis
TGF	Transforming growth factor
TNF	Tumor necrosis factor
TRC	Tuberculosis Research Centre
TST	Tuberculin skin test
TTD	Time to detection
WHO	World Health Organization
XDR	Extensively drug resistant
ZN	Zielh-Neelsen

I- Introduction

“Le microbe n’est rien. Le terrain est tout”

Louis Pasteur

Tuberculosis (TB) is an infectious disease caused by bacterial agents which belong to *Mycobacterium tuberculosis* complex (MTBC). TB is contagious and airborne. It ranks as the second leading cause of death from an infectious disease worldwide, after the human immunodeficiency virus (HIV).

According to the World Health Organization (WHO): in 2011, 8.7 million people fell ill with TB and 1.4 million died from TB. About one-third of the world's population has latent tuberculosis (LTB), which means people have been infected by TB bacteria but are not (yet) ill and cannot transmit the disease. People infected with TB bacteria have a 10% lifetime risk of falling ill with TB. When a person develops active TB (disease), the symptoms (cough, fever, night sweats, weight loss etc.) may be mild for many months. This can lead to delays in seeking care, and results in transmission of the bacteria to others. People ill with TB can infect up to 10-15 other people through close contact over the course of a year. Without proper treatment, up to two-thirds of people ill with TB will die. Persons with compromised immune systems, such as people living with HIV, malnutrition or diabetes, or people who use tobacco, have a much higher risk of falling ill.

Over 95% of TB deaths occur in low and middle-income countries. In 2011, the largest number of new TB cases occurred in Asia, accounting globally for 60% of new cases. However, Sub-Saharan Africa carried the greatest proportion of new cases per population with over 260 cases per 100 000 population in 2011.

In 2011, about 80% of reported TB cases occurred in 22 countries. Some countries are experiencing a major decline in cases. Brazil and China for example, are among the 22 countries that showed a sustained decline in TB cases over the past 20 years. In the last decade, the TB prevalence in Cambodia fell by almost 45%.

India is the second most populous country in the world and has more new TB cases annually than any other country, which makes this country with the highest TB burden in the world. TB is a social disease with medical implications. It has always occurred disproportionately among disadvantaged populations such as homeless, malnourished, and people living under crowded living conditions. The basis of TB control programs consists of timely diagnosis and correct treatment of patients with active TB. Early diagnosis is essential for controlling

the spread of the disease. Diagnosis of TB relies mostly on the detection of acid-fast bacilli by microscopy and less frequently by culture. The purpose of the "Tuberculosis Diagnostic Tool Box evaluation" in India was to assess the potential of conventional and newly developed TB diagnostic tests in a country highly endemic for TB. This kind of study requires a health care system allowing access to the patient recruitment with good clinical evaluation and laboratory infrastructures to run sophisticated diagnostic tools. India, where coexist high level of medical health care infrastructure and very limited health care resources, was an ideal country to conduct this kind of study. The study was supported as well by the Mérieux Foundation and the Indian Council Medical Research (ICMR).

II- Tuberculosis

1. Historical review

Few diseases have captured the imagination more than tuberculosis (TB). Tuberculosis fascinates many people: physicians, scientists, epidemiologists, humanitarians, sociologists and artists. Edward Munch's picture of his sister Sophie captures the pathos.

Figure 1: Edward Munch's the sick child (1)

Tuberculosis has been known to mankind since Antiquity. This disease has been called by numerous names including consumption (because of the severe weight loss and the way the infection appeared to "consume" the patients), phthisis pulmonary and the white plague (because of the extreme pallor seen among those infected).

The organism causing tuberculosis *Mycobacterium tuberculosis* existed 15,000 to 20,000 years ago. It has been found in relics from ancient Egypt, India and China. Among Egyptian mummies, spinal tuberculosis, known as Pott's disease has been detected by archaeologists. Evidence of tuberculosis of the cervical lymph nodes termed scrofula is found in the Middle Ages. It was termed as the "king's evil" and was widely believed that the kings of England and France could cure scrofula simply by touching those affected.

In the 18th century in Western Europe, tuberculosis reached its peak with a prevalence as high as 900 deaths per 100,000. Poorly ventilated and overcrowded housing, primitive sanitation, malnutrition and other risk factors led to the rise. The term "White Plague" emerged around this time.

The tubercle Bacillus or the causative organism of tuberculosis was demonstrated by Robert Koch in 1882. Its identification was possible until a specific stain called the Ziehl-Neelsen (ZN) stain was discovered.

The bacteria was called Koch's bacilli and since it took up the red acidic dye, it was called acid fast bacilli (AFB). Koch was awarded the Nobel Prize in 1905.

In the nineteenth century, the concept of keeping tuberculosis patients isolated in a sanatorium started. A German physician Hermann Brehmer opened the first sanatorium in Gröbersdorf (Silesia) in 1854.

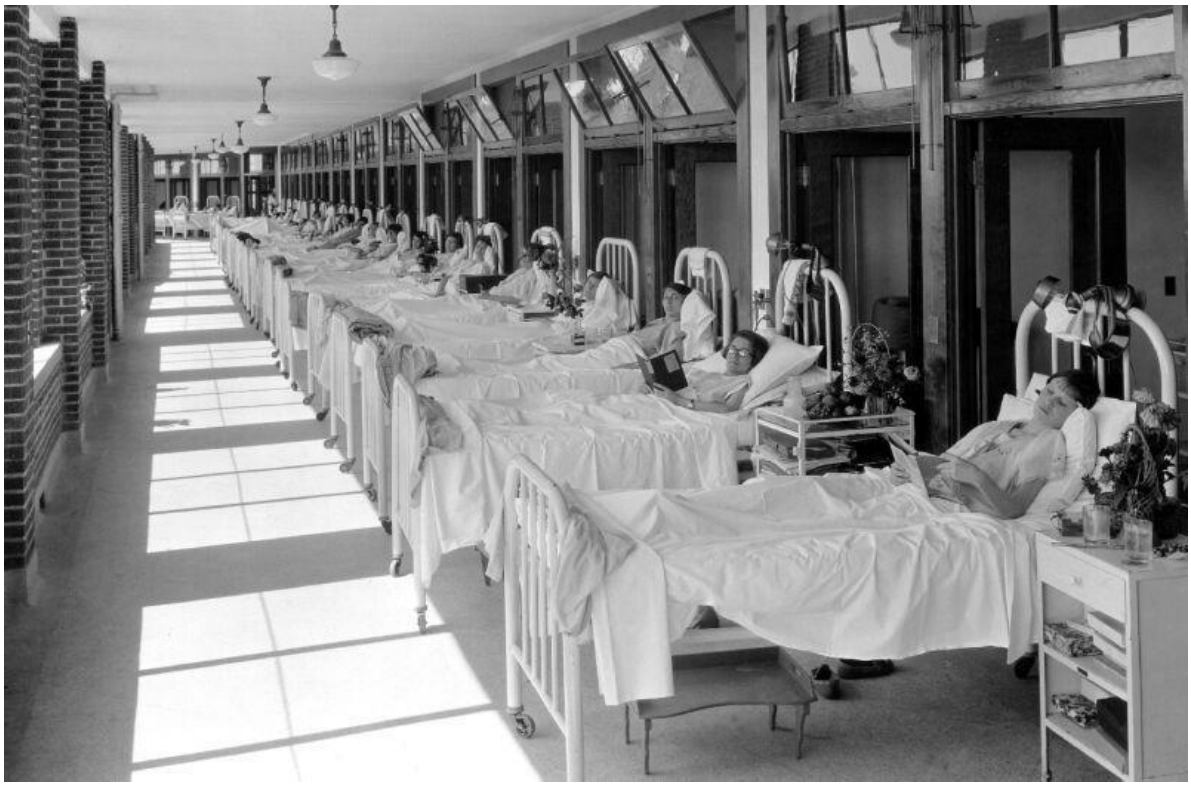


Figure 2: Warvely hills sanitorium (www.myoldkentuckyroadtrip.com)

In 1908, the French scientists Albert Calmette and Camille Guerin grew Koch's bacillus in several mediums to decrease its virulence and increase the capacity to produce immunity. This led to the famous vaccine called BCG "Bacille de Calmette et Guérin". Since its introduction in 1921, more than two billion doses of BCG have been delivered worldwide. BCG is thought to prevent the development of miliary and meningeal tuberculosis. It has not been shown to decrease the risk of pulmonary tuberculosis. Search of an effective tuberculosis vaccine remains one of the top priorities of public health organizations.

Before drug therapy, surgical treatment was common and often lifesaving. Its rationale came from an observation by Dr. James Carson, a Scottish physician who noted clinical improvement in patients who had a pleural effusion. In 1892, the Italian physician Carlo Forlanini introduced nitrogen into the pleural space based on an observation that pneumothorax led to decline in death.

Effective antibiotherapy began in 1944 with the discovery of streptomycin. It led to clinical improvement but also to the development of antibacterial resistance. Better results followed the development of PAS (para-aminosalicyclic acid), the first oral drug. The introduction of isoniazid (INH) in the early 1950s ushered in the modern era of effective chemotherapy. Modern anti-tuberculosis therapy now always requires the use of multiple drugs. Currently there are fewer than 20 agents with activity against *Mycobacterium tuberculosis* (1, 2, and 3).

2. General characteristics of *M. tuberculosis* bacilli

Mycobacterium tuberculosis (MTB) is a fairly large nonmotile rod-shaped bacterium distantly related to the Actinomycetes. Many nonpathogenic mycobacteria are components of the normal flora of humans, found most often in dry and oily locales. The rods are 2-4 micrometers in length and 0.2-0.5 um in width (4).

Mycobacterium tuberculosis is an obligate aerobe. For this reason, in the classic case of tuberculosis, MTB complexes are always found in the well-aerated upper lobes of the lungs. The bacterium is a facultative intracellular parasite, usually of macrophages, and has a slow generation time, 15-20 hours, a physiological characteristic that may contribute to its virulence.

Two media are used to grow MTB: Middlebrook's medium which is an agar based medium and Lowenstein-Jensen (LG) medium which is an egg based medium. MTB colonies are small and buff colored when grown on either medium. Both types of media contain inhibitors to keep contaminants from out-growing MTB. It takes 4-6 weeks to get visual colonies on either type of media.

Figure 3: Colonies of *Mycobacterium tuberculosis* on Lowenstein-Jensen medium (CDC web site).

Chains of cells in smears made from in vitro grown colonies often form distinctive serpentine cords. This observation was first made by Robert Koch who associated cord factor with virulent strains of the bacterium.

MTB is not classified as either Gram-positive or Gram-negative because it does not have the chemical characteristics of either, although the bacteria do contain peptidoglycan (murein) in their cell wall. If a Gram stain is performed on MTB, it stains very weakly Gram-positive or not at all (cells referred to as "ghosts").

Mycobacterium species, along with members of a related genus *Nocardia*, are classified as acid-fast bacteria due to their impermeability by certain dyes and stains. Despite this, once stained, acid-fast bacteria will retain dyes when heated and treated with acidified organic compounds. One acid-fast staining method for *Mycobacterium tuberculosis* is the Ziehl-Neelsen stain. When this method is used, the MTB smear is fixed, stained with carbol-fuchsin (a pink dye), and decolorized with acid-alcohol. The smear is counterstained with methylene-blue or certain other dyes. Acid-fast bacilli appear pink in a contrasting background.

In order to detect *Mycobacterium tuberculosis* in a sputum sample, an excess of 10,000 organisms per ml of sputum are needed to visualize the bacilli with a 100X microscope objective (1000X mag). One acid-fast bacillus/slide is regarded as "suspicious" of an MTB infection (4).

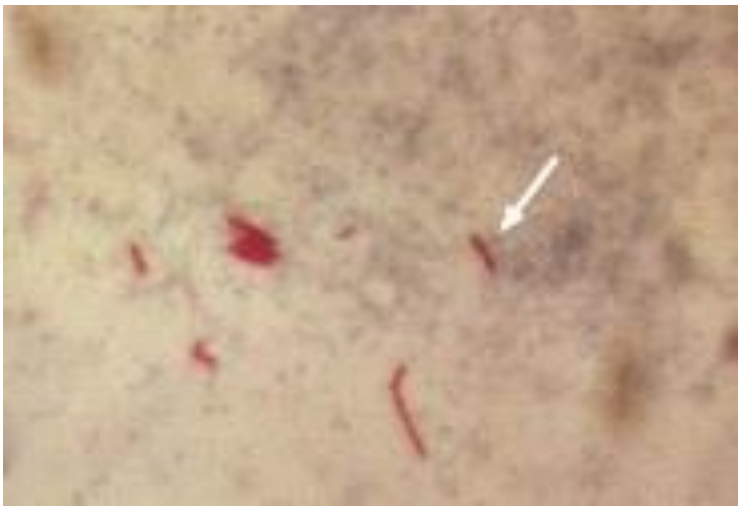


Figure 4: *Mycobacterium tuberculosis*. Acid-fast stain (CDC).

3. Tuberculosis physiopathology

Infection with TB requires inhalation of droplets, called droplet nuclei, generated by coughing of a person with pulmonary TB.

Once inhaled, the majority of the bacilli are trapped in the upper parts of the airways. The mucus produced catches foreign substances, and the cilia on the surface of the cells constantly beat the mucus and its entrapped particles upward for removal. This system provides the body an initial physical defense that prevents infection in most persons exposed to tuberculosis.

Bacteria that bypass the mucociliary system and reach the alveoli are engulfed by alveolar macrophage, but survive and multiply within the macrophages (5, 6).

Several mechanisms and macrophage receptors are involved in uptake of the mycobacteria. The mycobacterial lipoarabinomannan (LAM) is a key ligand for a macrophage receptor (7). The complement system also plays a role in the phagocytosis of the bacteria. The complement protein C3 binds to the cell wall and enhances recognition of the mycobacteria by macrophages.

The subsequent phagocytosis by macrophages initiates a cascade of events that results in either successful control of the infection, followed by latent tuberculosis, or progression to active disease, called primary progressive tuberculosis. The outcome is essentially determined by the quality of the host defenses and the balance that occurs between host defenses and the invading mycobacteria.

Successful containment of TB is dependent on the cellular immune system, mediated primarily through T-helper cells (TH1 response). T cells and macrophages form a granuloma with a center that contains necrotic material (caseous center). The granuloma serves to prevent further growth and spread of *M. tuberculosis.* These individuals are non-infectious and have latent TB infection; the majority of these patients will have a normal chest x ray and be tuberculin skin test (TST) positive (8).

Active TB typically occurs through a process of re-activation. Approximately 10% of individuals with latent infection will progress to active disease. The risk is greater within the 2 years following initial acquisition of *M. tuberculosis.* A number of conditions can alter this risk, particularly HIV infection. Immuno-compromised conditions and treatment with immunosuppressing medicines, including systemic corticosteroids and TNF-alpha antagonists, also contribute to reactivation (8).

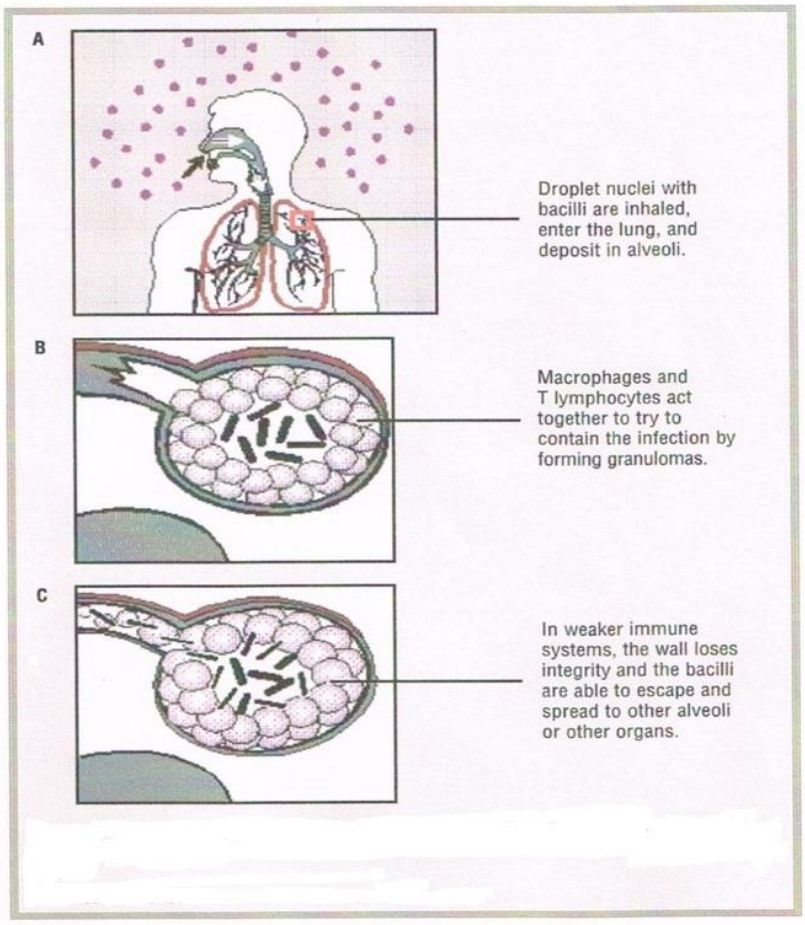


Figure 5: Pathophysiology of tuberculosis; inhalation of bacilli (A), containment in a granuloma (B), breakdown of the granuloma in less immunocompetent individuals (C). Image CDC

4. Biogenesis of the cell wall of MTB

Knowledge of the architecture of the cell envelope of mycobacteria is central to our understanding of some unsolved problems of mycobacterial diseases, such as recrudescence of tuberculosis and occurrence of the opportunistic mycobacterioses.

The cell wall structure of *Mycobacterium tuberculosis* deserves special attention because it is unique among prokaryotes, and it is a major determinant of virulence for the bacterium (4)

The mycobacterial cell wall differs from that of most other bacteria, and forms a diffusion barrier, which is 100-1000-fold less permeable to hydrophilic molecules than that of Escherichia coli. Therefore, due to the highly hydrophobic waxy cell wall, bacilli are

resistant to digestion by lysosomal enzymes and also resistant to the killing effects of macrophages.

The cell envelope distinguishes species of the *Mycobacterium* genus from the other prokaryotes. It consists of three major segments: the plasma membrane, the cell wall core and the outermost layer (9).

The cell wall core is made up of peptidoglycan in covalent attachment with arabinogalactan which is in turn esterified to α-alkyl, β-hydroxy long-chain (C_{70}-C_{90}) fatty acids known as mycolic acids (10).

The outermost layer consists of a variety of noncovalently attached (glycol) lipids, polysaccharides, lipoglycans and proteins, including pore forming proteins.
The surface exposed material of this outer layer, also called capsule, is essentially composed of polysaccharides and proteins with only minor amounts of lipids. This capsule surrounds only pathogenic mycobacteria and is postulated to be a defense barrier against the phagocytic cells (10, 11 and 12).
The capsular polysaccharides are represented by: α D-glucan, D-arabino-D-mannan and D-mannan corresponding to molecular masses of 120, 13 and 4 kDa, respectively. The α D-glucan is the major carbohydrate of the capsule of *M. tuberculosis*, representing up to 80% of the extracellular polysaccharides (13 and 14)).

The mycobacterial cell wall structure is represented in the Figure 6.

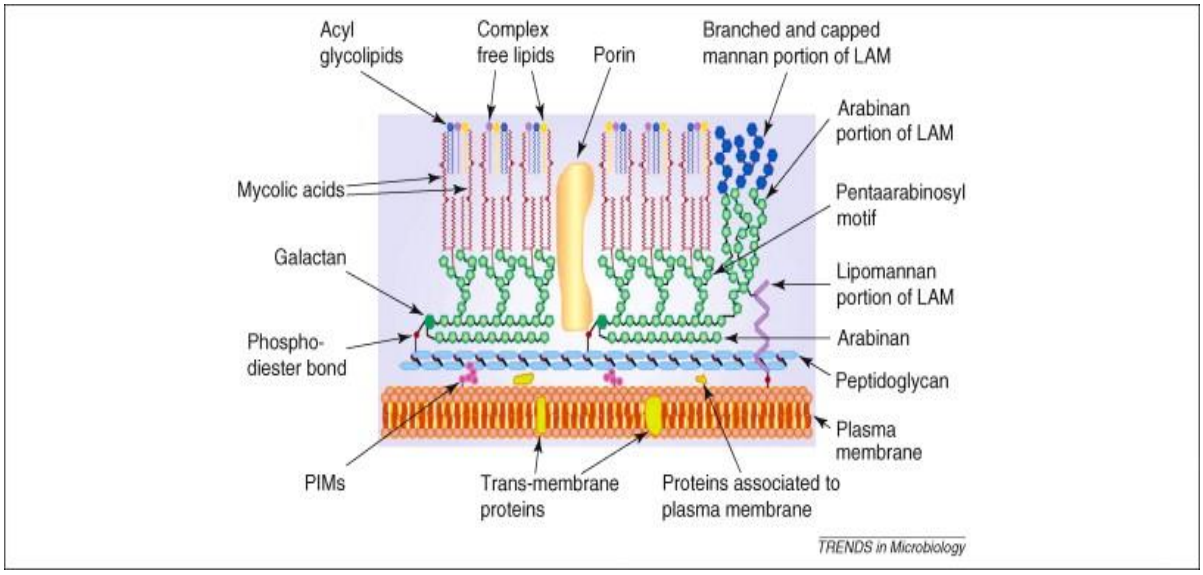


Figure 6: Mycobacteria cell wall structure (15)

5. Immunology of tuberculosis

5-1 Acquisition of immunity against *M. tuberculosis*

In 1880, R. Koch described a delayed hyper-sensitivity reaction to mycobacterial extracts, first in guinea pigs and then in human patients with active tuberculosis. In 1934, Seifert obtained a more purified extract of MTB proteins (PPD), which later became the reference used in tuberculin tests. Although these bacterial extracts are useful for the diagnosis of latent tuberculosis, as they produce a delayed hypersensitivity reaction in sensitized subjects, they do not confer immunity against the disease. Only infections with attenuated bacillus, such as *M. bovis* Calmette-Guérin, or with *M. tuberculosis* itself, confer some degree of protection against a secondary infection with MTB. Immunity cannot be transferred to animals by immune serum, but requires the transfer of lymphoid cells, as originally demonstrated by Chase in 1945. The transfer of CD4 but not CD8 T lymphocytes will protect immunodeficient mice; it is therefore clear that memory CD4 lymphocytes are required to maintain immune protection against MTB (16).

5-2 Cellular immunity in tuberculosis

Protective anti-mycobacterial immune response involves mainly T lymphocytes activating the macrophages and their microbicidal functions through the release of interferon γ (IFNγ). This leads to the formation of granulomas, crucial to the containment of mycobacteria.

Macrophages/dendritic cells (DC) are found in the center of these granulomas, along with mycobacteria surrounded by T lymphocytes which provide the proper activation. It has been shown that MTB enters DC after binding to the recently identified lectin DC specific inter-cellular adhesion molecular-3 grabbing non-integrin (DC-SIGN). The mycobacterial specific lipoglycan lipoarabinomannan (LAM) is identified as a key ligand of DC-SIGN (17).

CD4 T cells are most important in the protective response against *M. tuberculosis.* The primary effector function of CD4 T cells is the production of IFN-ϒ and possibly other cytokines, sufficient to activate macrophages. CD8 T cells are also capable of secreting cytokines such as IFN-ϒ and IL-4 and thus may play a role in regulating the balance Th1 and Th2 cells in the lung of patients with pulmonary TB. CD4 and CD8 populations have been analyzed from patients with rapid, slow and intermediate regression of the disease while receiving therapy and found that slow regression was associated with an increase in CD8 cells in Broncho alveolar lavage (5, 16 and 18).

5-3 Role of cytokines

5-3-1 Interferon ϒ (IFN-ϒ)

IFN-ϒ is a key cytokine in control of MtB infection and produced by both CD4 and CD8 cells, as well by natural killer (NK) cells. IFN-ϒ might augment antigen presentation, leading to recruitment of CD4 T-lymphocyte and/or cytotoxic T-lymphocytes which might participate in mycobacterial killing (5).

5-3-2 Interleukin 12 (IL-12)

IL-12 is induced following phagocytosis of *M. tuberculosis* bacilli by macrophages and dendritic cells, which leads to development of Th1 response with production of IFN-ϒ (5).

5-3-3 Tumor necrosis factor (TNF-α)

TNF-α play multiple roles in immune and pathologic responses in TB. It has been implicated in immunopathologic response and is often major factor in host-mediated destruction in lung tissue (5).

5-3-4 Interleukin 1 (IL-1)

IL-1 along with TNF-α plays an important role in the acute phase response such as fever and cachexia. IL-1 facilitates IL-2 release and has been implicated in immunosuppressive mechanisms which is an important feature in tuberculoimmunity (5).

5-3-5 Interleukin 2 (IL-2)

IL-2 has a pivotal role in generating an immune response by inducing an expansion of the pool of lymphocytes specific for an antigen (5).

5-3-6 Interleukin 6 (IL-6)

IL-6 is important in the initial innate response to the pathogen: inflammation, hematopoiesis and differentiation of T cells (5).

5-3-7 Interleukin 10

IL-10 is considered to be an anti- inflammatory cytokine. This cytokine, produced by macrophages and T cells during *M. tuberculosis* infection, possesses macrophage-deactivating properties, including down-regulation of IL-12 production, which in turn decreases IFN-γ production by T cells. IL-10 directly inhibits CD4+ T cell responses, as well as by inhibiting APC function of cells infected with mycobacteria (5).

5-3-8 Transforming growth factor-beta (TGF-β)

TGF-β is present in the granulomatous lesions of TB patients and is produced by human monocytes after stimulation with *M. tuberculosis* or lipoarabinomannan. TGF-β has important anti- inflammatory effects, including deactivation of macrophage production of reactive oxygen intermediates and reactive nitrogen intermediates, inhibition of T cell proliferation, interference with NK and CTL function and down-regulation of IFN-γ, TNF-α and IL-1 release (5,19).

5-4 Role of antibodies against mycobacteria

Antibodies against MTB may not allow the transfer of immunity against tuberculosis, they seem to have an opsoning role and thereby improve phagocytosis by macrophages or the cytotoxic actions of killer lymphocytes. Mycobacterium specific antibodies seem capable of enhancing both innate and cell-mediated immune responses to mycobacteria. It is possible that their absence in the late stage of acquired immunodeficiency syndrome (AIDS) could favor the dissemination of atypical mycobacteria, at least those belonging to the *M. avium* complex (16).

5-5 HIV-TB coinfection

Persons with HIV infection are at increased risk of rapid progression of a recently acquired infection, as well as of re-activation of latent infection. TB is the most common opportunistic infection occurring among HIV-positive persons in India, and studies from different parts of the country have estimated that 60 to 70 per cent of HIV positive patients will develop TB in their lifetime (20). Differences in HIV-positive TB, as opposed to HIV- negative TB, include a higher proportion of cases with extra-pulmonary or disseminated disease, a higher frequency of false-negative tuberculin skin tests, atypical features on chest radiographs, fewer cavitating lung lesions, a higher rate of adverse drug reactions, the presence of other AIDS-associated manifestations and a higher death rate.

TB and HIV infections are both intracellular and known to have profound influence on the progression of each other. HIV infection brings about the reduction in CD4+ T cells, which play a main role in immunity to TB. This is reflected in the integrity of the cellular immune response, namely the granuloma. Apart from the reduction in number, HIV also causes functional abnormality of CD4+ and CD8+ cells. Likewise, TB infection also accelerates the

progression of HIV disease from asymptomatic infection to AIDS to death. A potent activator of HIV replication within T cells is TNF-α, which is produced by activated macrophages within granuloma as a response to tubercle infection (5, 21).

6. Tuberculosis diagnostic

A complete medical evaluation for TB disease includes the following five components (22):

- medical history;
- physical examination;
- immunological test for *M. tuberculosis* infection;
- chest radiograph;
- bacteriologic examination of clinical specimens.

6-1 Medical history

When conducting a medical history, the clinician should ask if any symptoms of TB disease are present; if so, for how long, and if there has been known exposure to a person with infectious TB disease. Equally important is obtaining information on whether or not the person has been diagnosed in the past with latent tuberculosis infection (LTBI) or TB disease.

Clinicians should determine if the patient has underlying medical conditions, especially human immunodeficiency virus (HIV) infection or diabetes, that increase the risk for progression to TB disease in those latently infected with *M. tuberculosis* (22).

6-2 Physical examination

The clinical manifestations of tuberculosis are quite variable and depend on a number of factors. Before the beginning of the epidemic of infection with HIV, approximately 85% of reported tuberculosis cases were limited to the lungs, with the remaining 15% involving only non-pulmonary or both pulmonary and non-pulmonary sites. This proportional distribution is substantially different among persons with HIV infection.

A physical examination is an essential part of the evaluation of any patient. It cannot be used to confirm or rule out TB disease, but it can provide valuable information about the patient's overall condition, inform the method of diagnosis, and reveal other factors that may affect TB disease treatment, if diagnosed (22, 23).

6-2-1 Pulmonary tuberculosis

Cough is the most common symptom of pulmonary tuberculosis. Early in the course of the illness it may be nonproductive, but subsequently, as inflammation and tissue necrosis ensue, sputum is usually produced and is key to most of our diagnostic methods. Hemoptysis may rarely be a presenting symptom but usually is the result of previous disease and does not necessarily indicate active tuberculosis. The other symptoms encountered are chest pain, loss of appetite, unexplained weight of loss, night sweats, fever and fatigue (23).

6-2-2 Extra-pulmonary tuberculosis

Extra-pulmonary tuberculosis usually presents more of a diagnostic problem than pulmonary tuberculosis. In part this relates to its being less common and, therefore, less familiar to most clinicians. In addition, extra-pulmonary tuberculosis involves relatively inaccessible sites and, because of the nature of the sites involved, fewer bacilli can cause much greater damage. The combination of small numbers of bacilli and inaccessible sites causes bacteriologic confirmation of a diagnosis to be more difficult, and invasive procedures are frequently required to establish a diagnosis (23).

6-2-2-1 Disseminated tuberculosis

Occurs because of the inadequacy of host defenses in containing tuberculous infection.
The presenting symptoms and signs are generally nonspecific and are dominated by systemic effects, particularly fever, weight loss, night sweats, anorexia, and weakness. Other symptoms depend on the relative severity of disease in the organs involved. A productive cough is common because most patients with disseminated disease also have pulmonary involvement. The chest film is abnormal in most but not all patients with disseminated tuberculosis. Overall, it appears that at the time of diagnosis approximately 85% of patients have the characteristic radiographic findings of miliary tuberculosis. In patients with HIV infection the radiographic pattern is usually one of diffuse in- filtration rather than discrete nodules (23).

6-2-2-2 Lymph node tuberculosis

Tuberculous lymphadenitis usually presents as painless swelling of one or more lymph nodes. The nodes involved most commonly are those of the posterior or anterior cervical chain or those in the supraclavicular fossa. Frequently the process is bilateral and other noncontiguous groups of nodes can be involved. At least initially the nodes are discrete and

the overlying skin is normal. In HIV-infected persons, lymphadenitis is commonly associated with multiple organ involvement (23).

6-2-2-3 Pleural tuberculosis

Early in the course of a tuberculous infection a few organisms may gain access to the pleural space and, in the presence of cell-mediated immunity, cause a hypersensitivity response. Commonly, this form of tuberculous pleuritis goes unnoticed, and the process resolves spontaneously. In some patients, however, tuberculous involvement of the pleura is manifested as an acute illness with fever and pleuritic pain. If the effusion is large enough, dyspnea may occur, although the effusions generally are small and rarely bilateral (23).

6-2-2-4 Genitourinary tuberculosis

In patients with genitourinary tuberculosis, local symptoms predominate and systemic symptoms are less common. Dysuria, hematuria, and frequent urination are common, and flank pain may also be noted. However, the symptoms may be subtle, and, often, there is advanced destruction of the kidneys by the time a diagnosis is established (23).

6-2-2-5 Skeletal tuberculosis

The usual presenting symptom of skeletal tuberculosis is pain. Since the epiphyseal region of bones is highly vascularized in infants and young children, bone involvement with tuberculosis is much more common in children than adults (23).

6-2-2-6 Central nervous tuberculosis

Tuberculous meningitis is a particularly devastating disease. Meningitis can result from direct meningeal seeding and proliferation during a tuberculous bacillemia either at the time of initial infection or at the time of breakdown of an old pulmonary focus, or can result from breakdown of an old parameningeal focus with rupture into the subarachnoid space (23).

6-2-2-7 Abdominal tuberculosis

Tuberculosis can involve any intra-abdominal organ as well as the peritoneum, and the clinical manifestations depend on the areas of involvement. The most common sites of involvement are the terminal ileum and cecum, with other portions of the colon (23).

6-2-2-8 Pericardial tuberculosis

The symptoms, physical findings, and laboratory abnormalities associated with tuberculous pericarditis may be the result of either the infectious process itself or the pericardial inflammation causing pain, effusion, and eventually hemodynamic effects. The systemic symptoms produced by the infection are quite nonspecific. Fever, weight loss, and night sweats are common in reported series (23).

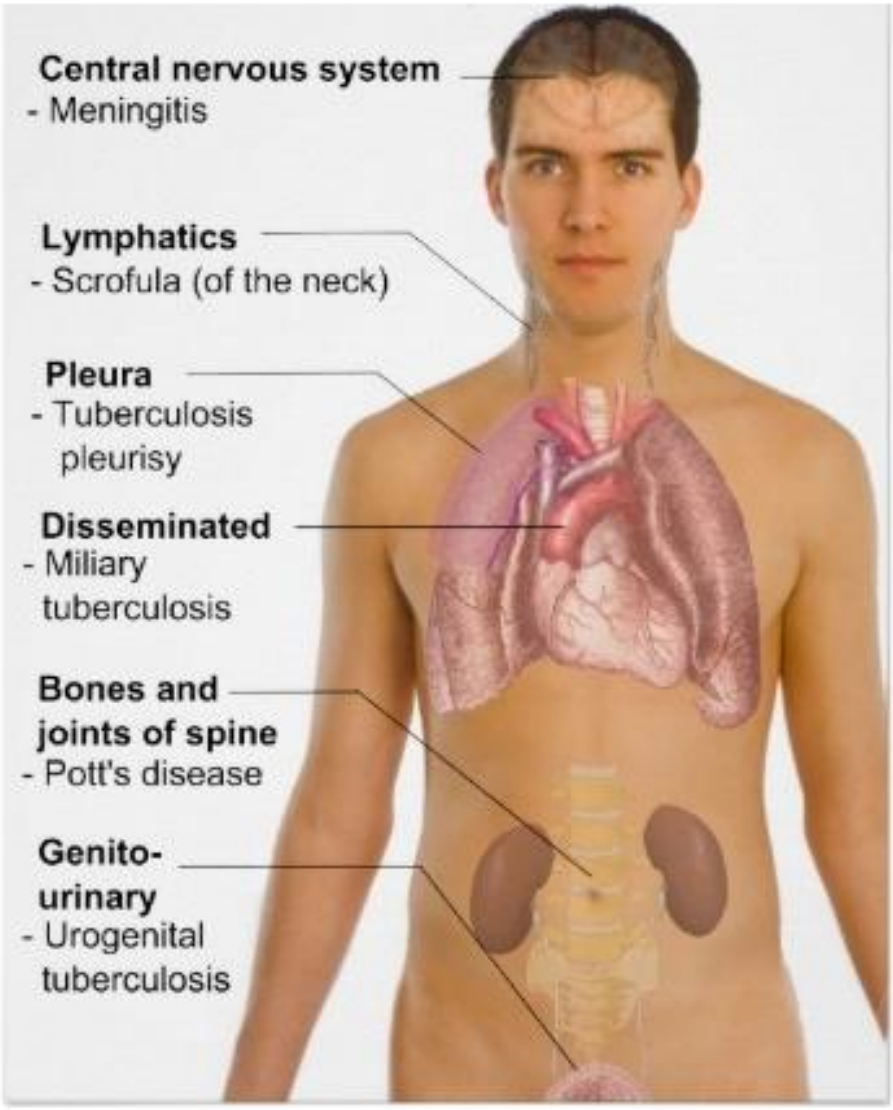


Figure 7: Main sites of extra pulmonary TB, source (http://commons.wikimedia.org/wiki/File:Extrapulmonary_tuberculosis_symptoms.png)

6-3 Immunological tests for *M. tuberculosis* infection

Currently, they are two methods available for the detection of *M. tuberculosis* infection: the Mantoux tuberculin skin test (TST) and Interferon-gamma release assays (IGRAs).
These tests help clinicians differentiate people infected with *M. tuberculosis* from those uninfected. However, a negative reaction to any of the test does not exclude the diagnosis of TB disease or LTBI.

6-3-1 Tuberculin skin test

The tuberculin test is based on the fact that infection with *M. tuberculosis* produces a delayed-type hypersensitivity reaction to certain antigenic components of the organism that are contained in extracts of culture filtrates called “tuberculins” (23, 24).

The tuberculin protein purified derivative (PPD) is isolated from a culture filtrate of tubercle bacilli by protein precipitation. The most commonly employed methods of precipitation use either ammonium sulfate or trichloroacetic acid (25, 26).

T cells sensitized by prior infection are recruited to the skin site where they release lymphokines. These lymphokines induce induration through local vasodilatation, edema, fibrin deposition, and recruitment of other inflammatory cells to the area. Features of the reaction include its delayed course, reaching a peak more than 24 h after injection of the antigen, its indurated character, and its occasional vesiculation and necrosis. Reactivity of the PPD provides a general measure of a person’s cellular immune responsiveness (23, 27).

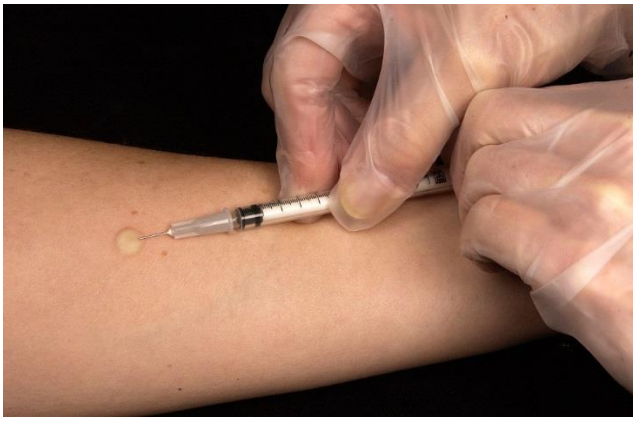


Figure 8: Mantoux skin test, source CDC

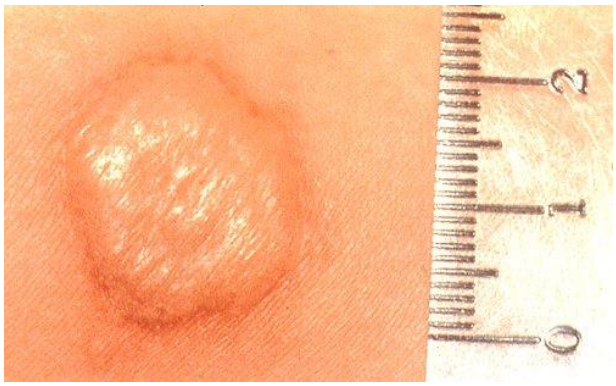


Figure 9: Size of induration measured 48-72 hours later, source (http://www.lung.ca/tb/tbtoday/tbdiagnosis/skin_test.html)

6-3-2 Interferon-gamma release assays (IGRA)

IGRAs measure a person's immune reactivity to *M. tuberculosis*. White blood cells from most persons that have been infected with *M. tuberculosis* will release interferon-gamma (IFN-ϒ) when mixed with antigens (substances that can produce an immune response) derived from *M. tuberculosis*. To conduct the tests, fresh blood samples are mixed with antigens and controls. The antigens, testing methods, and interpretation criteria for IGRAs differ. Today, there are 2 tests available on the market: the Quantiferon-TB Gold from Cellestis (Figure 10) and the T-SPOT.*TB* from Oxford Immunotech (Figure 11). The comparison between both tests is shown in table 2.

Figure 10: Diagnostic kit QuantiFERON-TB from Cellestis

Figure 11: Diagnostic kit T-SPOT.TB from Oxford Immunotech

What are the advantages?

- Requires a single patient visit to conduct the test.
- Results can be available within 24 hours.
- Does not boost responses measured by subsequent tests.
- Prior BCG (bacilli de Calmette-Guérin) vaccination does not cause a false-positive IGRA test result.

What are the disadvantages and limitations of IGRAs?

- Blood samples must be processed within 8-30 hours after collection while white blood cells are still viable.
- Errors in collecting or transporting blood specimens or in running and interpreting the assay can decrease the accuracy of IGRAs.
- Cannot predict who will progress to TB disease in the future.

	Q u a n t i F E R O N ® -TB Gold	T-SPOT ®.TB
Initial process	Process whole blood within 16 hours	Process peripheral blood mononuclear cells (PBMCs) within 8 hours or if T-cell Xtend® is used, within 30 hours.
M. tuberculosis Antigen	Single mixture of synthetic peptides representing ESAT-6, CFP-10 and TB7.7	Separate mixtures of synthetic peptides representing ESAT-6 and CFP-10
Measurement	IFN-ϒ concentration	Number of IFN-ϒ producing cells (spots)
Possible results	Positive, negative, indeterminate	Positive, negative, indeterminate, borderline

Table 1: Differences in currently available IGRAs tests

6-4 Chest radiograph

With pulmonary TB being the most common form of disease, the chest radiograph is useful for diagnosis of TB disease. Chest abnormalities can suggest pulmonary TB disease. A posterior-anterior radiograph of the chest is the standard view used for the detection of TB-related chest abnormalities (Figure 12). In some cases, especially in children, a lateral view may be helpful.

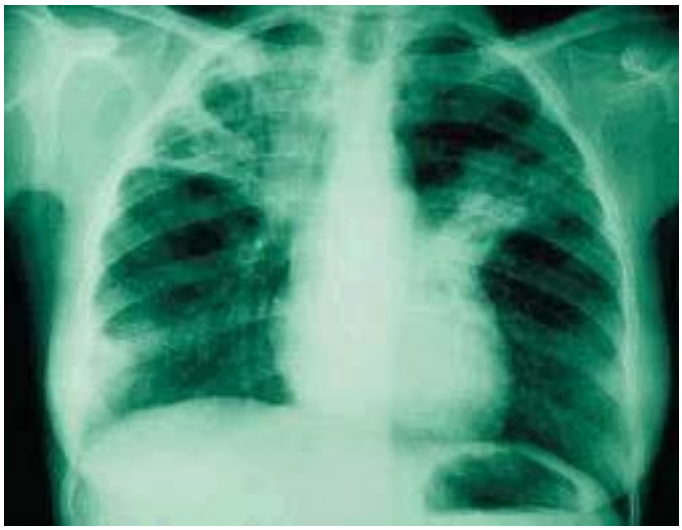


Figure 12: Chest X-ray from a patient with pulmonary TB

6-5 Bacteriologic examination of clinical specimens

Examinations of clinical specimens (e.g., sputum, urine, or cerebrospinal fluid) are of critical diagnostic importance. The specimens should be examined and cultured in a laboratory specialized in testing for *M. tuberculosis*. The bacteriologic examination has the following steps:

- specimen collection;
- specimen digestion and decontamination;
- staining and microscopic examination;
- direct detection of *M. tuberculosis* in clinical specimen using nucleic acid amplification (NAA);
- specimen culturing and identification;
- drug-susceptibility testing.

6-5-1 Specimen collection

For diagnostic purposes, all persons suspected of having TB disease at any site should have sputum specimens collected for an AFB smear and culture, even those without respiratory symptoms. At least three consecutive sputum specimens are needed, each collected in 8 to 24 hour intervals, with at least one being an early morning specimen. However, smears and cultures perform poorly in extra-pulmonary TB (American Thoracic Society, Chapter 4: Diagnosis of TB disease).

For pulmonary tuberculosis, specimen collection can be obtained with the following methods (Table 2):

- coughing, the most commonly used method of sputum collection;
- sputum induction, for patients unable to cough up sputum; deep sputum producing coughing may be induced by inhalation of an aerosol of warm, sterile, hypertonic saline;
- bronchoscopy, if possible avoid bronchoscopy on patients with suspected TB or confirmed TB disease;
- gastric aspiration, procedure sometimes used to obtain specimen for culture when a patient cannot cough up adequate sputum; this procedure is particularly useful for diagnosis in children, who are often unable to cough up sputum.

Method	Description	Advantage	Disadvantage
Spontaneous sputum sample	Patient coughs up sputum into a sterile container	Inexpensive Easy to do	Patient may not be able to cough up sputum without assistance or may spit up saliva instead of sputum Health care worker has to coach and supervise the patient when collecting sputum
Sputum induction	Patient inhales saline mist which can cause a deep cough	Easy to do Use to obtain sputum when coughing sputum is not productive	Specimens may be watery and may be confused with saliva (should be labeled "induced specimen") Requires special equipment May cause bronchospasm
Bronchoscopy	Bronchoscope is passed through the mouse or nose directly into the diseased portion of the lung, and sputum or lung tissue is removed	Use to obtain sputum when coughing or inducing sputum is not productive or other diagnoses are being considered	Most expensive and invasive procedure Requires special equipment Must be done by a specialist in a hospital or clinic Requires anesthesia
Gastric washing	Tube is inserted through the patient's mouth or nose and passed into the stomach to get a sample of gastric secretions that contain sputum that has been coughed into the throat and then swallowed	Use to obtain samples in children, who do not produce sputum when they cough	Must be done as soon as patient wakes up in the morning; patient may be required to stay in hospital Can be uncomfortable for the patient

Table 2: Methods of obtaining a sputum specimen (22)

6-5-2 Specimen digestion and decontamination

Most clinical specimens contain an abundance of non-mycobacterial organisms. On arrival in the laboratory, most specimens are homogenized with a mucolytic agent (such as N-acetyl-L-cysteine) and decontaminant (such as a 1-2% sodium hydroxide solution) to render the bacterial contaminants nonviable (23).

6-5-3 Staining and microscopic examination

Smears may be prepared directly from clinical specimens or from concentrated preparations. The acid-fast staining procedure depends on the ability of mycobacteria to retain dye when treated with mineral acid or an acid–alcohol solution. Two procedures are commonly used for acid–fast staining: the carbolfuchsin methods, which include the Ziehl-Neelsen (Figure 13) and Kinyoun methods, and a fluorochrome procedure using auramine-O or auramine–rhodamine dyes (Figure 14).

Several quantitative studies have shown that there must be 5,000 to 10,000 bacilli per milliliter of specimen to allow the detection of bacteria in stained smears. In contrast, 10 to 100 organisms are needed for a positive culture.

Figure 13: Ziehl–Neelsen acid-fast stain of some sputum positive for tuberculosis. Arrows indicate mycobacteria which appear as red rods. [Image taken from http://www.courses.ahc.umn.edu/medical-school/IDis/Images/M.tuberculosis.gif.]

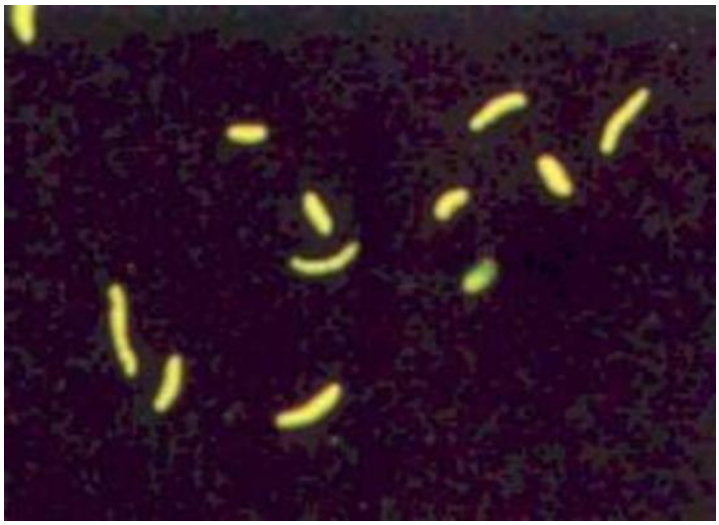


Figure 14: *Mycobacterium*-auramine stain
http://www.lung.ca/tb/abouttb/what/causes_tb.html

Concentration procedures in which a liquefied specimen is centrifuged and the sediment is used for staining increases the sensitivity of the test; thus, smears of concentrated material are preferred. Negative smears, however, do not preclude tuberculosis disease. Various studies have indicated that 50 to 80% of patients with pulmonary tuberculosis will have positive sputum smears. Factors influencing the sensitivity of smears include staining technique, centrifugation speed, reader experience, and the prevalence of tuberculosis disease in the population being tested. In reading smears, the microscopist should provide the clinician with a rough estimate of the number of AFB detected.
The World Health Organization (WHO) recently revised its policies on smear microscopy. It now recommends that the number of specimens to be examined for screening of TB cases be reduced from three to two, in places where a well- functioning external quality assurance system exists, where the workload is very high, and where human resources are limited. The revised WHO definition of a new sputum smear- positive pulmonary TB case is based on the presence of at least one acid fast bacillus in at least one sputum sample in countries with a well-functioning external quality assurance system. These new policies have major implications for resource-poor settings with high TB prevalence where sputum microscopy is the main or only diagnostic test available.

Light-emitting diodes (LED) have been developed to offer the benefits of fluorescence microscopy without the associated costs (28). In 2009, the evidence for the efficacy of LED microscopy was assessed by the World Health Organization (WHO), on the basis of standards appropriate for evaluating both the accuracy and the effect of new TB diagnostics

on patients and public health. The results showed that the accuracy of LED microscopy was equivalent to that of international reference standards, it was more sensitive than conventional Ziehl-Neelsen microscopy and it had qualitative, operational and cost advantages over both conventional fluorescence and Ziehl-Neelsen microscopy (29).

On the basis of these findings, WHO recommends that conventional fluorescence microscopy be replaced by LED microscopy, and that LED microscopy be phased in as an alternative for conventional Ziehl-Neelsen light microscopy (30).

Figure 15: Smear examination with LED microscopy in an Indian decentralized health care center (http://www.imaging-git.com/news/led-fluorescence-microscopy-detection-tuberculosis).

6-5-4 Direct detection of *M. tuberculosis* in clinical specimen using nucleic acid amplification tests (NAAT)

Microscopy is rapid, specific and inexpensive but often lack of sensitivity. NAAT which can give results in 3 to 6 hours have been developed to address these issues. These test amplify DNA or RNA segment of the microorganisms. The PCR is the most common NAAT. Tests include "in house" methods based on a protocol developed in a noncommercial laboratory (home brew) and commercial kits. Systemic reviews of different studies indicate that the accuracy of NAATs varies more among in-house NAATs than commercial products. If these tests are widely used in many advanced countries, it is not routinely applicable in developing countries due to its high cost, complicated procedures, insufficient laboratory facilities and shortage of skilled technologists (31, 32).

Recently new tests have been developed to simplify the methods:

- Loop-mediated isothermal amplification (LAMP);
- Xpert® MTB/RIF from Cepheid.

A new Lamp test kit including a simple DNA extraction device has been developed for commercial use. The test is designed to be performed on untreated sputum. It is a simple method that does not require expensive devices or detection system (33, 34).

The Gene Xpert MTB/RIF is a novel automated molecular diagnostic system recently endorsed by the World Health Organization (35, 36, 37). This system allows the simultaneous detection of *M. tuberculosis complex* and resistance to rifampicin directly from the sputum (38).

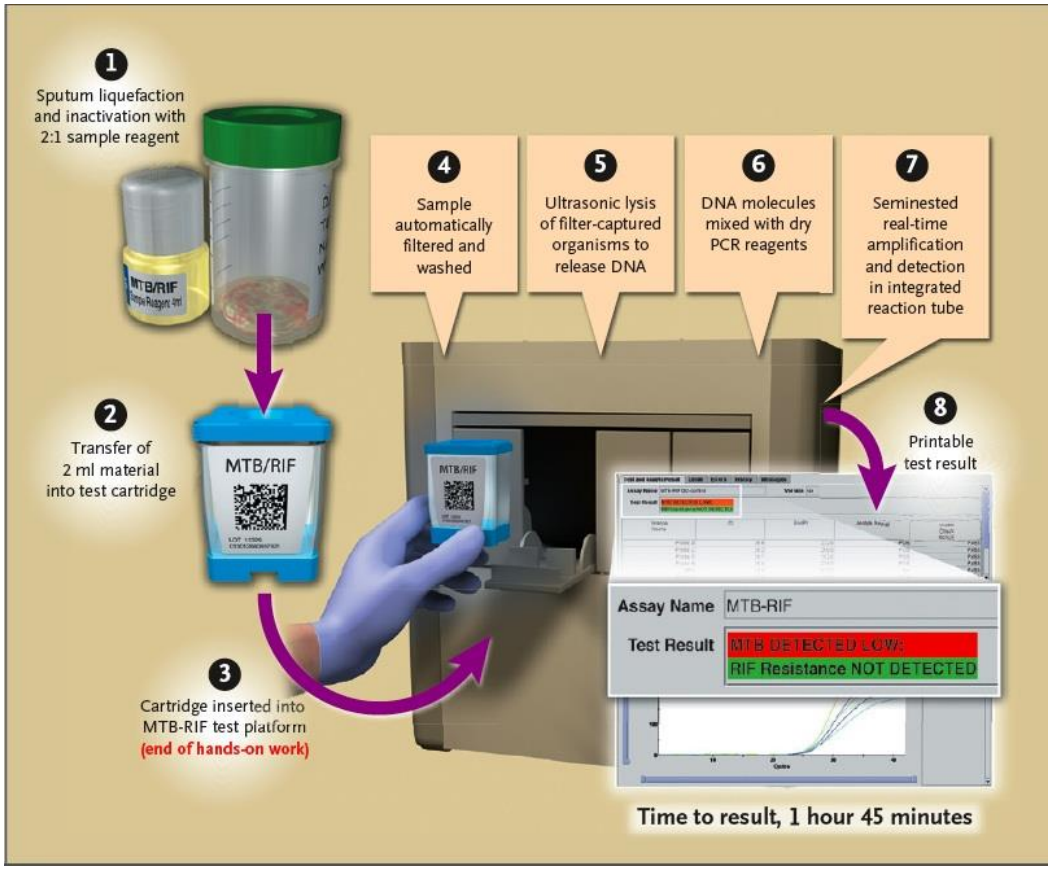


Figure 16: Gene Xpert system http://www.biotechniques.com/news/Affordable-PCR-TB-diagnostic-tool-developed/biotechniques-303309.html

6-5-5 Specimen culturing and identification

All clinical specimens suspected of containing mycobacteria should be inoculated (after appropriate digestion and decontamination) onto culture media for four reasons (23):

- culture is much more sensitive than microscopy, being able to detect as few as 10 bacteria/ml of material;
- growth of the organisms is necessary for precise species identification;
- drug susceptibility testing requires culture of the organisms;
- genotyping of cultured organisms may be useful to identify epidemiological links between patients or to detect laboratory cross-contamination.

Three different types of traditional culture media are available: egg based (Löwenstein–Jensen), agar based (Middlebrook 7H10 or 7H11 medium), and liquid (Middlebrook 7H12 and other commercially available broths), and each can be made into selective media by adding antibiotics. Of the solid media, growth of mycobacteria tends to be slightly better on the egg-based medium but more rapid on the agar medium. Growth in liquid media is faster than growth on solid media. However, liquid media can be used for primary isolation of mycobacteria from non-sterile sites only if supplemented with an antibiotic cocktail.

A major improvement in mycobacteriology has been the development of commercial broth systems for mycobacterial growth detection. Automated culture systems such as BACTEC MGIT Becton Dickinson (Figure 17 and BacT/ALERT 3D from bioMérieux (Figure 18).

Liquid systems allow for rapid growth detection of mycobacterial growth within 1-3 weeks compared with solid media, where growth takes 3–8 weeks, whereas agar media provides an opportunity to examine colony morphology and detect mixed cultures (39, 40, 41, 42, 43).

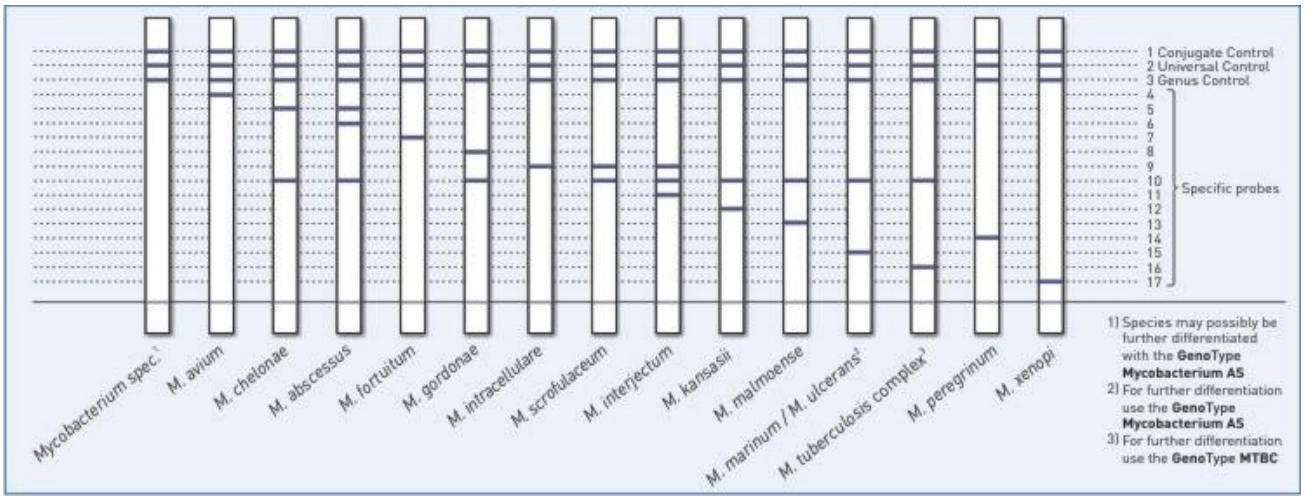
Figure 17: BACTEC MGIT system from Becton & Dickinson

Figure 18: BacT/Alert 3 D system from bioMérieux

Traditionally, mycobacteria are identified by phenotypic methods, based on culture, such as morphological characteristics, growth rates, preferred growth temperature, pigmentation and on a series of biochemical tests. Testing is laborious, difficult and time-consuming, requiring several weeks for adequate growth, and sometimes misidentification may occur because different species may have indistinguishable morphological and biochemical profiles.

In the last decade, advances in molecular methods have facilitated the rapid and reliable identification of many mycobacterial species. Nucleic acid probes, species-specific PCR, reverse hybridization and 16S rRNA sequencing have been evaluated for application in clinical laboratories. The first commercially available method was the AccuProbe (Gen- Probe Inc.), based on species-specific DNA probes that hybridize to rRNA for the identification of several important mycobacteria, including the *M. tuberculosis* complex, *M. avium*, *M. intracellulare*, the *M. avium* complex, *M. kansasii* and *M. gordonae*.

More recently, other molecular commercial systems have also been introduced for the rapid identification of *M. tuberculosis* complex: the INNO- LiPA MYCOBACTERIA v2 (Innogenetics NV, Ghent, Belgium), and the Geno- Type MTBC and GenoType Mycobacterium (Figure 26) (Hain Lifesciences, Nehren, Germany). INNO-LiPA MYCOBACTERIA v2 is a line probe assay that simultaneously detects and identifies the genus *Mycobacterium* and 16 different mycobacterial species. It is based on nucleotide differences in the 16S-23S rRNA gene spacers. The GenoType MTBC and GenoType *Mycobacterium* are also based on the reverse line probe hybridization assay and are intended for the differentiation of members of the *M. tuberculosis* complex and for the identification of 35 mycobacteria species including *M. tuberculosis*, respectively. The GenoType MTBC is based on a 23S rRNA gene fragment specific for the *M. tuberculosis* complex, together with gyrB sequence polymorphisms, and the RD1 deletion for identification of *M. bovis* BCG. Several “in-house” techniques are also available with sequencing of the 16S rRNA gene as the reference standard to which all other new techniques are generally compared.



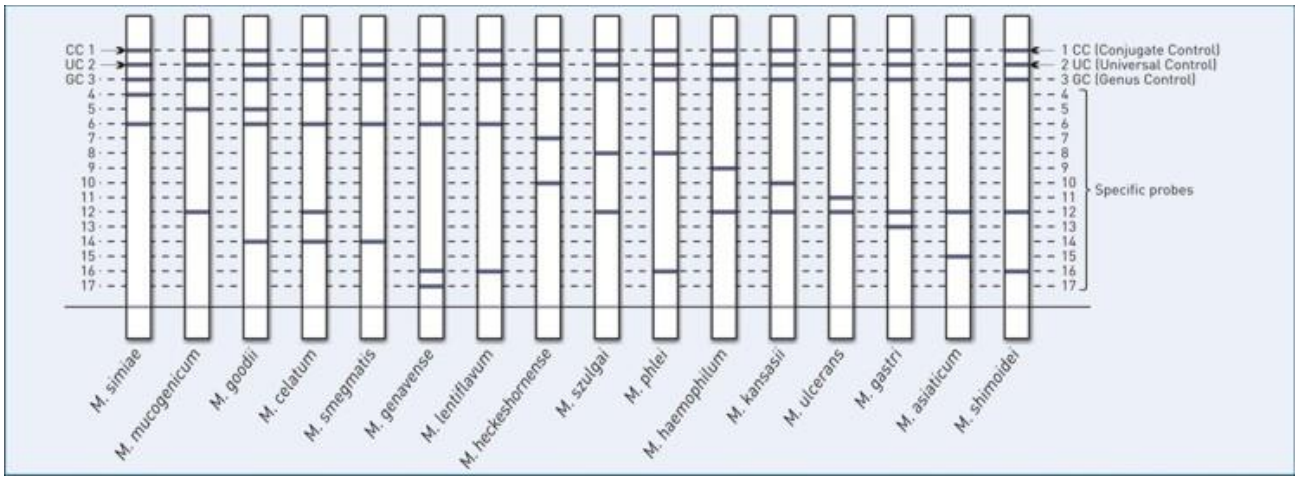


Figure 19: GenoType® Mycobacterium CM/AS: Identification of the *M. tuberculosis* complex and 40 of the most common NTM species from cultivated samples

6-5-6 Drug-susceptibility testing

Drug susceptibility tests (DST) should be performed on initial isolates from all patients in order to identify what should be an effective antituberculous regimen. There are a variety of methods to determine the susceptibility *of Mycobacterium tuberculosis* to antituberculosis drugs, none of them is perfect. DST can be determined either by conventional culture methods or by molecular assays (44). Conventional culture methods using egg- or agar-based media are still the most utilized in many countries. Although the long turnaround time of DST results displeases physicians for the purpose of case management. The standard methods using Löwenstein-Jensen medium include the proportion method, the absolute concentration method and the resistant ratio method, which are fairly well standardized with clinical samples, at least for the major antituberculosis drugs. Among conventional methods,

the proportion method is the most preferred choice, but the absolute concentration method is also commonly used on account of its technical simplicity for inoculum preparation and for reading the results.

Newer rapid phenotypic techniques based on liquid culture have been developed. There are methods that can detect bacterial growth based on CO_2 production (BACTEC 460) or oxygen consumption (MGIT) (45).

Growing concerns about the spread of multidrug-resistant tuberculosis (MDR-TB) and the emergence of extensively drug resistant TB (XDR-TB) have triggered interest in the development of rapid tests for the detection of drug resistant TB based on molecular test. New molecular commercial tests have been developed to detect rifampicin (RIF) and insoniazid (INH) resistance. These assays detect mutations in the *rpoB* gene for RIF resistance, the *katG* gene for high level INH resistance and the *inhA* gene for the low level INH resistance directly from smear positive sputum. Results are available within 1 day (46, 47).

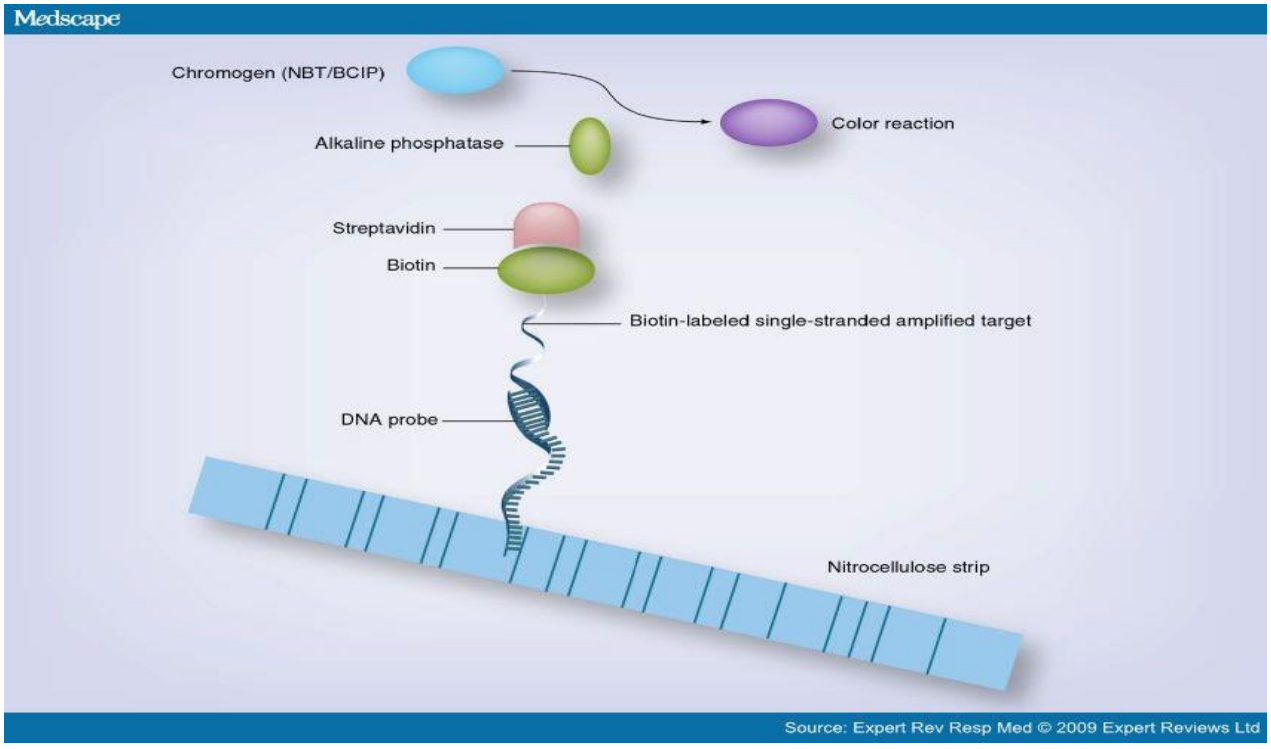


Figure 20: Overview Genotype MTBDR plus assay, hybridization principle

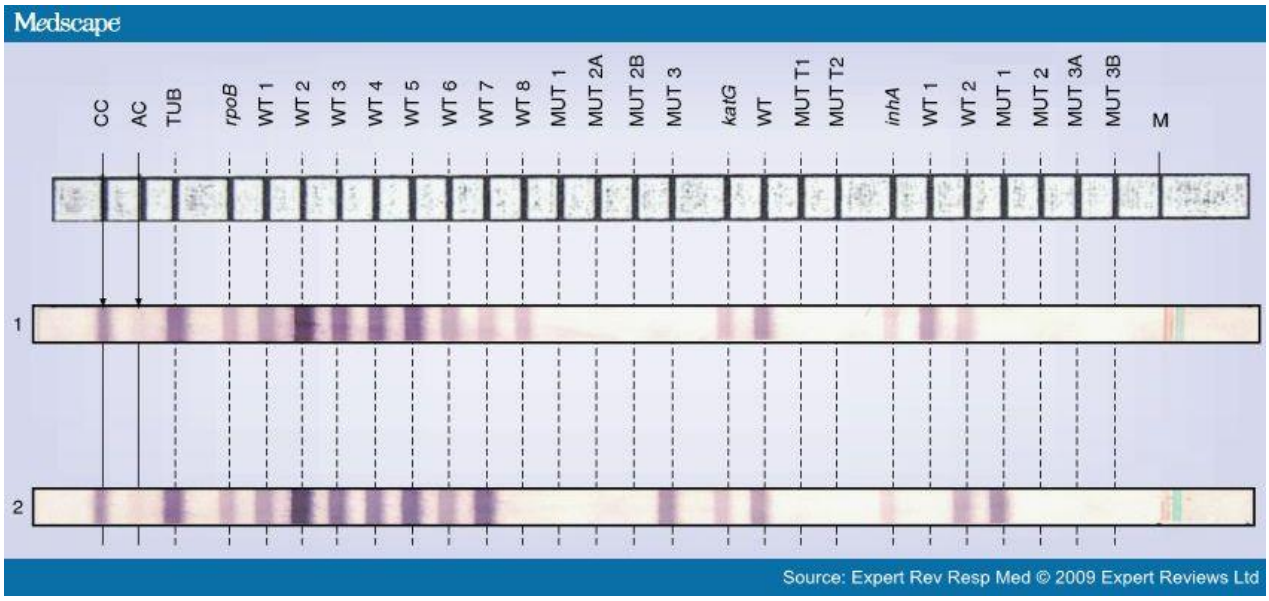


Figure 21: Genotype MTBDR plus assay, interpretation

MDR-TB is TB that is resistant to at least two of the most important first line antimycobacterial drugs: isoniazid (INH) and rifampicin (RIF).

XDR-TB is defined as TB that is resistant to INH and RIF, plus resistant to any fluoroquinolone and at least three injectable second line drugs (amikacin, kanamycin and capreomycin).

7. Treatment

The aims of tuberculosis treatment are:

- cure patient and restore quality of life and productivity;
- prevent death from active TB or its late effects;
- prevent relapse of TB;
- reduce transmission of TB;
- prevent development and transmission of drug resistance.

The latest WHO guidelines for the treatment on Tuberculosis (4th edition) has been released in 2010. Major progress in global TB control follows the wide spread implementation of DOTs (Direct Observed Treatment) strategy. In 2006 stop TB strategy was launched with the objective of universal access to patient treatment and protection of population from TB/HIV co-infection and multidrug resistance tuberculosis (49).

The standard treatment regimens will depend upon the group the patient belongs (49, 50).

The different group of patients are as followed:

- new patients;
    - new patients presumed or known to have drug-susceptible TB;
    - new patients known with isoniazid-resistant TB or in settings with high isoniazid resistance;
- previously treated patients and multidrug resistance;
- treatment of TB/HIV co-infection;
- management of latent tuberculosis.

7-1 Treatment of new cases

For initial empiric treatment of TB, start patients with the first line antituberculosis drug regimen: isoniazid (H), rifampicin (R), pyrazinamide (Z) and ethambutol (E) during intensive phase and 4 months of isoniazid and rifampicin in continuation phase (Figure 22).

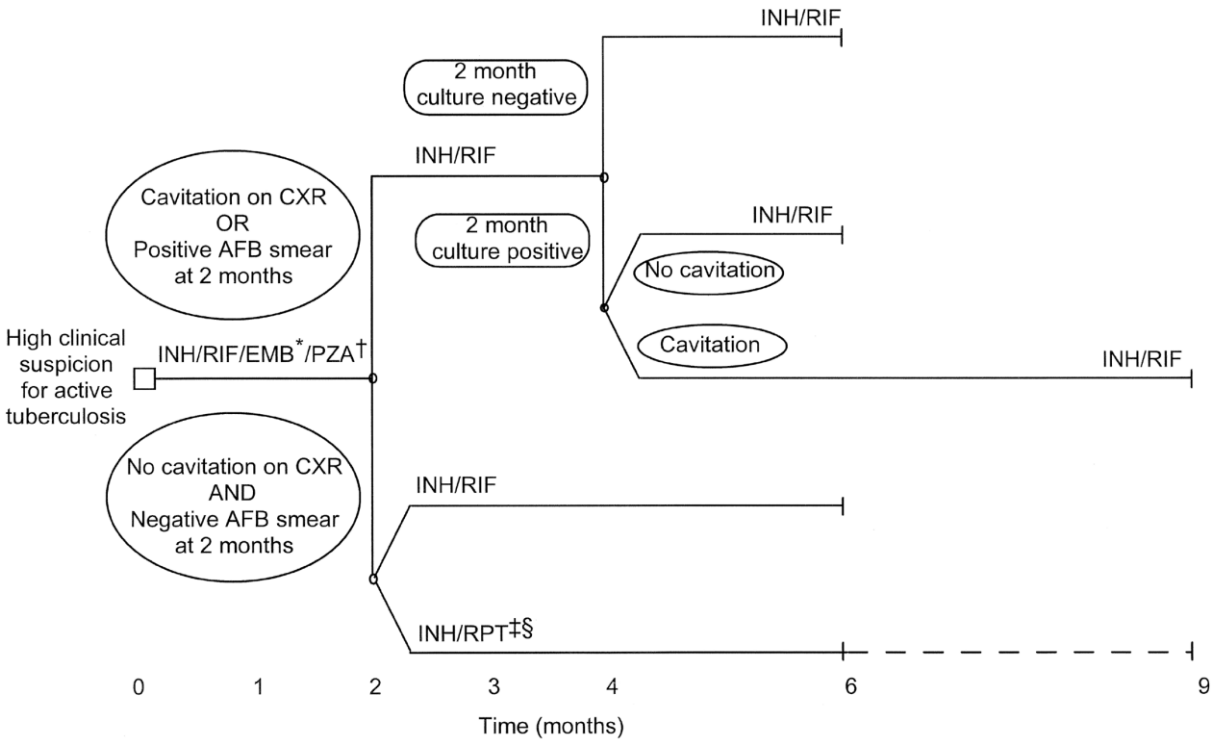


Figure 22: Treatment algorithm for tuberculosis (48)

The duration of treatment will be increased in TB meningitis (9-12 months) and bone and joint TB (9 months). In TB meningitis, ethambutol should be replaced by streptomycin.

For new patients known with isoniazid resistant TB or the local prevalence of isolates resistant to isoniazid is high, new TB patients may receive the following regimen: 2 months of isoniazid, rifampicin, pyrazinamide and ethambutol (HRZE) and 4 months of isoniazid, rifampicin and ethambutol (HRE).

7-2 Treatment of previously treated patients and multidrug resistance

Previous TB treatment is a strong determinant of drug resistance. All cases should have culture and DST of sputum at the start of the treatment. Prompt identification of MDR and initiation of MDR treatment with second line drugs give better chance of cure and prevent the spread of further resistance. At the global level, 15% of previously treated patients have MDR, which is five time higher than the global average of 3% in new patients.

In a retrospective study conducted in India on 2816 patients with treatment failure and chronic cases, the rate of MDR-TB was 53% (51). In India, all antituberculosis drugs, first line drugs and second line drugs, are widely available and used, in the private and public sectors outside of the Revised National Tuberculosis Control Program (RNTCP).

MDR Regimen

There are 5 groups of drugs to treat MDR (Table 4). Include at least 4 drugs certain to be effective by taking one drug from each group from 1-5 in a hierarchical order.

In India, 6 drugs are given in intensive phase (Z + E + K + L/Ofx + Cs + Eto) and 4 drugs during continuation phase (E + L/Ofx + Eto + Cs).

The duration of the treatment should be a minimum of 6 months during the intensive phase and for at least 4 months after the patient first becomes and remains smear and culture negative. The continuation phase should continue for minimum 18 months to 24 months after culture conversion (49).

Group	Drugs (abbreviations)
Group 1 First line	- Pyrazinamide (Z) - Ethambutol (E) - Rifabutin (rfb)
Group 2 Injectable drugs Aminoglycoside or polypeptide	- Kanamycin (Km) - Amikacin (AM) - Capreomycin (Cm) - Streptomycin (S)
Group 3 Fluoroquinolone	- Levofloxacin (Lfx) - Moxifloxacin (Mfx) - Ofloxacin (Ofx)
Group 4 Oral bacteriostatic second line agents	- Para-aminosalicylic acid (PAS) - Cyclocerine (Cs) - Terizidone (Trd) - Ethionamide (Eto) - Protionamide (Pto)
Group 5	- Clofazimine (Cfz) - Linezolid (Lzd) - Amoxicillin/clavulinate (Amx/Cln) - Thioacetazone (Thz) - Imipenen/cilastatin (lpm/Cln) - High dose isoniazid (high-dose H) - Clarithromycin (Clr)

Table 3: Groups of drugs to Treat MDR-TB (49)

7-3 Treatment of TB with HIV infection

The approach to the treatment of tuberculosis in the HIV-infected patient is complex and must address the patient's requirement for antiretroviral therapy (ART), potential drug reactions, and complications related to the immune reconstitution inflammatory syndrome (IRIS) (52).

The decision to give ART co-treatment in a TB patient must be made by a TB-HIV trained doctor or medical officer. The health worker at the first-level health facility, however, needs to decide whether and when to consult with or refer the patient to this doctor or medical officer at the district hospital.

For this, use the clinical stage, whether already on ART, and CD4 count if available.

The preferred recommendation for many TB-HIV patients is to start and complete TB treatment, and then start ART. However, if the patient's clinical status is poor (other signs of HIV clinical stage 3 or 4 or CD4 count less than 350/mm^3), it may be necessary to refer the patient for ART treatment sooner. If patient is not on ART, start TB treatment immediately, or if already started, continue TB treatment. ART improves survival in HIV cases with TB. It reduces TB recurrence rate by 50%. ART should be used in all persons living with HIV with active TB disease irrespective of CD4 cell count (Tuberculosis care with TB-HIV co-management, WHO, 2007).

Cotrimoxazole therapy is to be given throughout TB treatment as it reduces mortality in HIV-TB cases. The exact mode of activity is not clear but co-trimoxazole is known to prevent *Pneumocystis jirovecii* and malaria and is likely to have an impact on a range of bacterial infections in HIV-positive TB patients (49, 50).

7-4 Treatment of latent tuberculosis infection (LTBI)

Treatment of latent tuberculosis infection is important for controlling and eliminating TB. Indeed it reduces the risk that TB infection will progress to TB disease. Once the diagnosis of LTBI has been made by TST or IGRA test, health care providers must choose the appropriate treatment regimen. 4 regimens are proposed (Table 4). The association Isoniazid and rifapentine was proposed in a new CDC guideline released in December 2011.

Drugs	Duration	Interval	Minimum doses
Isoniazid	9 months	Daily	Adult : 5mg/kg Children : 10-20mg/kg
		Twice weekly*	Adult : 15mg/kg Children : 20_40mg/kg
Isoniazid	6 months	Daily	Adult : 5 mg/kg Children : not recommended
		Twice weekly*	Adult : 15mg/kg Children : not recommended
Isoniazid (INH) and Rifapentine (RPT)	3 months	Once weekly*	INH : 15mg/kg RPT : 10-14mg/kg
Rifampin	4 months	Daily	Adult : 10 mg/kg

*Use Directly Observed Therapy (DOT)

Table 4: Proposed regimen for latent TB treatment (http://www.cdc.gov/tb/topic/treatment/ltbi.htm)

Isoniazid may not be the most rational choice of drug for latent TB. Isoniazid inhibits synthesis of mycolic acids, key cell wall constituents and displays a biphasic killing with rapid early bactericidal activity against actively replicating bacilli but much less efficacy in killing bacilli with low metabolic activity (53).

Current diagnostics identify many individuals as having latent tuberculosis particularly in endemic countries that will never develop active disease, and though efficacious treatments strategies exist, their length, inconvenience and poor targeting mean they are often not implemented in practice. Fundamental questions about the adaptation and fate of the tubercle bacillus within its human host still need to be addressed to allow the development of practical diagnostics to identify just those at risk of developing active TB (54).

III- Tuberculosis in India

1. Overview of India country

India is one of the oldest civilizations in the world with a kaleidoscopic variety and a rich cultural heritage. It has achieved all-round socio-economic progress during the last 60 years of its independence. India has become self-sufficient in agricultural production and is now in the top 10 industrialized nations in the world and the sixth nation to have gone into outer space to conquer nature for the benefit of the people. It covers an area of 32,87,2631 sq. km, extending from the snow-covered Himalayan heights to the tropical rain forests of the south. As the 7th largest country in the world, India stands apart from the rest of Asia, marked off as it is by mountains and the sea, which give the country a distinct geographical entity. Bounded by the Great Himalayas in the north, it stretches southwards and at the Tropic of Cancer, tapers off into the Indian Ocean between the Bay of Bengal on the east and the Arabian Sea on the west (55). India is a union of twenty-eight states and seven territories (Figure 23) with a total population estimated at 1, 21 billion.

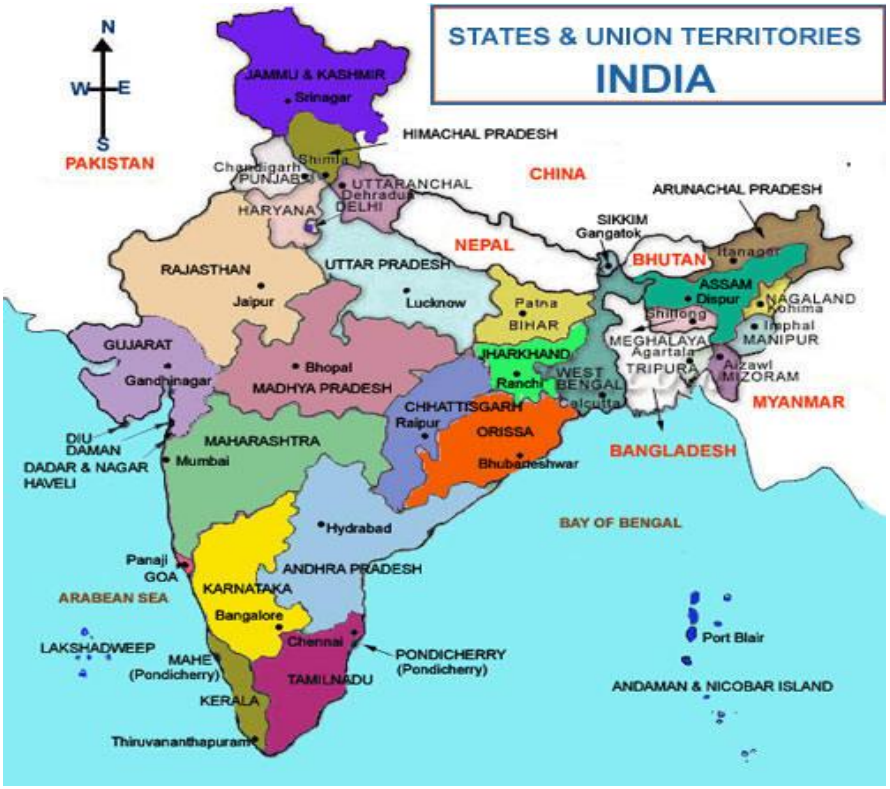


Figure 23: India map (Wikipedia.org)

India's history is intimately tied to its geography; it has always been an invader's paradise.

The civilization that laid the bricks, one of the world's oldest, was known as the Indus. They had a written language and were highly sophisticated. Dating back to 3000 BC, they originated in the south and moved north, building complex, mathematically-planned cities.

The first group to invade India were the Aryans, who came out of the north in about 1500 BC. The Aryans brought with them strong cultural traditions that, miraculously, still remain in force today. They spoke and wrote in a language call Sanskrit. The Aryans introduced the *caste* system and established the basis of the Indian religions. The second great invasion into India occurred around 500 BC, when the Persian kings Cyrus and Darius conquered the Indus valley. Compared to Aryans, the Persian influence was marginal, they occupied the region for a brief period of 150 years. Persian were conquered by the Greeks under Alexander the Great. In the 5th century BC, Siddhartha Gautama founded the Buddhism religion. The muslin influence started to make itself keenly felt in 1001. In that year, Arab armies swept down the Khyber Pass and hit like a storm. The Mughal Empire which was a Muslim Persianate imperial power, started in 1556 with the ascension of Akbar the great. Akbar tolerated local religions and married a Hindu princess. Shah Jahan, Akbar grandson, left behind the colossal monuments of the Munghal empire, including the Taj Majal, the Pearl Mosque, the Royal Mosque and the Red Fort.

Figure 24: Taj Mahal (National Geographic Channel)

The Mughal Empire crumbled just as the Europeans were beginning to flex their own imperialistic muscles. The Portuguese started to trade in Goa in 1510 and founded three

colonies on the west coast. In 1610, the British chased away a Portuguese naval squadron, and the East India Company created its own outpost at Surat. This small outpost marked the beginning of a remarkable presence that would last over 300 years. The British came to administer a huge territory of 300 million people through a highly effective and organized system called the "*Raj*". India had become a profitable venture and the British were no willing to allow the Indian population any power in a system that they viewed as their own accomplishment. The Indians didn't appreciate this much, and as the 20th century dawned there were increasing movements towards self-rule. Along with the desire of independence, tensions between Hindus and Muslims has also been developing over the years. The Muslims had always been a minority, and the prospect of an exclusively Hindu government made them wary of independence; they were as inclined to mistrust Hindu rule as they were to resist the Raj. In 1915, Mohandas Karamchand Gandhi called for unity between the two groups in an astonishing display of leadership. The profound impact Gandhi had on India and his ability to gain independence through a totally non-violent mass movement made him one of the most remarkable leaders in the world.

While Gandhi was leading a largely Hindu movement, Mahammed Ali Jinnah was fronting a Muslim one through a group called the Muslim league. Jinnah advocates for the division of India into two states: Muslim and Indu. When the British left they created the separate states of Pakistan and Bangladesh (56, 57). Through the British Parliament of the Indian Independence Act 1947, on 14 August 1947, Pakistan was declared a separate nation and on 15 August 1947 India became also a separate nation. The Republic of India was officially proclaimed on 26 January 1950.

The economy of India is the ninth-largest in the world by nominal Gross Domestic Product (GDP) and the third-largest by purchasing power parity (PPP). The country is one of the G-20 major economies and a member of BRICS (Brazil, Russia, India, China and South Africa). As predicted by Goldman Sachs, the Global Investment Bank, by 2035 India would be the third largest economy of the world just after US and China. It will grow to 60% of size of the US economy.

India is developing into an open-market economy, yet traces of its past autarkic policies remain. Economic liberalization, including industrial deregulation, privatization of state-owned enterprises, and reduced controls on foreign trade and investment, began in the early 1990s and has served to accelerate the country's growth, which has averaged more than 7%

per year since 1997. India's diverse economy encompasses traditional village farming, modern agriculture, handicrafts, a wide range of modern industries, and a multitude of services. Slightly more than half of the work force is in agriculture, but services are the major source of economic growth, accounting for nearly two-thirds of India's output, with less than one-third of its labor force. India has capitalized on its large educated English-speaking population to become a major exporter of information technology services and software workers.

India has many long-term challenges that it has not yet fully addressed, including poverty, inadequate physical and social infrastructure, limited non-agricultural employment opportunities, inadequate availability of quality basic and higher education, and accommodating rural-to-urban migration (58).

India which is the world's largest democracy with a strong economic development is facing strong contradictions like being the world's worst failures in health and education. If democracy works there, why are so many Indian live so wretched?

Last summer, the country's eastern and northern electricity grids have collapsed, leaving an estimated 600 million people in 13 states without power, yet 400 million Indians had no electricity. Sanitation and public hygiene are awful, especially in the north: half of all Indians still defecate in the open, resulting in many deaths from diarrhea and encephalitis. Polio may be gone, but immunization rates for most diseases are lower than in sub-Saharan Africa. Twice as many Indian children (43%) as African ones go hungry. Many adults, especially women, are also undernourished, even as obesity and diabetes spread among wealthier Indians. Despite gains, extreme poverty is rife and death in childbirth all too common. Prejudice kills on an immense scale: as many as 600,000 fetuses are aborted each year because they are female (59).

Indian public health is especially disturbing. Southern states like Tamil Nadu do well, but generally the country gets what it pays for: a pitiful $39 per person per year for public health, compared with China's $203 or Brazil's $483. In India the total amounts to 1.2% of GDP, as against a global average of 6.5%. Some of the shortfall is made up privately, as both rich and poor Indians pay doctors and quacks. But even good private providers do nothing for preventive care and better health education (59).

Figure 25: Street view in New-Delhi (personal picture)

2. India health care system/India TB program

2-1 India health care system

The heath care system in India is a complex organization with a mix of private and public sector and an important disparity inside the population. There is an important contrast of access to the Indian Healthcare within the population. The 1.2 billion Indians are split into 2 categories: the middle and upper classes, which generally live in the urban areas and a large majority of the population who lives in rural areas (70%). There is an important gap in the healthcare system between urban and rural areas. Consequently, only a quarter of the Indian population has access to allopathic medicine, and most of them live in urban areas. The majority of the hospitals are privately owned and located in cities due to the sector's awareness of the health related issues and financial viability (60, 61, 62).

The WHO' 2000 global healthcare profile ranked India's healthcare system 112th out of 190 countries. This survey highlighted the following major health concerns for India that still are prominent today:

- ✓ vulnerability of young children; 43.5% less than five years old are underweight;
- ✓ poor sanitation: only 30% of the population uses improved sanitation and less than 20% in rural population;
- ✓ infectious diseases: the top 3 being: malaria, tuberculosis and diarrhea.

The Health care system is organized in 6 levels (63):

- ✓ national level: consist of the Union Ministry of Health and Family Welfare;
- ✓ state level: organization under the state department of health and family welfare;
- ✓ regional level: covers 3 to 5 districts and acts under authority delegated by the state directory of health services;
- ✓ district level: middle level management organization and is a link between the state and the peripheral structure such as Primary Health Center (PHC) and sub-centre;
- ✓ sub-divisional/Taluk level: healthcare services are rendered through the office of assistant district health and family welfare officer;
- ✓ community level: one Community Health Centre (CHC) has been established for every 80,000 to 120,000 population. This centre provides basic specialty services in general medicine, pediatrics, surgery, obstetrics and gynecology.

The rural Indian health care system (64) has three levels: community health care, primary health care and sub-centre (Figure 26). The total number of different centres (65) is presented in the Figure 27.

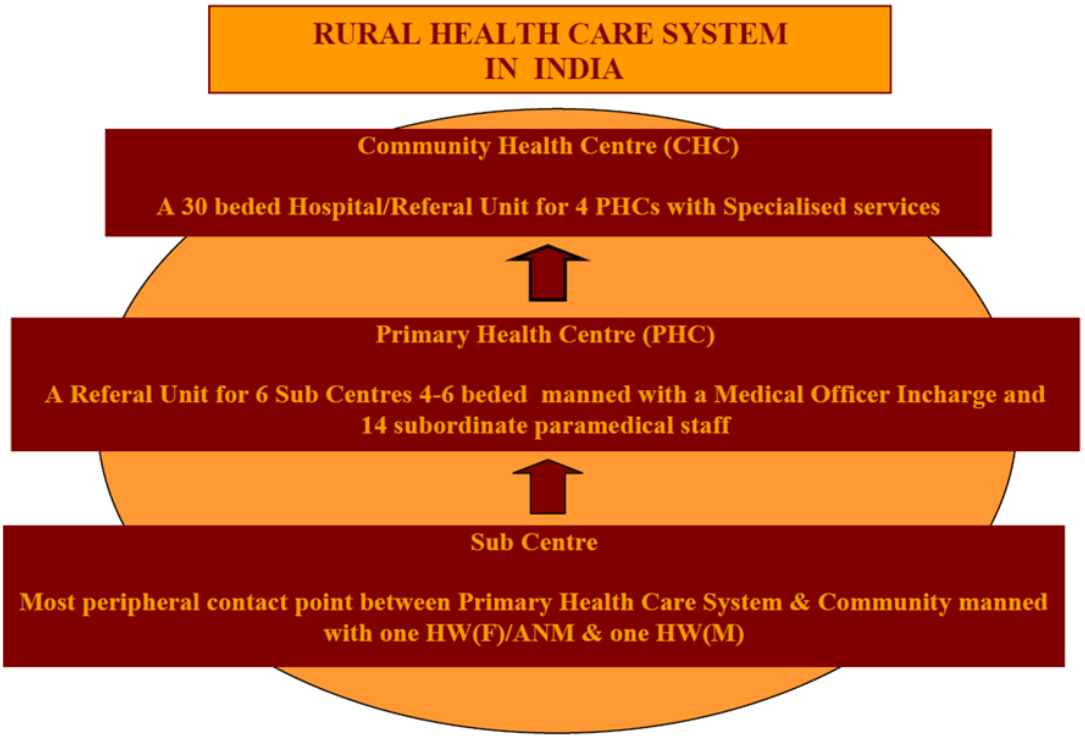


Figure 26: Rural health care system in India (64)

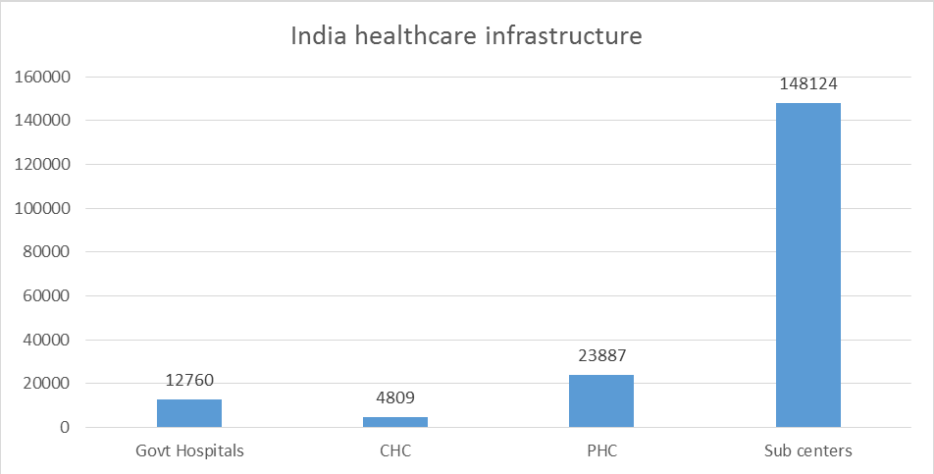


Figure 27: Number of different centres (Ministry of Health and Family welfare, https://nrhm-mis.nic.in)

Sub-Health Centre (sub-centre) is the most peripheral and first point of contact between the primary health care system and the community (66).

Keeping in view the current varied situation of sub-centres in different parts of the country, sub-centres have been categorized into 2 types (Types A and B).

Type A Sub Centre will provide all recommended services except that the facilities for conducting delivery will not be available here. Type B sub-centre, will provide all recommended services including facilities for conducting deliveries at the sub-centre itself.

Figure 28: Sub-centre type A; with such unhygienic and inadequate delivery facilities pose a great risk to the mother and newborn (Indian Public Health Standards (IPHS), guidelines for sub-centres, Revised 2012)

Figure 29: Sub-centre type B (Indian Public Health Standards (IPHS), guidelines for sub-Centres, Revised 2012)

Sub-centres are expected to provide promotive, preventive and few curative primary health care services. Keeping in view the changing epidemiological situation in the country, both types of sub-centres should lay emphasis on non-communicable diseases related services. All the activities related to the sub-centres are described in the "Indian Public Health Standards (IPHS) Guidelines for sub-Ccntres Revised 2012" (66). Sub-centres are also involved in different national programmes.

Regarding the Revised National Tuberculosis Control Programme (RNTCP) (67), the main missions of the Sub-centers are:

- ✓ identify persons especially with fever for 15 days and above with prolonged cough or spitting blood and take sputum smears from these individuals. Refer these cases to the PHC medical officer for further investigations;
- ✓ check whether all cases under treatment for Tuberculosis are taking regular treatment, motivate defaulters to take regular treatment and bring them to the notice of the medical officer PHC;
- ✓ educate the community on various health education aspects of tuberculosis programme;

- ✓ assist the ASHA (accredited social health activist)/similar village health volunteer to motivate the TB patients in taking regular treatment.

Primary Health Centre is the cornerstone of rural health services. It is the first port of call to a qualified doctor of the public sector in rural areas for the sick and those who directly report or referred from Sub-Centres for curative, preventive and promotive Health care (68). A typical Primary Health Centre covers a population of 20,000 in hilly, tribal, or difficult areas and 30,000 population in plain areas with 6 indoor/observation beds. It acts as a referral unit for 6 sub-centres and refers out cases to CHC (30 bedded hospital) and higher orders to public hospitals located at sub-district and district level C (Indian Public Health Standards (IPHS) Guidelines for Primary Health Centres Revised 2012).

Community Health Centre constitutes the First Referral Units and the sub-district and District Hospitals (69). The CHCs were designed to provide referral health care for cases from the Primary Health Centres level and for cases in need of specialist care approaching the centre directly. 4 PHCs are included under each CHC thus catering to approximately 80,000 populations in tribal/hilly/desert areas and 120,000 population for plain areas. CHC is a 30-bedded hospital providing specialist care in medicine (Indian Public Health Standards (IPHS) Guidelines for Community Health Centres Revised 2012).

The service deliveries in CHCs are:

- ✓ outpatient and inpatient services: general medicine, surgery, obstetrics & gynecology, pediatrics, dental and AYUSH (Ayurveda, Yoga & Naturopathy, Unani, Siddha and Homoeopathy);
- ✓ eye specialist services;
- ✓ emergency services;
- ✓ laboratory services;
- ✓ national health programmes.

Concerning the RNTCP, both PHCs and CHCs are involved in laboratory TB diagnostic which is limited to smear microscopy.

2-2 India TB programme

India has had a National Tuberculosis Programme (NTP) in place since 1962 (19). However, the treatment success rates were unacceptably low and the death & default rates remained high. Further the HIV-AIDS epidemic and the spread of multi-drug resistance TB were threatening to further worsen the situation. In view of this, in 1992, the Government of India with WHO and AIDS reviewed the TB situation.

The Revised National TB Control Programme (RNTCP) adopted in 1993 the internationally recommended Directly Observed Treatment Short-course (DOTS) strategy. Political and administrative commitment to set up an organized TB control services was obtained. Smear microscopy was introduced in decentralized health services (PHC and CHC). DOTS was adopted to increase the treatment completion. Supply of drugs was strengthened to provide assured supply of drugs. After pilot studies, large scale implementation of the RNTCP began in 1997.

In early 2002, the World Bank assisted the TB control project. The Government of India took up the nation-wide expansion of the RNTCP in 2005.

Then the RNTCP phase II was developed based on the lessons learnt from implementation of the program over 12 years. The phase II programme was almost similar than phase I: quality was implemented in the diagnosis, private sector was involved, DOTS+ was implemented for MDR TB and treatment for XDR TB was offered (TB India 2012 Revised National TB Control programme, Annual Status Report).

Every quarter, Central TB Division receives aggregate case-finding, programme management, sputum conversion, and treatment outcome information for patients registered under the programme from over 2,600 tuberculosis units nationwide. RNTCP follows the global method of cohort analysis for describing case finding and treatment outcomes. Timely data collection and dissemination are hallmarks of the RNTCP surveillance and data management systems. The data from the quarterly reports are analyzed and disseminated in the public domain as quarterly performance reports before the end of the subsequent quarter and as an annual report.

As an example of the data collection reported by the RNTCP, table 1 reports the cases finding and notification rates over 11 years analysis period. We can notice that the population covered increased from 139 million to 1.21 billion.

Year	Total population of India covered under RNTCP (millions)	Sputum Microscopy Services				Case Notification			
		Suspects examined		Sputum smear positive cases diagnosed		Total TB cases notified		Total sputum smear positive cases notified	
		Number	Rate	Number	Rate	Number	Rate	Number	Rate
1999	139	n/a		n/a		133,918	101	61,103	46
2000	241	956,113	421	148,610	65	240,835	106	131,100	58
2001	441	2,046,039	517	286,789	73	468,360	118	252,878	64
2002	528	2,507,455	524	356,409	75	619,259	129	327,519	68
2003	761	3,955,395	576	555,250	81	906,638	132	473,378	69
2004	920	5,128,852	599	711,661	83	1,188,545	139	615,343	72
2005	1058	5,684,860	569	762,619	76	1,294,550	129	676,542	68
2006	1105	6,216,509	566	834,628	76	1,400,340	127	746,149	68
2007	1,138	6,483,312	570	879,741	77	1,474,605	130	790,463	69
2008	1,156	6,817,390	590	911,821	79	1,517,363	131	815,254	71
2009	1,174	7,247,895	617	930,453	79	1,533,309	131	825,397	70
2010	1,192	7,550,522	633	939062	79	1,522,147	128	831,429	70
2011	1,210	7,875,158	651	953032	79	1,515,872	125	844,920	70

Population is total covered at the year end of each year till 2006
Estimated population based on 2001 & 2011 Census
Rates are adjusted for the number of days of implementation till 2006

Table 5: TB cases finding activities and notification rates (1999-2011) (67)

3. Tuberculosis epidemiology in India

Estimation and regular measurement of TB disease burden is important for reviewing the progress towards the Millennium Development Goals (MDG) related to TB.

3-1 Global TB disease burden

As per the WHO Global TB Report 2011, there were an estimated 8.8 million incident cases of TB (range, 8.5 million-9.2 million) globally in 2010, 1.1 million deaths (range, 0.9 million-1.2 million) among HIV-negative cases of TB and an additional 0.35 million deaths (range, 0.32 million-0.39 million) among people who were HIV-positive. In 2009, there were an estimated 9.7 million (range, 8.5-11 million) children who were orphans as a result of parental deaths caused by tuberculosis.

Globally, the absolute number of incident TB cases per year has been falling since 2006 and the incidence rate (per 100 000 population) has been falling by 1.3% per year since 2002. If these trends are sustained, the MDG target that TB incidence should be falling by 2015 will be achieved.

TB mortality is falling globally and the Stop TB Partnership target of a 50% reduction by 2015 compared with 1990 will be met if the current trend is sustained. The target could also be achieved in all WHO regions with the exception of the African Region.

Although TB prevalence is falling globally and in all regions, it is unlikely that the Stop TB Partnership target of a 50% reduction by 2015 compared with 1990 will be reached. However, the target has already been achieved in the Region of the Americas and the Western Pacific Region is very close to reaching the target.

Dramatic reductions in TB cases and deaths have been achieved in China. Between 1990 and 2010, prevalence rates were halved, mortality rates were cut by almost 80% and incidence rates fell by 3.4% per year. In addition, methods for measuring trends in disease burden in China provide a model for many other countries.

Between 2009 and 2011, consultations with 96 countries that account for 89% of the world's TB cases have led to a major updating of estimates of TB incidence, mortality and prevalence, particularly for countries in the African Region.

Estimates of TB mortality have substantially improved in the past three years, following increased availability and use of direct measurements from vital registration systems and mortality surveys. In this report, direct measurements of mortality are used for 91 countries (including China and India for the first time).

3-2 TB burden in India

Though India is the second-most populous country in the world, it has more new TB cases annually than any other country. In 2009, out of the estimated global annual incidence of 9.4 million TB cases, 2 million were estimated to have occurred in India, thus contributing to a fifth of the global burden of TB. It is estimated that about 40% of Indian population is infected with TB bacillus. The incidence of TB in India is estimated based on findings of the nationwide annual risk of tuberculosis infection (ARTI) study conducted in 2000-2003.

The national ARTI being 1.5%, the incidence of new smear positive TB cases in the country is estimated as 75 new smear positive cases per 100,000 population. The prevalence of TB has been estimated at 3.8 million bacillary cases for the year 2000, by an expert group of the government of India. However the recent estimate by WHO gives a prevalence of 3 million. On a national scale, the high burden of TB in India is illustrated by the estimate that TB accounts for 17.6% of deaths from communicable diseases and for 3.5% of all causes of

mortality (WHO, 2004). More than 80% of the burden of tuberculosis is due to premature death, as measured in terms of disability-adjusted life years (DALYs) lost. WHO estimated TB mortality in India as 280,000 (23/100,000 population) in 2009. With RNTCP implementation, death due to TB has come down to half in the country. It was estimated that the TB mortality was over 5 million annually at the beginning of the revised national TB control programme (RNTCP). Data from specific surveys, however, suggest that case fatality rates prior to RNTCP were generally greater than 25%. In RNTCP era, case fatality has remained less than 5% for new cases registered under programme.

The indicator 23 of the MDGs mentions to halve the prevalence of TB disease and deaths due to TB between 1990 and 2015. With respect to the progress towards indicator 23, as per the recent WHO estimates, in the year 1990, the prevalence rate of TB in India was 338 per 100,000 populations (best estimates) and the mortality due to TB was 42 per 100,000 populations. In comparison, in the year 2009, the prevalence of TB in India was estimated to be 249 per 100,000 populations, and the mortality due to TB is 23 per 100,000 populations [WHO Global TB Report, 2010]. These estimates are derived based on mathematical and have its own inherent limitations.

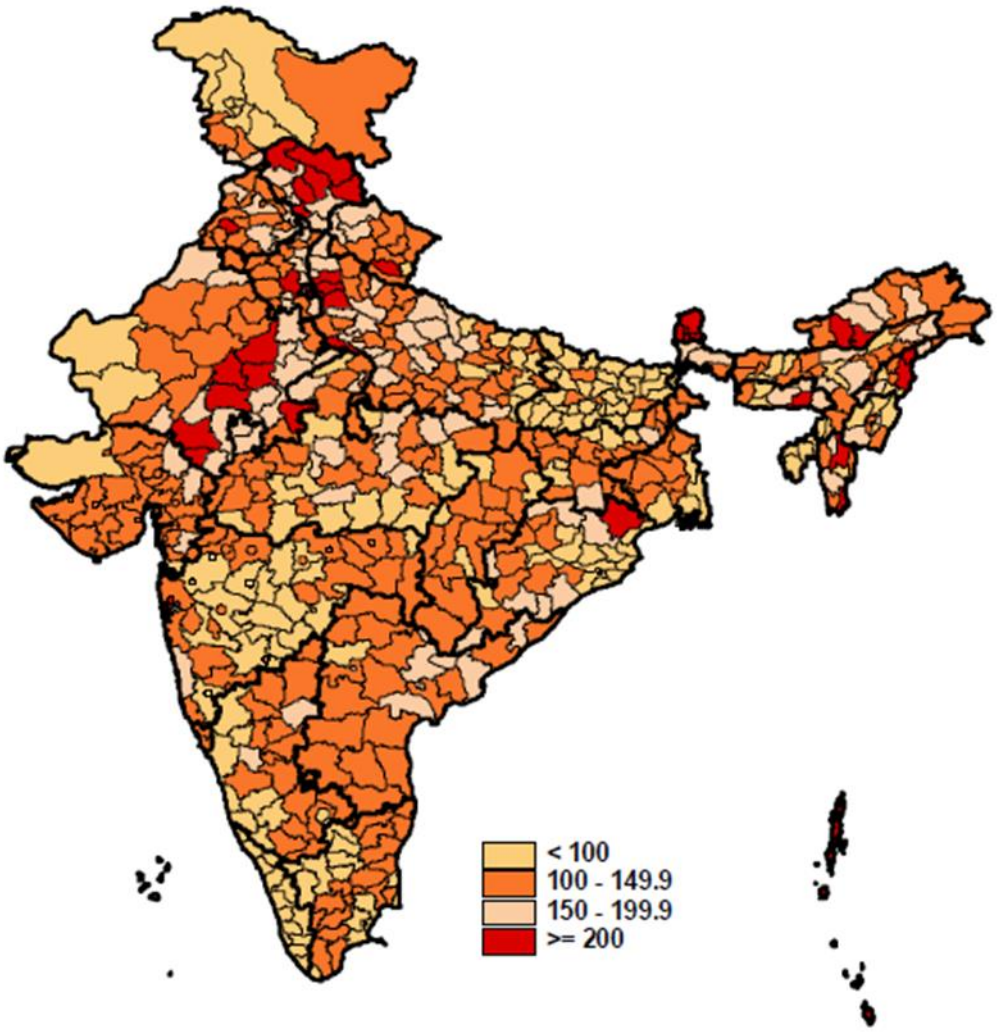


Figure 30: Annual total TB case notification rate, India, 2010 (63)

It is important to note that the TB prevalence is link to the poverty. Figure 31 shows that TB prevalence increases linearly with wealth quintile (70), with estimates ranging from 201 per 100,000 population (95% CI 142–260 per 100,000) among the wealthiest to 1105 per 100,000 population (95% CI 919–1291 per 100,000) in the poorest (71).

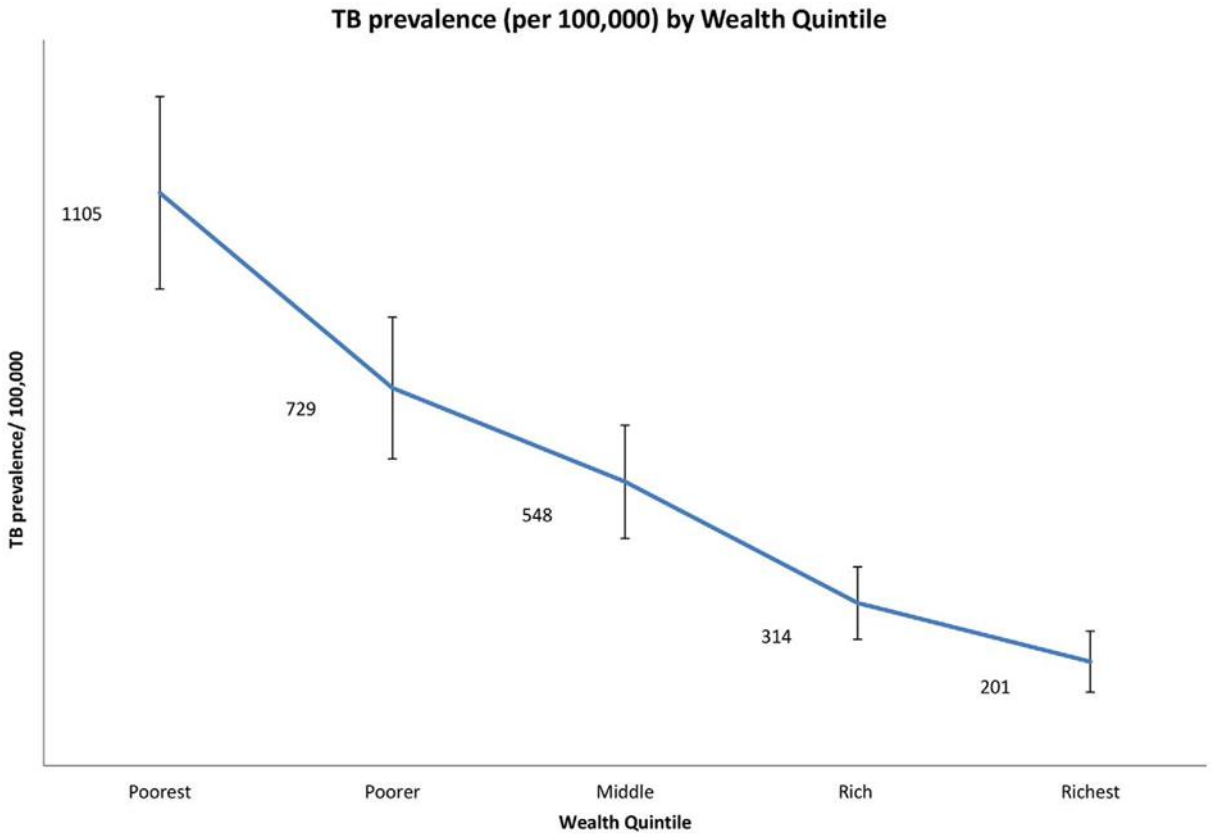


Figure 31: Self-reported TB prevalence (per 100,000) by wealth quintile (71)

3-3 Burden of TB-HIV

TB and HIV act in deadly synergy. HIV infection increases the risk of TB infection on exposure, progression from latent infection to active TB, risk of death if not timely treated for both TB and HIV and risk of recurrence even if successfully treated. Correspondingly, TB is the most common opportunistic infection and cause of mortality among people living with HIV (PLHIV), difficult to diagnose and treat owing to challenges related to co-morbidity, pill burden, co-toxicity and drug interactions. Though only 5% of TB patients are HIV- infected, in absolute terms it ranks 2nd in the world and accounts for about 10% of the global burden of HIV-associated TB. This coupled with heterogeneous distribution within country is a challenge for joint delivery of integrated services. National and international studies indicate that an integrated approach to TB and HIV services can be extremely effective in managing the epidemic. A modelling study by Williams *et al* predicts that RNTCP should be able to reverse the increase in TB burden due to HIV; but to reduce mortality by 50% or more by 2015 (20), universal access to coordinate TB and HIV care is essential (72). Studies also indicate that emphasis needs to be on early diagnosis linked to TB and HIV treatment.

Global estimation of burden of HIV positive incident TB cases is 1,000,000 while the estimates of HIV positive incident TB cases in India is 75,000. As per WHO's Global TB Report of 2011, HIV prevalence amongst incident TB cases is estimated to be 3.3% (5%-7.1%).

3-4 Burden of pediatric TB in the country

The actual burden of pediatric TB is not known due to diagnostic difficulties but has been assumed that 10% of total TB load is found in children. Globally, about 1 million cases of pediatric TB are estimated to occur every year accounting for 10-15% of all TB; with more than 100,000 estimated deaths every year, it is one of the top 10 causes of childhood mortality. Though MDR-TB and XDR- TB is documented among pediatric age group, there are no estimates of overall burden, mainly because of diagnostic difficulties and exclusion of children in most of the drug resistance surveys.

3-5 Prevalence of drug resistant TB in India

The emergence of mycobacteria which are resistant to drugs used to treat tuberculosis has become a significant public health problem over creating an obstacle to effective TB control. Its presence has been known virtually from the time anti-tuberculosis drugs were introduced for the treatment of TB but drug resistant tuberculosis is being encountered more frequently in most countries including India. There have been a number of reports on drug resistance TB in India, but most studies were undertaken using non-standardized methodologies with bias and small samples usually from tertiary level care facilities.

To obtain a more precise estimate of Multi-Drug Resistant TB (MDR-TB) burden in the country, RNTCP carried out drug resistance surveillance (DRS) surveys in accordance with global guidelines in selected states: Gujarat (56 million population) and Maharashtra (107 million) in 2005-2006, and Andhra Pradesh (81 million) in 2007-2008. The results of these surveys indicate prevalence of MDR-TB to be low: less than 3% amongst new cases and 12-17% in re-treatment cases. These surveys also indicate that the prevalence of MDR-TB is stable in the country. The previous studies conducted by the Tuberculosis Research Centre (TRC) in Chennai and the National Tuberculosis Institute (NTI) in Bangalore have shown a similar prevalence figures. To substantiate the findings of the earlier surveys, two more DRS surveys are presently ongoing in Western UP (85 million) and Tamil Nadu and it is planned in Rajasthan and Madhya Pradesh in the near future. These surveys will be undertaken periodically to monitor and study the trend of MDR prevalence in the community.

Based on the results of Gujarat, Maharashtra and Andhra Pradesh DRS Survey, estimated proportion of MDR-TB is 2.1% (1.5% - 2.7%) in new TB cases and 15% (13% - 17%) in previously treated cases. As compared to global rates, the proportions of MDR-TB is inferior in India.

As per the WHO Global TB Report 2011, estimated number of MDR-TB cases out of notified pulmonary TB cases in India was 64,000 (range 44,000 to 84,000) emerge annually.

3-6 Extensively drug resistant tuberculosis

Extensively drug resistant TB (XDR-TB), subset of MDR-TB with resistance to second line drugs i.e. any fluoroquinolone and to at least 1 of the 3 second line injectable drugs (capreomycin, kanamycin and amikacin), has been reported in India. However, the extent and magnitude of this problem is yet to be determined. Results of the second line DST on MDR isolates from Gujarat DRS survey have shown that there is no XDR amongst new cases and the prevalence amongst re-treatment cases is 0.5%. The extent of fluoroquinolone resistance observed is of great concern, and may compromise MDR-TB treatment outcomes. Efforts to expand surveillance to second-line anti-TB drugs are underway.

No separate DRS surveys have been undertaken to estimate the burden of XDR-TB in the country. However, DRS surveys to estimate burden of MDR- TB conducted in Gujarat and Andhra Pradesh reported 14 XDR-TB cases. 112 XDR-TB patients have been diagnosed at National Reference Laboratories as reported by the states from 2008 till Sept 2011. Programme have formulated guidelines for treatment of XDR-TB patients with category V regimen (TB INDIA 2012, Revised National TB control India, Annual Status Report).

IV-Personal work

IV-A Development of a tuberculosis immunoserodiagnostic test

1. Objective

The vast majority of tuberculosis patients live in developing countries, where the diagnosis of tuberculosis relies on the identification of acid-fast bacilli on unprocessed sputum smears using conventional light microscopy. Microscopy has high specificity in tuberculosis-endemic countries, but modest sensitivity which varies among laboratories (range 20% to 80%). Moreover, the sensitivity is poor for paucibacillary disease (e.g., pediatric and HIV associated tuberculosis). This lack of sensitivity of the sole diagnostic test in many parts of the world results in delays in diagnosis, enabling the disease to progress and increasing the potential for transmission of *M. tuberculosis*. To ensure appropriate care for patients and to improve control of the global TB epidemic, simple, accurate, inexpensive and ideally point of-care diagnostic tools for TB are urgently needed. The relative importance of the different characteristics of a diagnostic test depends upon the setting in which the test is to be performed and the intended use of the results.

Technical simplicity, for example, is essential if a test is to be used in a primary health-care clinic or basic health laboratory in low-income countries. Immune-based tests would seem to offer the potential to improve case detection as currently performed, as some of the test formats (e.g., immunochromatographic test) are suitable for resource-limited areas. The major advantages of immune-based tests are their speed (results may be available within hours) and simplicity compared with microscopy. The development of immune-based tests for the detection of antibodies, antigens, and immune complexes has been attempted for decades, and their performance has been critically appraised in several descriptive reviews and textbook chapters.

2. Antigen selection

Several *M. tuberculosis* specific antigens have been identified. Apart from using many protein antigens for the serodiagnosis of TB, considerable amount of work has been done with glycolipids molecule of *M. tuberculosis*. There are no commercial available serodiagnostic tests for tuberculosis with acceptable sensitivity and specificity for routine laboratory use. Development of serodiagnostic tests must take into consideration individual variation in antibody responses, differential gene expression and subsequent protein

production by *M. tuberculosis*, depending on the strain and stage of disease. Results from different studies suggest that a cocktail of different antigens is needed in order to obtain the desired performances.

2-1 Protein antigens

Several hundred different proteins have been identified from culture filtrate preparation of *M. tuberculosis* that replicate in liquid media.

The main protein antigens which has been widely studied for TB serodiagnostic are: the 38-kDa Antigen, Antigen 85, the 16 kDa antigen, Antigens encoded by the RD1 region: ESAT6, CFP10, TB 6.7. (73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84).

- ✓ The 38-kDa antigen has been the most frequently studied and included in a number of studies. The sensitivities of the assays with this antigen have been reported to range from 16 - 80%, depending on the smear status of the patient and the patient populations used in the studies.
- ✓ The secreted Ag85B belonging to the Ag 85 complex, a family of proteins (Ag85A, Ag 85B and Ag85C) has been isolated, purified and evaluated by many investigators for its potential for serodiagnosis. The reported sensitivity varies from 41% to 94%. The Ag 85 complex possesses mycosyl transferases activity required for biosynthesis of cord factor.
- ✓ The Ag MTB81 is a 88-kDa malate synthetase protein. It elicits strong antibody responses in TB patients.
- ✓ The 16 kDa antigen is a cytosolic regulatory protein; specific to *M. tuberculosis* complex. This antigen is used in several commercial products along with other antigens: PATHOZYME® TB COMPLEX PLUS (Omega Diagnostics), TB Enzyme (kreatech), Serocheck-MTB (Zephyr Biomedicals), etc…
- ✓ The antigen A 60 is a heat stable component of PPD and *M. tuberculosis* culture filtrate that is defined by crossed immunoelectrophoresis. This antigen, however, is not specific for mycobacteria because it is also present in *Nocardia* and *Corynebacterium*.
- ✓ Two proteins: ESAT-6 and CFP-10 coded by the RD1 region of *M. tuberculosis* have shown a great interest for TB serodiagnostic. The RD1 region corresponds to 9.5-kb section of DNA present only in virulent strains

of *M. tuberculosis* and is deleted in all attenuated *Mycobacterium bovis* BCG vaccine strains.

2-2 Glycolipid antigens

Extensive work has been done with lipid antigens to understand the humoral response for developing a serodiagnostic tool. Four lipid antigens have been widely studied as a TB serological marker: the LAM Ag, The diacyl trehalose (DAT), the lipooligosaccharide (LOS II) and the phenol glycolipid of *M. tuberculosis* (PGL-Tb1).

2-2-1 LAM Ag

Sada conducted the first study in which LAM from *M. tuberculosis* was applied for serodiagnosis of tuberculosis (85, 86).

Several commercial TB serodiagnostic tests employ LAM antigens. MycoDot ™ is one of the well-known product (Figure 32). Performances of these tests have been published in many articles with a wide range of performances (85, 86, 87, 88, 89, 90, 91).

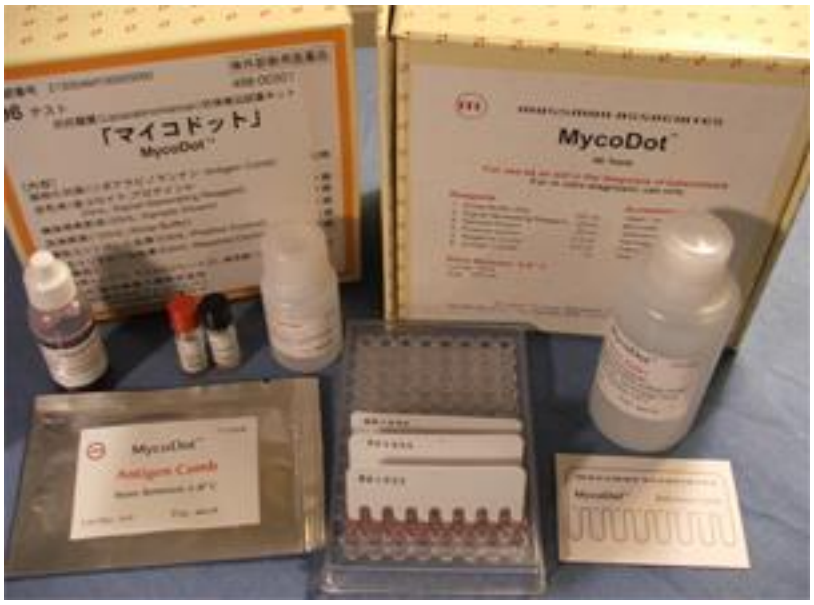


Figure 32: Mycodot TB serological test

2-2-2 Diacyl trehalose (DAT) Ag

Papa *et al* were the first to describe the antigenicity of the sulfo lipid diacyl trehalose-2'-sulphate (SL-IV) in rabbits (92, 93). The structure of this glycolipid has been established by Daffe *et al* and was called DAT (Figure 33) (94, 95).

It was found that the DATs from *M. tuberculosis* and from *M. fortuitum* were almost identical and have the same serological reactivity. The DAT from *M. fortuitum* is more abundant and easier to produce (96).

The performances of this antigen as a serological marker has been studied by different teams showing variable performances as well among HIV negative and HIV positive patients. (97, 98, 99, 100, 101)

It has been reported that positive results in HIV sera obtained 12 months preceding the onset of TB was significantly associated with later development of the disease (102).

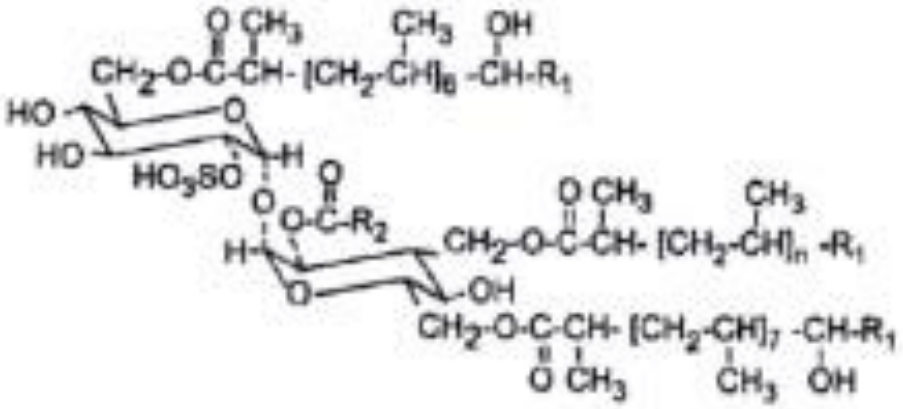


Figure 33: DAT structure (95)

2-2-3 Lipoolygosaccharide (LOS) Ag

The LOS corresponds to alkali-labile and phosphorus-free neutral glycolipids based on glycosidically linked acyl trehalose (103). Daffe *et al* described a new class of highly antigenic lipooligosacharides obtained from the *M. tuberculosis* Canetti strain call LOS-II. This antigen was found typifying the relatively smooth Canetti strain but was not detected in two typical rough strains of *M. tuberculosis*, namely H37Ra and H37Rv (104, 105).

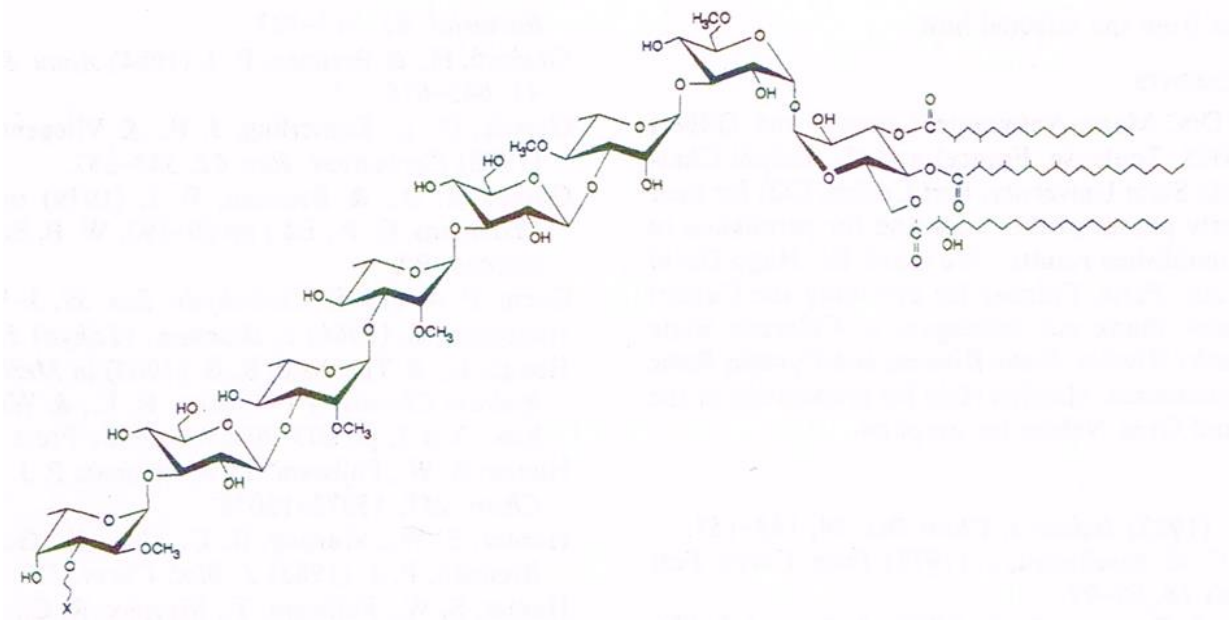


Figure 34: LOS II structure (104)

The LOS-II glycolipid extracted from the *M. tuberculosis* Canetti strain has been evaluated as a potential source of antigen for the serological diagnosis of tuberculosis in HIV seropositive and seronegative patients by Daleine and Lagrange (1995). The results suggested that the LOS antigen is a potential marker for detecting the development of TB in HIV patients (106).

The extraction and purification of the LOS-II antigen is very cumbersome. The yield is low, requiring large production of *M. tuberculosis* Canetti strain. This process is not well adapted for mass production.

2-2-4 Phenol glycolipid Ag: PGL -Tb1

Phenol-phtiocerol glycolipids have been found previously in *Mycobacterium leprae, M. kansasii, M. bovis* and *M. marinum,* but not in *M. tuberculosis.*

Daffe *et al* (1987) discovered a major phenol-phthiocerol trisaccharide, accompanied by minor amounts of a phenol-phtiocerol monosaccharide in Canetti strains of *M. tuberculosis* (Figure 35) (107).

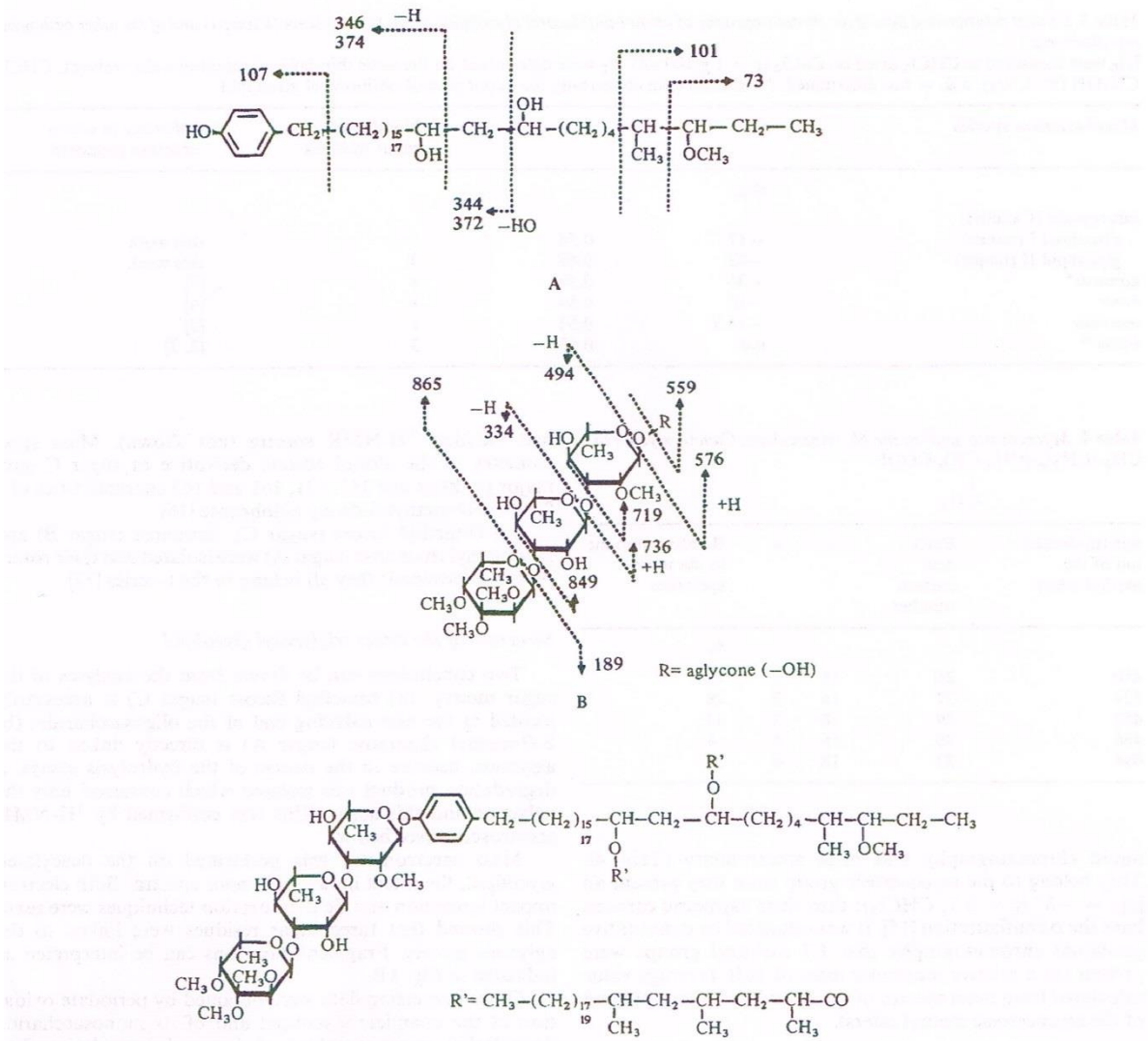


Figure 35: PGL-Tb1 structure (107)

A strong immunoreactivity of this glycolipid has been demonstrated with antisera prepared in rabbits against this phenol glycolipid (108).

Several studies have been conducted to evaluate the interest of this antigen as a TB serological marker. Casabona *et al* published a specific IgG detection against PGL-Tb1 with a sensitivity of 94.7% and a specificity of 96.8%. These results were obtained on a limited patient panel: 38 tuberculous patients and 62 healthy controls (109).

Further studies have been done to evaluate the diagnostic value of the PGL-Tb1 on HIV positive patients. Berlie *et al* observed a significant high antibody titers on a serial assays in sera collected from 11 AIDS patients before tuberculosis was diagnosed (110). Simmoney *et al* evaluated the B-cell immune responses in HIV positive and negative patients with

tuberculosis (111). In another paper they demonstrated that a low level of anti PGL-Tb1 in a HIV-TB patients could be an indicator of developing an immune restoration syndrome (IRIS) (112).


Tuberculosis (2007) 87, 109–122

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B-cell immune responses in HIV positive and HIV negative patients with tuberculosis evaluated with an ELISA using a glycolipid antigen

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Tuberculosis (2008) xx, 1–9

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Anti-PGL-Tb1 responses as an indicator of the immune restoration syndrome in HIV-TB patients☆

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Received 16 September 2007; received in revised form 8 January 2008; accepted 12 January 2008

3. Development of an enzyme linked immunosorbent assay (ELISA) serological test

In order to develop the most performing TB serological test, based on the literature and knowledge from every potential antigens described above, our attention has been brought particularly to the RD1 secretory proteins: ESAT6 and CFP10 and the glycolipid PGL-Tb1. These antigens present the advantage to be the more specific of *M. tuberculosis*. The combination of proteins and glycolipid PGL-Tb1 presents the advantage to improve the sensitivity regarding HIV-TB patients (113).

Concerning the protein Ag, we selected the ESAT6-CFP10 fusion recombinant Ag produced on *E. coli*, provided by the Staten Serum Institute of Copenhagen, Denmark.

3-1 PGL-Tb1 production

The PGL-Tb1 Ag was produced by our-self according to the protocol described by Daffé (Annex 1).

Briefly the production and purification of PGL-Tb1 has the following steps summarized in Figure 34:

- ✓ mycobacterial culture of the *M. tuberculosis* Canetti strain in Sauton media for 3 weeks;
- ✓ glycolipid extraction in chloroform/methanol solution;
- ✓ PLT-Tb purification on silica gel in solvent mixture of chloroform/methanol at different ratios;
- ✓ thin layer chromatography in order to select the fraction containing the purified PGL-Tb1;
- ✓ nuclear magnetic resonance (NMR) analysis of the purified antigen;
- ✓ immunoreactivity control of the purified antigen in ELISA microtiter plate.

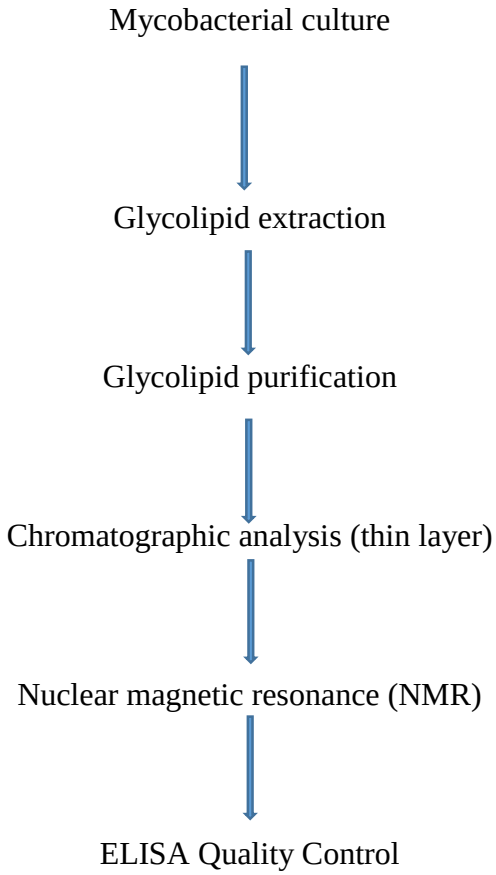

Figure 36: PGL-Tb1 production and purification flow chart

3-2 Design of the ELISA TB serodiagnostic Kit

Many experiments were performed to optimize the TB serological assay. The final design of the TB diagnostic test was frozen following a limited clinical evaluation conducted at the Nizam's Hospital in Hyderabad (India).

The kit contains the following components:

- microtiter plate coated with PGL-Tb1 Ag and recombinant ESAT6-CFP10 Ag; in two separate wells;
- serum diluent;
- wash buffer;
- anti human IgG-HRP conjugate (HRP=horseradish peroxidase);
- TMB Substrate (3,3′,5,5′-tetramethylbenzidine);
- stopping reagent.

3.2.1 Microtiter plate design

The PGL-Tb1 Ag is not soluble in aqueous media, therefore in order to combine the association of this glycolipid antigen with the ESAT6-CFP10 protein antigen we had to coat both selected antigens in 2 separated wells.

The purified PGL-Tb1 in dry powder was first dissolved in chloroform and then the concentrated antigen was diluted in PBS buffer at the coating concentration of 25 µg/ml. The coating plate was done in polystyrene flat bottom microtiter plates (Polysorb, Nunc A/S, Denmark) by dispensing 100 µl per well of prepared antigen. The plates were incubated for 2 hours at 37°C followed by an overnight incubation at room temperature. Antigen was then removed, the wells were blocked with 0.5% of bovine serum albumin (BSA) and the plates were washed and dried. The ESAT6-CFP10 was coated at 2.5 µg/ml after optimization experiments in carbonate buffer. Then the process was similar to the PGL-Tb1.

3.2.2 Serum dilution

After performing sequence of optimization experiments, the serum dilution was optimized at 1/ 200. For the optimization, known TB positive serum samples and sera of the healthy blood donors were used.

3.2.3. Wash buffer and wash protocol

The tween 20 is widely used as detergent in ELISA washing buffer, however, due to the interference of Tween-20 with PGL-Tb1, we intended to find an alternative for this detergent. Tween 20 has a hydrophilic and hydrophobic part. The hydrophobic part of Tween-20 is responsible to form the detergent micelle. In ELISA, the unbound molecules entrapped inside this micelle are removed during the wash step. Though the mechanism is not fully understood, it is suspected that the hydrophilic part of Tween-20 could make a binding to the phthiocerol part of the PGL-Tb1, thus the coated molecules are removed from the polystyrene surface. The binding between Tween 20 and PGL-Tb1 must be strong enough to remove the Van der Walls force between PGL-Tb1 and polystyrene surface. After experimenting with other non-ionic detergents like Triton-X 100 without much success, we have tried with PEG 6000 in the wash buffer for PGL-Tb1 and ESAT6-CFP10 ELISA. The results obtained with the preliminary standardization experiments were promising. Further we have optimized the new wash buffer with 50mM Tris (Sigma-Aldrich, USA), 0.9% NaCl (CDH, Germany) and 0.01% PEG 6000 (CDH, Germany) . The pH of the buffer was set at 7.0.

4. Preliminary data

The preliminary data obtained with this ELISA serological test are described in the thesis of T. Satheesh Kumar: "Study on selected *M. tuberculosis* specific antigens for IgG antibody response for the development of ELISA based diagnostic test for tuberculosis".

Sensitivity and specificity obtained with the optimized prototype ELISA test are reported in Table 6 and 7.

Study Group / Sub group	n	Number of Positives (Sensitivity)		
		PGL-Tb1	ESAT6-CFP10	Combined*
TB Patients	**90**	**67 (74%)**	**43 (48%)**	**72 (80%)**
HIV Negative TB patients	79	57 (72%)	34 (43%)	61 (77%)
HIV/TB Patients	11	10 (91%)	9 (82%)	11 (100%)
Pulmonary TB Patients	75	55 (73%)	35 (47%)	58 (77%)
Extra-Pulmonary TB Patients	15	12 (80%)	8 (53%)	14 (93%)
Smear Positive TB Patients	58	44 (76%)	25 (43%)	46 (79%)
Smear Negative TB Patients	32	23 (72%)	18 (56%)	26 (81%)

Table 6: Sensitivity obtained with the ELISA prototype kit

Study Group / Sub group	n	Number of Negatives (Specificity)		
		PGL-Tb1	ESAT6-CFP10	Combined*
All Control Group	136	127 (93%)	131 (96%)	122 (90%)
Healthy Blood donors	105	99 (94%)	100 (95%)	94 (90%)
Non-TB Patients	31	28 (90%)	31 (100%)	28 (90%)

Table 7: Specificity obtained with the ELISA prototype kit

In this study, we obtained the same specificity on both populations (healthy donors and non-TB patients which were TB suspected but the final diagnostic excluded a TB disease): 90%. However the number of samples tested was small.

We noticed that the sensitivity obtained on TB HIV positive patients were excellent and higher than TB-HIV negative patients. The sensitivity among smear positive and smear

negative patients was quite similar and was significantly higher among extra-pulmonary TB compared to pulmonary TB.

The encouraging results obtained on this preliminary study conducted to introduce this new test for a further large clinical trial described in chapter IV-B.

IV-B Multicenter Clinical Evaluation of a TB tool box

1. Introduction

The basis of TB control programs consists of timely diagnosis and correct treatment of patients with active TB: early diagnosis is essential for controlling the spread of this disease. The role of laboratory in the management of tuberculosis is not fully established. Diagnosis of TB in India rely mostly on the detection of acid-fast bacilli by microscopy and less frequently by culture.

Currently no single diagnostic tool can help to achieve these goals and it is evident that using carefully selected diagnostic tools (tool box) may come to help.

The aim of the multicentric prospective study in India was to assess the value of several selected diagnostic tools to understand which one or which combination of diagnostic tools may serve better for the maximum case detection.

The study was conducted in nine centers in 5 different cities (Figure 37):

- 4 tertiary care centers: 3 public (AIMS and Safdarjung Hospital, New Delhi; Nizam's Institute of Medical Sciences, Hyderabad) and 1 private (P D Hinduja Hospital and Medical Research Center, Mumbai);
- 2 referral centers (JALMA, Agra; Tuberculosis Research Centre-TRC, Chennai);
- 1 pediatric hospital (SN Medical College, Agra);
- 2 centers caring for HIV-infected patients (JJ hospital, Mumbai; Government Hospital for Thoracic Medicine, Chennnai).

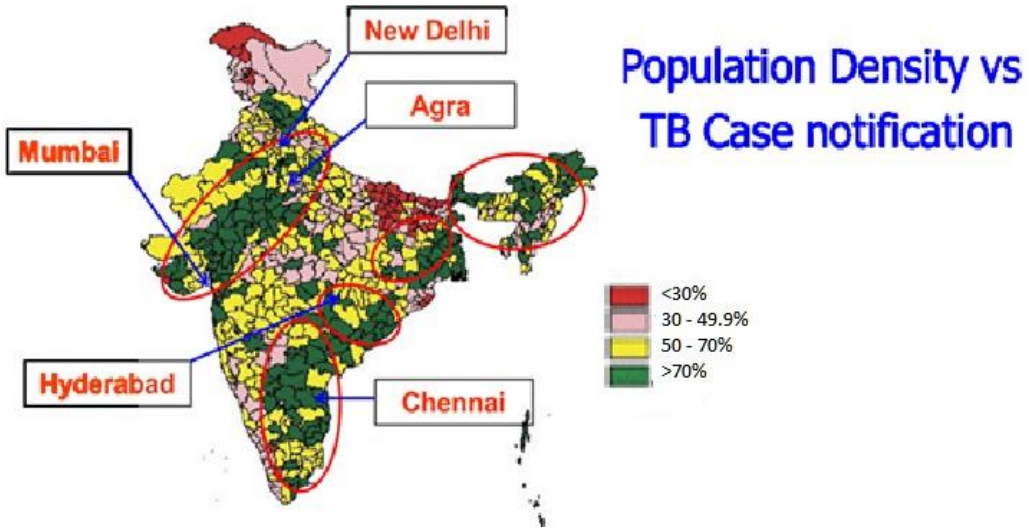

Figure 37: Centers involved in the clinical studies

The protocol was divided into two parts: the main study and the satellite study. The purpose of the main study was to evaluate the most conventional methods used in specialized TB laboratories along with the prototype serological diagnostic test. We selected the following diagnostic tools in the main study:

- clinical based diagnosis that includes Clinical suspicion and Chest X ray;
- smear Microscopy;
- conventional culture method on Lowenstein-Jensen (LJ) solid media;
- automated culture with identification (BacT/ALERT + Accuprobe MTD) on liquid media;
- antibody response for the *Mycobacterium tuberculosis* complex specific antigens by using the ELISA based kit described above;
- Direct detection based on Molecular diagnosis (Gen-Probe MTD and PCR).

The purpose of the satellite study was to evaluate in some selected sites:

a. key interferences which may occur with other major infectious diseases encountered in India: leishmaniasis, leprosy and filariasis;
b. the accuracy of "in house" molecular technics versus commercial tests. Indeed, most laboratories using molecular methods in India for TB diagnosis, use "in house" methods for cost issue. We wanted to evaluate the accuracy of these "in house" methods versus commercial kits;
c. the performances of a new IGRA test developed by the Lazzaro Spallanzani National Institute for Infectious Diseases (INMI) in Rome in comparison to the commercial QuantiFERON-TB Gold test;
d. the interest of a new sputum sample processing (USP) method versus the standard N-acetyl L-cysteine (NALC)-NaOH method (114);
e. the antibody response among healthy family contacts;
f. the level of remaining antibodies on TB cases treated and followed for at least 5 years without relapse;
g. the influence of the serological test when using fresh versus frozen sera;
h. the possibility of using dry blood spot versus serum for the serological test. Indeed dry blot spot is more and more used in developing countries where laboratory structures are very weak. It allows to collect the blood directly on a paper and forward it to a reference center for analysis.

i. Antibody response in skin tuberculosis.

2. Material and methods

The study protocol (Annex 2) was submitted and approved by the local ethical committees of each institution. Written informed consent was obtained from all patients before enrollment.

The patients tested included: TB suspected cases and several control populations. For each enrolled individual, using Epi Info™ software, a file was done including patient's characteristics (serial number, study center, date of enrolment, specimen collected, patient study group, age, sex, address), TB risk information (history of TB in family, close contact with a TB case, personal history of TB and treatment, immunological debilitating condition), clinical symptoms (cough for more than 2 weeks, persisting low grade fever, weight loss, night sweats, anorexia, fatigue and haemoptysis….), tuberculin skin test (TST) performed or not (if performed the diameter of induration), chest X ray observation, effect of a non TB antibiotic treatment, the final clinical diagnosis and the clinical suspicion score given by the clinician in charge of the patient (only 3 categories were selected : very high, high, low), the final therapeutic intervention with the date of therapy initiation and the anti-TB drugs being prescribed. The following diagnostic tools were tested: smear examination, culture (liquid and solid) and immunological tests (ELISA serological test and IGRAs).

2-1 Smear examination

Microscopy using the Ziehl-Neelsen stain was used.

2-2 Culture

2-2.1 Liquid culture on BacT/ALERT® MP Bottle

Samples (5 ml) were transferred into a 50 ml sterile, conical bottom, graduated centrifuge tube. An equal volume of NALC-NaOH reagent were added to the specimen. The mix was vortexed for 15 to 30 seconds followed by an incubation at room temperature for 15 minutes. Sterile phosphate buffer was added up to 45 ml mark of centrifuge tube. The tube was then centrifuged at 3000 g for 20 minutes. The pellet was re-suspended with little above 1 ml of phosphate buffer with phenol red. Five hundred microliters were transferred to the prepared

BacT/ALERT® MP bottle which was then placed into BacT/ALERT® 3D (bioMérieux) or MB/BacT® system (bioMérieux).

2-2.2 Solid culture on LJ media

Two hundred microliters were inoculated to a Lowenstein Jensen (LJ) solid media (bioMérieux) and then incubated in slanting position facing the LJ's slant position up for first 2 days. Then incubation was done vertically until 42 days. The inoculated LJ media were periodically observed for microbial growth.

2-2.3 Identification on positive culture by Accuprobe MTBC test

The identification was done following the "Intended for Use manual" of the Accuprobe MTBC test (Genprobe).

2-3 Immunological tests

2-3.1 Serological TB ELISA test

The TB ELISA test is a serological test for the detection of specific IgG towards 2 different *Mycobacterium tuberculosis* antigens: PGL-Tb1 and ESAT6-CFP10 which were coated into 2 consecutive wells in a micro-ELISA test format.

Serum from patient was diluted at 1:200 in sample dilution buffer and 100 µL were dispense into the 2 consecutive wells. After an incubation of 60 minutes at 37°C, the plates were washed 6 times using the wash buffer. One hundred microliters of diluted anti-human IgG-HRP conjugate were added to each well and the plates were incubated for 60 minutes at 37°C. After 6 washes, 100 µL substrate TMB were added into each well. The reaction was stopped using 100 µL stop solution after 30 minutes incubation in dark. The reading was performed on a spectrophotometer at 450 nm with a reference at 620 nm.

2-3.2 IGRA tests

- QuantiFERON®-CMI test

QuantiFERON®-CMI (Cellestis) is an in-vitro laboratory test for measuring cell-mediated immune responses (CMI) in humans. Undiluted human whole blood is stimulated with test antigen(s) or mitogen, followed by the quantitative measurement of interferon-gamma (IFN-γ) in plasma by enzyme-linked immunosorbent assay (ELISA).

- RD1 selected peptides test

The RD1 selected peptides test has been developed by the Lazzaro Spallanzani National Institute for Infectious Diseases (INMI) in Rome using RD1 peptides as T response stimulator following by a quantitative measurement of IFN- γ by ELISA.

3. Result analysis

3-1 Enrolled population

2,213 patients were prospectively enrolled from January 2006 to July 2008; 75 patients were excluded for insufficient data. 1,787 patients were included in the main study and 351 included in the satellite study according to the protocol. Among the patients included in the main study, 807 were diagnosed with active tuberculosis and 980 individuals with non-active tuberculosis (Figure 38).

Active TB includes microbiologically confirmed and clinical TB.
Microbiologically confirmed TB corresponds to identification of *M. tuberculosis* in LJ and/or BacT/Alert MP culture, molecular tests or histology.
Patients were classified with clinical TB if the diagnosis was based on clinical and radiologic criteria (after excluding other diseases) including responses to anti-tuberculosis therapy.

The non-active tuberculosis were classified into 6 subgroups: hospitalized non-TB patients, cured TB patients, health care workers, family contacts, blood donors and healthy community adults (Figure 39).

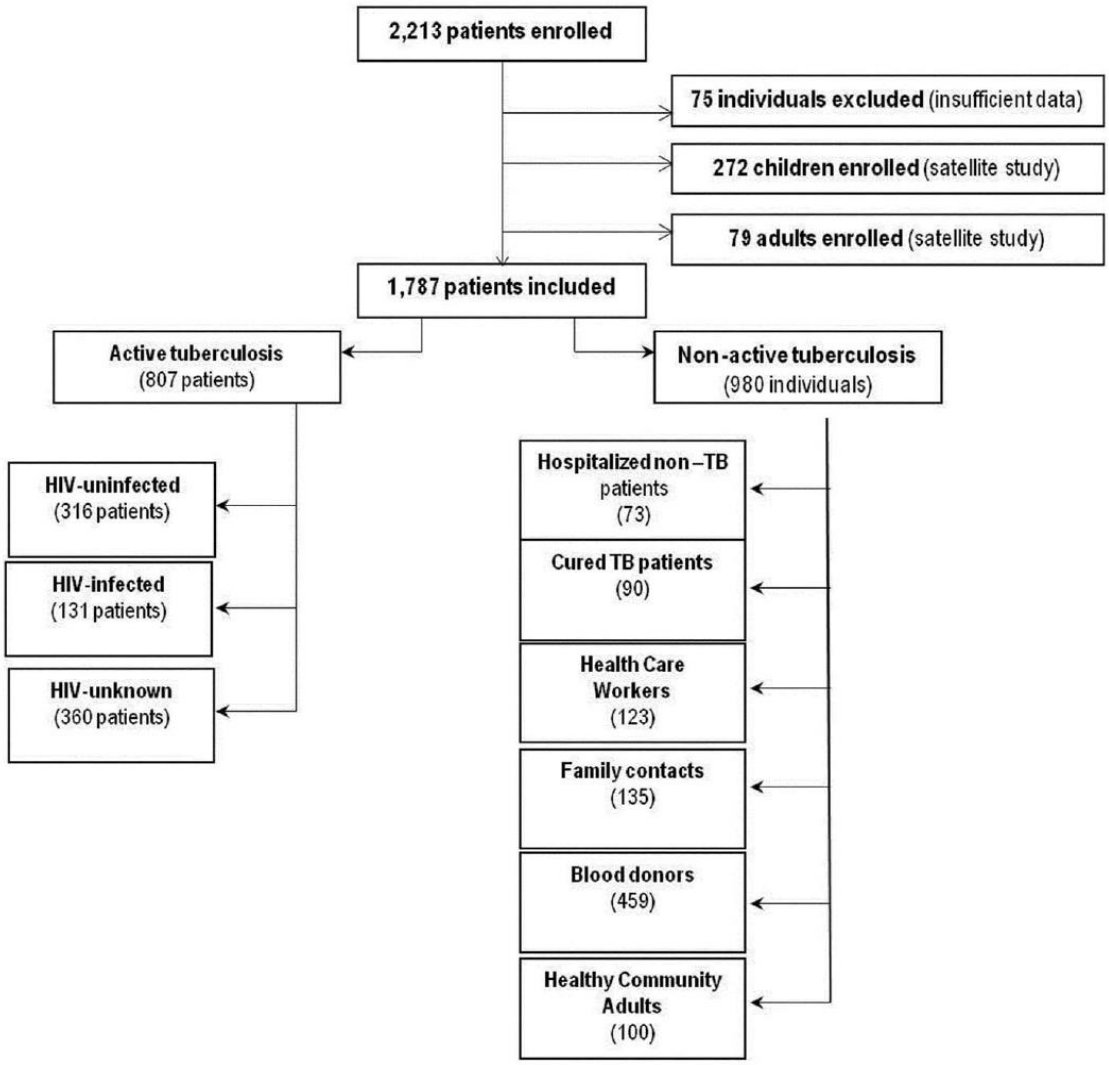

Figure 38: Flow chart of patients recruited to the multicentric study stratified by patients subgroups

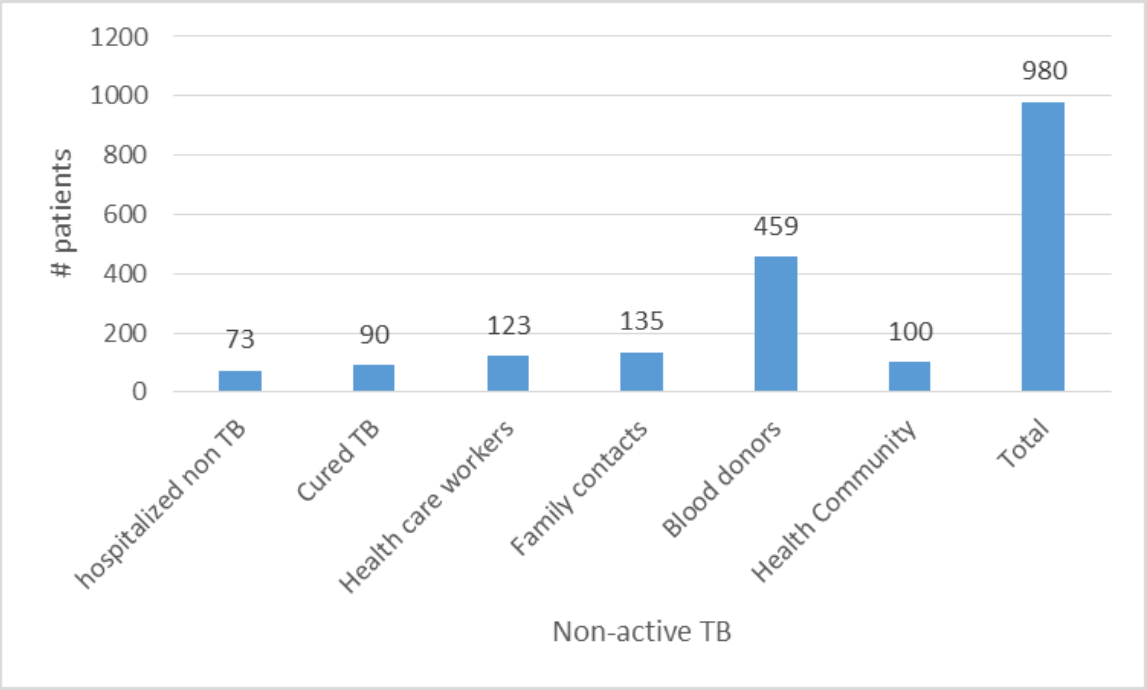

Figure 39: Non-active TB subgroups

3-2 Demographic and clinical characteristics of TB patients

The TB patients have been mainly classified according to the disease localization (pulmonary and extra-pulmonary) and their HIV status. Median age, male/female ratio, diabete, poor nutrition and history of past TB are also indicated in the table 8.

	Patients with active tuberculosis (n = 807)					
	Pulmonary tuberculosis (n = 656)			Extrapulmonary tuberculosis (n = 151)		
	HIV-infected	HIV-uninfected	HIV-unknown	HIV-infected	HIV-uninfected	HIV-unknown
	(n = 93)	(n = 270)	(n = 293)	(n = 38)	(n = 46)	(n = 67)
	Percentage					
Disease Localization	71.0	85.4	81.4	29.0	14.6	18.6
Median Age (IQR)	35.0	34.0	30.0	36.0	30.0	28.0
Ratio M/F	4.8	2.3	2.3	5.3	1.7	1.0
Male prevalence	82.8	66.3	70.0	84.2	63.0	50.8
Diabetes	0.0	11.1	2.4	0.0	6.5	1.5
Poor Nutrition	0.0	1.1	10.9	0.0	0.0	4.5
Past TB	28.4[a]	15.2	21.4	26.5[c]	10.9	5.9
TB <2 years	13.4	6.0	9.2	11.8	4.4	4.4
TB 2 to10 years	14.9	7.1	9.2	14.7	4.4	1.5
TB>10 years	0.0	2.2	3.1	0.0	2.2	0.0
Contact with TB	12.9[b]	8.5	8.2	12.5[d]	19.6	6.0

[a]26/93 patients have no record;
[b]23/93 patients have no record;
[c]4/38 patients have no record;
[d]6/38 patients have no record.

Table 8: Demographic and clinical characteristics of patients with active tuberculosis

Regarding extra-pulmonary population, table 9 indicates the localization of the infection according to the HIV status. We observed that the most frequent localization was pleural and abdominal as well among HIV positive and negative patients. Disseminated infection was mostly encountered among HIV positive population.

	HIV-infected	HIV-uninfected	HIV-unknown	Total
	(n = 38)	(n = 46)	(n = 67)	(n = 151)
	Percentage (95% CI)			
Cervical lymphadenopathy	5.3 (1.4–17.6)	30.4 (18.9–45.1)	32.8 (22.6–45.0)	25.2 (18.8–32.8)
Mediastinal lymphadenopathy	7.9 (2.7–21;1)	0.0 (0.0–8.0)	0.0 (0.0–5.6)	2.0 (0.7–5.8)
Pleural	27.8 (13.7–44.3)	26.1 (15.4–40.6)	19.4 (11.6–30.7)	23.2 (17.1–30.7)
Abdominal	29.0 (16.8–45.1)	17.4 (9.0–31.0)	7.5 (3.2–16.5)	15.9 (10.8–22.7)
CNS	15.8 (7.3–30.8)	15.2 (7.5–28.5)	0.0 (0.0–5.6)	8.6 (5.1–14.3)
Disseminated	10.5 (4.1–27.5)	2.2 (0.4–11.6)	0.0 (0.0–5.6)	3.3 (1.4–7.6)
Skeletal	5.3 (1.4–17.6)	2.2 (0.4–11.6)	38.8 (27.9–51.0)	19.2 (13.6–26.4)
Genito-urinary	0.0 (0.0–9.5)	2.2 (0.4–11.6)	0.0 (0.0–5.6)	0.7 (0.1–3.8)
Skin	0.0 (0.0–9.5)	0.0 (0.0–8.0)	2.2 (0.4–11.6)	0.7 (0.1–3.8)
Rectal	0.0 (0.0–9.5)	2.2 (0.4–11.6)	0.0 (0.0–5.6)	0.7 (0.1–3.8)
Pericarditis	0.0 (0.0–9.5)	2.2 (0.4–11.6)	0.0 (0.0–5.6)	0.7 (0.1–3.8)

Table 9: Localization of infection among extra-pulmonary TB according to their HIV status

The prevalence of the clinical sympoms varies between TB Pulmonary and TB extra-pulmonary infection. Fever and weight loss are commonly encoutered in both cases (table 10).

Total	Pulmonary	Extra Pulmonary
Cough > 2 weeks	91.3%	21.9%
Fever	81.4%	65.7%
Night sweat	14.8%	5.6%
Weight loss	72.4%	46.6%
Anorexia	42.3%	3.9%
Fatigue	43.4%	27.5%
Hémoptysie	12.7%	0.6%

Table 10: Clinical symptoms prelalence among pulmonary and extra-pulmonary TB

3-3 Tuberculin skin test results

TST has been performed in 43.5% (350/804) of enrolled adult TB patients, with a higher proportion in HIV-negative/unknown patients with pulmonary TB. The proportion was lower in patients with extra pulmonary TB and the lowest proportion was observed in HIV-positive patients with extra pulmonary TB (Table 11).

Tuberculosis patients		HIV-negative/unknown	HIV-positive	Total
Pulmonary TB	Number tested (%)	275/560 (49.1%)	27/93 (29.0%)	302/653 (46.2% %)
	Sensitivity	175/275 **(63.6%)**	15/27 **(55.6%)**	190/302 **(62,9%)**
Extra Pulmonary TB	Number tested (%)	44/113 (38.9%)	4/38 (10.5%)	48/151 (31.8%)
	Sensitivity	30/44 (**68.2%**)	3/4 **(75.0%)**	33/48 **(68.8%)**
Total	Number tested (%)	319/673 (47.4%)	31/131 (23.6%)	350/804 (43.5%)
	Sensitivity	205/319 **(64.3%)**	18/31 **(58.1%)**	223/350 **(63.7%)**

Table 11: Tuberculin skin test results (TST positive results with a cut off: diameter ≥ 10 mm) in the different groups of TB patients according to their HIV status and TB localization

3-4 Chest X ray results

Chest X ray has been performed on 88.4% of the pulmonary TB suspected patients included in that study, only 50% of the patients with extra pulmonary TB had no radiological report. This proportion was higher in HIV-negative patients as compared to HIV-positive patients. About two-thirds of the HIV-positive patients had a radiological record. The various radiological abnormalities notified by the chest X ray are reported in table 12.

X ray abnormalities	HIV-negative/unknown		HIV-positive		Total	
	Pulmonary TB	Extra pulmonary TB	Pulmonary TB	Extra pulmonary TB	Pulmonary TB	Extra pulmonary TB
Massive lesions	5.5	2.9	6.3	0.0	5.6	2.2
Infiltrate	**56.0**	**17.7**	**18.8**	0.0	**53.1**	**13.3**
Cavity	**23.3**	0.0	**29.2**	4.6	**23.8**	1.1
Consolidation	6.2	1.5	6.3	0.0	6.2	1.1
Med. adenopathy	2.8	**20.6**	2.1	0.0	2.7	**15.6**
Pleural effusion	1.9	**39.7**	**20.8**	**54.6**	3.8	**43.3**
Miliary	1.0	1.5	**10.4**	**13.6**	1.8	4.4
Fibrosis	0.9	0.0	2.1	0.0	1.0	0.0
Other	2.6	**17.7**	4.2*	**37.3***	2.7	**20.0**

Table 12: Percentage of most frequent chest X ray abnormalities reported in the different groups of TB patients according to their HIV status and TB localization *About half of this chest X ray did not present any abnormality

The main radiological abnormalities in pulmonary TB patients were infiltrates followed by the presence of cavities. The frequency of cavities was similar in HIV-negative/unknown and HIV-positive TB patients and infiltrates were less prevalent in HIV-positive as compared to HIV-negative TB patients. Pleural effusion and miliary tuberculosis were much more prevalent in HIV positive pulmonary TB as compared to pulmonary HIV negative TB patients. In patients with extra pulmonary TB, the main radiological abnormality was represented by the presence of pleural effusion in more than 40% of the patients; such abnormality was observed even more frequently in HIV-positive patients with a prevalence of 55%. Again in such group the presence of miliary tuberculosis was observed in 14% of these patients. In HIV-negative TB patients with extra-pulmonary disease it was also observed that about one-fifth of them presented minimal lesions such as mediastinal adenopathy. In the HIV-TB coinfected patients, it was also observed that several subjects

did not present any abnormality on their chest X ray. Among them one had high yield of AFB in the sputum.

3-5 Microbiological results (Annex 3) (Lagrange *et al*, Plos one 2012)

3-5-1 Smear microscopy

All enrolled patients had smear examination for at least one specimen: 43% had two specimens and 23% had three. The sensitivity of the smear was higher in pulmonary HIV negative patients compared to the pulmonary HIV positive patients, respectively 75.9% and 42.2%. We observed a much lower sensitivity of the smear in extra-pulmonary TB patients as well in HIV positive and negative patients as expected (Table 13).

	Pulmonary TB		Extrapulmonary-TB		Pulmonary vs Extrapulmonary
	AFB-positive/total number of patients tested per group (Percentage)	Comparison by HIV Status *p* value	AFB-positive/total number of patients tested per group (Percentage)	Comparison by HIV Status *p* value	*p* value
HIV-infected	38/90* (**42.2%**)	0.0001 vs HIV-uninfected	3/31** (**9.7%**)	0.34 vs HIV-uninfected	0.0004
		0.0047 vs HIV-unknown		0.37 vs HIV-unknown	
HIV-uninfected	205/270 (**75.9%**)	0.0002 vs HIV- unknown	9/46 (**19.6%**)	1.00 vs HIV-uninfected	0.002
HIV-unknown	180/293 (**61.4%**)	–	13/67 (**19.4%**)	–	0.0001
Total	423/653* (**64.8%**)		25/144** (**17.4%**)		0.0001

3 patients had no AFB records;
**7 patients had no AFB records.

Table 13: Sensitivity of smear microscopy (AFB positive) in the different groups of TB patients according to their HIV status, TB localization and culture results on BacT Alert (BTA) and Lowenstein Jensen (LJ) solid media.

3-5-2 Culture

Culture was performed on all specimens using two culture media (liquid and solid media), identification was performed on the isolated mycobacteria with the AccuProbe test.

- ✓ *Culture sensitivity*

The performances of both culture systems have been calculated according to the localization of the infection (pulmonary and extra-pulmonary) and the HIV status (Table 14 and 15).

	Pulmonary TB			
	Liquid cuture	**Solid culture**	**Total**	**P *value of* liquid vs solid cultures**
HIV-infected	55/86	38/87	56/87	0.3
	64%	47%	64.4%	
HIV-unifected	218/269	184/268	225/270	0.0001
	81%	68.7%	83.3%	
HIV-unknown	238/293	164/293	245/293	0.0001
	81.2%	56%	83.6%	
Total	511/648	386/648	526/650	0.0001
	78.9%	59.6%	80.9%	

Table 14: Comparative frequency of positive results of liquid versus solid culture in pulmonary active tuberculosis patients according to HIV status.

	Extra-pulmonary TB			
	Liquid cuture	**Solid culture**	**Total**	**P *value of* liquid vs solid cultures**
HIV-infected	14/30	1/33	15/33	0.2
	46.7%	30.3%	45.5%	
HIV-unifected	27/46	20/45	29/46	0.2
	58.7%	44.4%	63%	
HIV-unknown	39/67	27/67	39/67	0.03
	58.2%	403%	48.2%	
Total	80/143	57/145	83/146	0.004
	55.9%	39.3%	56.8%	

Table 15: Comparative frequency of positive results of liquid versus solid culture in extra-pulmonary active tuberculosis patients according to HIV status.

✓ *Comparison of time to detection (TTD)*

The TTD between liquid and solid culture media was significantly higher in solid media. The median TTD are (Figure 40):

- 34 days for solid culture
- 12 days for liquid culture

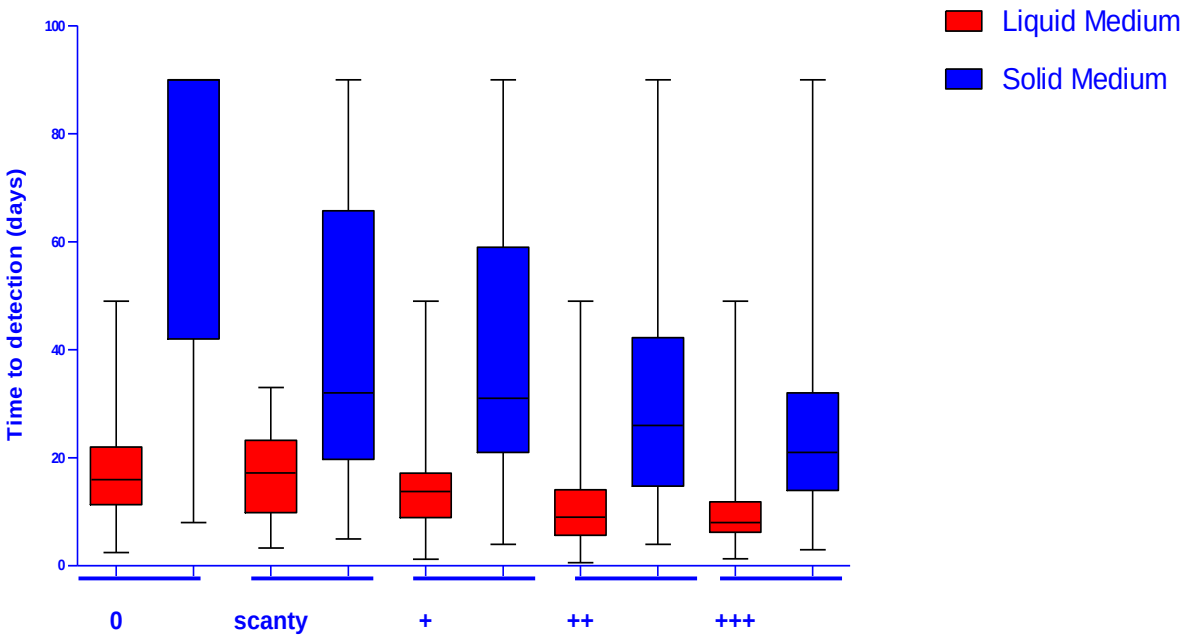

Figure 40: Time to detection (TTD) of mycobacteria between liquid and solid medium

✓ *Contamination rate*

The overall contamination rate was very low for liquid (1.9%) and solid cultures (1.4%).

✓ *Prevalence of mycobacteria other than tuberculosis (MOTT)*

The frequency of MOTTs isolated in positive cultures according to the HIV status and localization of the infection is indicated in table 16.

	Positive MOTTs Pulmonary TB	**Positive MOTTs Extra-pulmonary TB**	**Total**
HIV-infected	7/56	4/15	11/71
	12.5%	26.7%	15.5%
HIV-unifected	11/224	2/29	13/253
	4.9%	6.9%	5.1%
HIV-unknown	5/245	0/39	5/284
	2%	0%	1.8%
Total	23/525	6/83	29/608
	5.4%	7.2%	4.8%

Table 16: MOTT prevalence among pulmonary and extra-pulmonary TB

Note

The results of the standard microbiological tools are described in the following paper: "A toolbox for tuberculosis Diagnosis: An Indian Multicentric Study (2006-2008): Microbiological results. Lagrange *et al*, PLOS one, vol 7: 2012" (Annex 3)

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PLOS | ONE

A Toolbox for Tuberculosis Diagnosis: An Indian Multicentric Study (2006-2008): Microbiological Results

Philippe H. Lagrange[1]*, Satheesh K. Thangaraj[2], Rajeshwar Dayal[3], Alka Despande[4], Nirmal K. Ganguly[5], Enrico Girardi[6], Beenu Joshi[7], Kiran Katoch[7], Vishwa M. Katoch[7], Manoj Kumar[8], Vemu Lakshmi[9], Marc Leportier[2], Christophe Longuet[10], Subbalaxmi V. S. Malladi[9], Deepali Mukerjee[5], Deepthi Nair[11], Alamelu Raja[12], Balambal Raman[12], Camilla Rodrigues[13], Pratibha Sharma[11], Amit Singh[3], Sarman Singh[8], Archana Sodha[13], Basirudeen Syed Ahamed Kabeer[12], Guy Vernet[10], Delia Goletti[6]

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3-6 Molecular tests

In this study, the purpose of the evaluation of molecular tests was mainly to compare the performances between home brew PRC and a commercial test: Gene probe TMA kit. Due to cost issue, many laboratories in India developped their own PCR techniques in order to have access to this technology at a much cheaper price.

The study was performed on 3 sites (Jalma, AIMS and Hinduja).

Every site used its own method.

- Jalma used:

PCR for IS6110 (115)
IS1 5'CCTGCGAGCGTAGGCG3'
IS2 CTCGTCCAGCGCCGCTTCGG3'

- AIMS used:

Species specific PCR targetting internal transcribed spacer (ITS) region for identifying mycobacteria at the species level. Detection of *M. tuberculosis* was based on PCR targetting the ESAT-6 region (116).

- Hinduja used:

a single tube multiplex nested PCR targeting insertion sequence 6110, *mpb64*, *rrs* and *rpo*B genes (117).

The comparative sensitivity results between home brew PCR and TMA are reported in the table 17.

Site		# samples	PCR sensitivity	TMA sensitivity
Jalma	PTB	58	79.3%	79.3%
	Extra PTB	4	75%	25%
AIMS	PTB	30	90%	90%
Hinduja	Extra PTB	51	74.5%	50.9%
Total	PTB	88	82.9%	82.9%
	Extra PTB	54	75.9%	50%

Table 17: Molecular results; PTB: pulmonary tuberculosis; Extra PTB: extra pulmonary tuberculosis

3-7 Immunological tests results

3-7-1 Serological ELISA test

The cut off has been established using the receiver operating characteristic (ROC) curve analysis on the low risk population (healthy adults and blood donors) with a specificity of 91.8% for each antigen.

The overall specificity with both antigens is 85.4% (Table 18).

Specificity on the low risk TB population			
	Blood donors	**Healthy adults**	**Total**
# individuals	459	155	614
PGL-Tb1	91.5%	93%	91.8%
ESAT6-CFP10	91.9%	91%	91.8%
PGL-Tb1 + ESAT6/CFP10	85.5%	85%	**85.4%**

Table 18: Specificity on low risk TB population

✓ *Sensitivity of the ELISA test*

The sensitivity of the ELISA test has been calculated according to the HIV status, the localization of the infection, among the smear negative (AFB) and clinical TB patients (microbiologically negative) (Tables 19, 20 and 21).

Sensitivity among TB patients (clinical + microbiologically confirmed TB)			
HIV negative/unknown	**Pulmonary**	**Extra-pulmonary**	**Total**
PGL-Tb1	**41.6%**	**29.9%**	**39.5%**
ESAT6/CFP10	**29.6%**	**16.7%**	**27.2%**
Both	**51.6%**	**36.8%**	**48.9%**
# Patients	**639**	**144**	**783**
HIV positive	**Pulmonary**	**Extra-pulmonary**	**Total**
PGL-Tb1	**72.9%**	**67.7%**	**71.2%**
ESAT6/CFP10	**60.0%**	**67.7%**	**63.5%**
Both	**75.7%**	**73.5%**	**75.0%**
# Patients	**70**	**34**	**104**

Table 19: Sensitivity among TB patients microbiologically confirmed

Sensitivity among AFB negative TB patients			
HIV negative/unknown	Pulmonary	Extra-pulmonary	Total
PGL-Tb1	35.9%	30.7%	33.7%
ESAT6/CFP10	23.9%	16.1	21.0%
Both	43.1%	37.1%	40.8%
# Patients	209	125	334
HIV positive	Pulmonary	Extra-pulmonary	Total
PGL-Tb1	74.4%	74.1%	74.2%
ESAT6/CFP10	66.7%	70.4%	68.2%
Both	79.5%	74.1%	78.8%
# Patients	39	27	66

Table 20: Sensitivity among AFB negative TB patients

Sensitivity among Clinical TB patients (microbiologically negative)			
HIV negative/unknown	Pulmonary	Extra-pulmonary	Total
PGL-Tb1	32.1%	35.9%	33.5%
ESAT6/CFP10	22.0%	21.9%	19.1%
Both	40.4%	39.1%	39.9%
# Patients	109	64	173
HIV positive	Pulmonary	Extra-pulmonary	Total
PGL-Tb1	70.6%	75.0%	72.73%
ESAT6/CFP10	58.9%	81.3%	69.7
Both	76.5%	81.3%	78.8%
# Patients	17	16	33

Table 21: Sensitivity among clinical TB patients (microbiologically negative)

The sensitivity of the serological test was significantly higher in the HIV acute TB population (75% for HIV positive/ 48.9% HIV negative-unkown).

The sensitivity was similar in all 3 TB patient categories: AFB negative, clinical TB and microbiologically confirmed TB.

- ✓ *Specificity of the ELISA test*

The specificity of the ELISA test has been calculated for the following non TB high risk population: laboratory staffs, healthy contacts, non TB patients and leprosy patients (Table 22). Among the high risk population, the highest specificity was obtained with the laboratory staffs; however, this specificity was significantly lower than the low risk patients (Table 18) (85.4% and 76% respectively). A poor specificity was obtained with the non TB patients.
For leprosy patients, the specificity of the ESAT6/CFP10 antigen was significantly higher than the PGL-Tb1 (90% and 76.7% respectively).

Regarding the healthy contacts, the specificity of the ESAT6/CFP10 antigen was significantly higher than the PGL-Tb1 (85.8% and 69.8% respectively).

Specificity among the non TB high risk individuals		
	Laboratory staff	**Non TB patients**
PGL-Tb1	**81.8%**	**69%**
ESAT6/CFP10	**87.6%**	**59.5%**
Both	**76%**	**50%**
# individuals	**121**	**42**
	Healthy contacts	**Leprosy patients**
PGL-Tb1	**69.8%**	**76.7%**
ESAT6/CFP10	**85.8%**	**90%**
Both	**67.9%**	**70%**
# individuals	**106**	**30**

Table 22: Specificity among non TB high risk individuals

✓ *Combined sensitivity smear and ELISA*

Most TB diagnostics in India rely on clinical examination and smear microscopy (SM), culture is realized on a very limited number of patients. As ELISA tests are widely performed in India in many laboratories, therefore, we evaluated the medical interest to combine the serological test with the smear microscopy examination.

The sensitivity of the smear microscopy alone and combined with the serological ELISA test according to the HIV status and TB localization (pulmonary and extra-pulmonary) are shown in figures 41 & 42 and table 23.

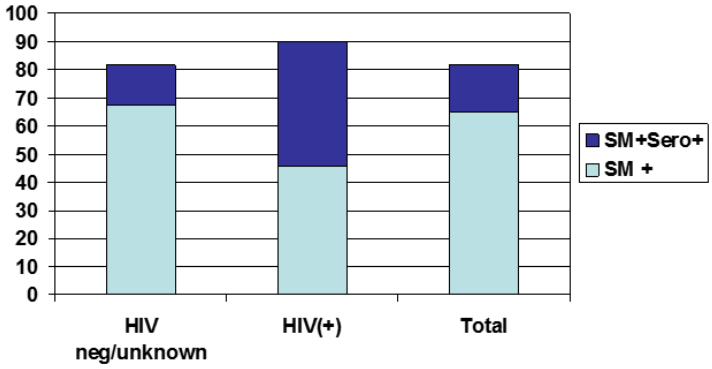

Figure 41: Combined sensitivity smear positive and ELISA among pulmonary TB

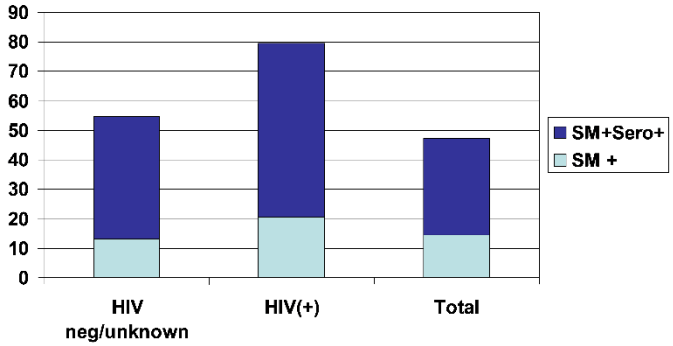

Figure 42: Combined sensitivity smear positive and ELISA among extra-pulmonary TB

TB patients	Test	HIV neg/unknown	HIV +	Total
Pulmonary	**Smear +**	430/639 (67.3%)	32/70 (45.7%)	462/609 (65.2%)
	Combined tests	520/639 (81.4%)	63/70 (90%)	583/709 (82.2%)
Extra Pulmonary	**Smear +**	19/144 (13.2%)	7/34 (20.6%)	26/178 (14.6%)
	Combined tests	65/144 (45.1%)	28/34 (82.4%)	93/178 (52.3%)
Total	**Smear +**	449/783 (57.3%)	39/104 (37.5%)	488/887 (55%)
	Combined test	585/783 (74.7%)	91/104 (87.5%)	676/887 (76.2%)

Table 23: Comparative sensitivity results of smear positive and ELISA serological test on all TB patients (pulmonary and extra-pulmonary)

3-7-2 Interferon-Gamma release assay

- ✓ ***Results obtained with the QuantiFERON-TB Gold in tube (QFT-GIT)***

291 individuals have been tested by GFT-GIT: 111 active TB and 180 non-active TB patients. The results are shown in tables 24 and 25.

		# Patients tested	# Indeterminate	# Positive	Sensitivity %
HIV +	**Pulmonary**	38	12	23	88.5%
	Extra pulmonary	4	2	2	100%
	Total	42	14 (33.3%)	25	89.2%
HIV -	**Pulmonary**	58	2	53	94.6
	Extra pulmonary	2	0	0	0
	Total	60	2 (3.3%)	53	91.4%
HIV unknown	**Pulmonary**	8	4	4	100%
	Extra pulmonary	1	0	1	100%
	Total	9	4 (44.4%)	5	100%
Total patients tested		111	20 (18%)	83	91.2%

Table 24: GFT-GIT results among the active TB patients

	# Patients tested	# Indeterminate	# Positive	# positive/ # Interpretable
Health community adults (HIV-)	55	0	23	41.8%
Health community adults (HIV+)	54	6	19	39.6%
Health care workers	5	0	3	60%
Healthy family contact	54	1	33	62.3%
Cured TB patients	12	0	11	91.7%
Total	180	7	89	51.5%

Table 25: GFT-GIT results among the non-active TB patients

Among the individuals at low risk to be exposed (healthy community adults) we observed a percentage of positivity around 40% whatever the HIV status.
As the GFT-GIT is not a diagnostic tool to differentiate between active TB from latent TB, this data indicates the percentage of the Indian population which has been exposed to TB.

The percentage of positivity was higher with the population at higher risk to be exposed to TB (health care workers and healthy family contacts), around 60%.

The percentage of positivity in the cured TB patients was almost equal to what we observed among the active TB population (around 91%).

- ✓ ***Concordance between GFT-GIT and TST***

The concordance between the GFT-GIT and the TST has been done on 57 active pulmonary tuberculosis and 122 non active TB. The TST was read with a cut off at 10 mm (Tables 26 & 27).

			GFT-GIT			
			Positive	**Negative**	**Total**	**Agreement**
TST	**HIV+**	**Positive**	17	2 (2*)	19 (2*)	81.1%
		Negative	5	13 (10*)	18 (10*)	
		Total	22	15 (12*)	37 (12*)	
	HIV -	**Positive**	16	1	17	80%
		Negative	3	0	3	
		Total	19	1	20	

***indeterminate by GFT-GIT**

Table 26: GFT-GIT/TST concordance on 57 active pulmonary TB according to their HIV status.

			GFT-GIT			
			Positive	**Negative**	**Total**	**Agreement**
TST	**HIV+**	**Positive**	8	1	9	77.8%
		Negative	11	34 (6*)	45 (6*)	
		Total	19	35 (6*)	54 (6*)	
	HIV -	**Positive**	32	8	40	70.6%
		Negative	12	16	28	
		Total	44	24	68	

***indeterminate by GFT-GIT**

Table 27: GFT-GIT/TST concordance on 122 non active TB individuals according to their HIV status

The sensitivity on active pulmonary TB between GFT-GIT and TST was not significantly different. We observed a significant amount of indeterminate results among the HIV positive population with the GFT-GIT (32.4%), 10 of them were TST negative. In most cases the percentage of indeterminate results was related to the percentage of patients with a low CD4 level. In this study, the level of CD4 was available for a very small amount of patients, therefore no correlation could be performed.

In HIV-infected non active tuberculosis, the percentage of positive was higher with the IGRA test compare to the TST: respectively 39.5% and 18.7% (excluding indeterminate results).

- ✓ ***RD1 selected peptides***

The response to RD1 selected peptides has been evaluated under three different angles:

- ✓ After stimulation of the lymphocytes with specific RD1 selected peptides, 4 cytokines (IFN-ϒ, IP-10, MCP-2 and IL-2) productions were measured in order to evaluate their usefulness as biomarkers of active tuberculosis in a country with a high incidence of TB. This study was conducted on 129 individuals: 41 active pulmonary TB and 88 non active TB (household contacts and community controls) (Annex 4).

- ✓ Evaluation of the IP10 and IFN-ϒ response to RD1 selected on HIV infected individuals: 28 with active TB and 38 without (Annex 5).

- ✓ Evaluation of the IP10 and IFN-ϒ response to RD1 selected peptides as diagnostic marker to monitor the efficacy of tuberculosis therapy (Annex 6).

These studies has been published in the following 3 papers.

Journal of Infection (2010) 61, 133–143

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IFN-γ, but not IP-10, MCP-2 or IL-2 response to RD1 selected peptides associates to active tuberculosis

Delia Goletti [a,*], Alamelu Raja [b], Basirudeen Syed Ahamed Kabeer [b], Camilla Rodrigues [c], Archana Sodha [c], Ornella Butera [a], Stefania Carrara [a], Guy Vernet [d], Christophe Longuet [d], Giuseppe Ippolito [e], Satheesh Thangaraj [f], Marc Leportier [f], Enrico Girardi [g], Philippe Henri Lagrange [h]

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PLoS one

Is IP-10 an Accurate Marker for Detecting *M. tuberculosis*-Specific Response in HIV-Infected Persons?

Delia Goletti[1]*, Alamelu Raja[2], Basirudeen Syed Ahamed Kabeer[2], Camilla Rodrigues[3], Archana Sodha[3], Stefania Carrara[1], Guy Vernet[4], Christophe Longuet[4], Giuseppe Ippolito[5], Satheesh Thangaraj[6], Marc Leportier[6], Enrico Girardi[7], Philippe Henri Lagrange[8]

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Kabeer *et al. BMC Infectious Diseases* 2011, **11**:135
http://www.biomedcentral.com/1471-2334/11/135

BMC Infectious Diseases

RESEARCH ARTICLE Open Access

IP-10 response to RD1 antigens might be a useful biomarker for monitoring tuberculosis therapy

Basirudeen Syed Ahamed Kabeer[1], Alamelu Raja[1], Balambal Raman[2], Satheesh Thangaraj[3], Marc Leportier[3], Giuseppe Ippolito[4], Enrico Girardi[5], Philippe Henri Lagrange[6] and Delia Goletti[7*]

V- Discussion

This prospective multicenter clinical evaluation (MCE) was designed to evaluate the diagnostic value of several TB diagnostic tools in a highly endemic country.
Several diagnostic tools were systematically tested to each patient with suspected TB: 2 conventional tools (smear microscopy and solid culture) and 2 relatively new tools: automated liquid culture system and ELISA serological test. Two other categories of new tools were applied on a portion of patients: direct nucleic acid amplification and interferon gamma release assays (IGRAs) tests.
The total number of patients enrolled in that study was high (2,213). Among them, 1,787 patients were included in the main study with a proportion of 45% with active TB. Among the active TB, the HIV status was known for only 55% of the patients.
In India, smear microscopy (SM) is most of the time the only laboratory diagnostic test realized for TB diagnostic as recommended in the Indian tuberculosis control programme. Detection of acid fast bacilli (AFB) has therefore a crucial epidemiological importance in this country. The sensitivity of SM detecting 2/3 of active pulmonary TB in HIV-uninfected and half in HIV infected is within the expected range. SM were performed by very well trained technicians in outstanding laboratories. We have to consider that the SM performance varies according to the site involved, the type of population recruited (prevalence of HIV infected patients) and the localization of the infection. We confirmed the poor sensitivity of SM among extra-pulmonary TB patients: 14.6%.

Lowenstein-Jensen (LJ) media has traditionally been the gold standard for TB diagnosis in India and in other resource-limited countries. Liquid culture is the gold standard in developed countries. As demonstrated in the study, the sensitivity of the liquid culture is significantly higher than the LJ media in TB pulmonary and extra pulmonary patients, liquid culture also allows to reduce significantly the time to detection (TTD). These data confirmed those obtained in other studies.

Our study showed a very low overall contamination rate on both liquid and LJ media, whereas other studies reported a higher contamination rate with liquid culture (118). These good results obtained in our study may be due to the quality of the process to decontaminate the clinical specimens and the expertise of the technicians in charge of the evaluation.

Another added value of the liquid culture is the capability to detect mycobacteria directly from blood specimens. This advantage is very important to better diagnose disseminated TB often encountered among HIV- infected patients. WHO issued a report regarding the

use of liquid culture in low and medium income settings, recognizing the performance of this method.
(http://www.who.int/tb/laboratory/use_of_liquid_tb_culture_summary_report.pdf).
The identification of mycobacteria using the molecular technique (Accuprobe) on all positive culture samples showed that frequency of *mycobacterium* other than tuberculosis complex (MOTT) was higher in HIV-infected than HIV-uninfected and HIV-unknown TB patients, respectively 15.5%, 5.1% and 1.8%. These results were in agreement with other reports. MOTTs were isolated from around 10% of hospitalized non active TB patients. These patients were cured using second line macrolides. Such a high prevalence has not been previously reported in India.

When molecular tests for direct detection of mycobacteria in clinical specimens were performed in India, many laboratories used home brew methods. The advantage of commercial product is the standardization of the method and therefore less variability between laboratories. In our study the results obtained from 3 different sites showed equivalent performances between PCR and Gene probe TMA and better results with PCR for extra-pulmonary tuberculosis. From these results, it seems that the use of home brew PCR may be used instead of the Geneprobe commercial kit. Recently, new automated system from Cepheid has been released on the market, allowing direct detection of *mycobacterium tuberculosis* complex and rifampicin resistance. This system has been widely studied by the Foundation for Innovative New Diagnostics (FIND) and is strongly supported by WHO to be implemented in low income countries (WHO, Nucleic Acid Amplification Technology for Rapid and Simultaneous Detection of Tuberculosis and Rifampicin Resistance: Xpert MTB/RIF System Policy Statement, WHO/HTM/TB/2011.4).

The serological data obtained previously on a limited patient panel were very encouraging (Tables 6 & 7). However, as already described, the performances of TB serological diagnostic test may vary according to the population tested and often studies were conducted on a very limited number of patients (119). This multicenter clinical evaluation allowed to evaluate our ELISA prototype serological test on a large number of patients from different parts of India.
First, we observed using, the same mathematical model to establish the cut off, that the specificity on the healthy blood donors is lower in this MCE compared to the previous data, respectively 85.4% and 90%. The specificity in the previous study was identical between healthy blood donors and the non TB patients (non TB high risk), while the specificity in the

non TB high risk population is lower compared to the healthy blood donors in the MCE. The sample size in the preliminary study was low (31 individuals); in the MCE, the number of non TB high risk patients tested was much higher (299 individuals). In a country like India with a high level a latent tuberculosis, we may suspect that a proportion of patients exposed to TB may develop an antibody response.
The higher level of false positive results observed in the non TB patients, suspected of TB, but confirmed to have another infectious disease, might be the result of a polyclonal antibody reactivation involving previous anti specific TB antibodies raised during a primary TB infection.

Regarding leprosy patients, the overall specificity is similar to the healthy contact. We noticed a relatively high specificity with the ESAT-6/CFP-10 antigen (90%). The RD1 region coding for the ESAT-6 and CFP-10 protein antigens in *M. tuberculosis* is not present in *M. leprae*. In contrario, *M. leprae* produces a phenol glycolipid antigen (PGL1) known to induce antibody response. The structure of the PGL1 from *M. leprae* shares common sugar with *M. tuberculosis* PGL-Tb1. Therefore, we can suspect some level of cross reactivity with the PGL-Tb1 used in the ELISA test.

The sensitivity among TB-HIV negative/unknown was significantly lower than in TB-HIV positive population, respectively 48.9% and 75%; these results confirmed the data observed during the preliminary study. The population of patients with unknown HIV status has been combined with the HIV negative; indeed, there are good indications that the percentage of HIV positive among this population is very low according to the clinical information and the prevalence of MOTT which is extremely low: 1.8% for HIV unknown, 5.1% for HIV negative and 15.5% for HIV positive. Therefore combining HIV negative and HIV unknown will not change the statistical analysis.

Among HIV positive patients the sensitivity was similar between pulmonary and extra-pulmonary TB. On the other hand, for the HIV negative/unknown population, the sensitivity was lower in extra-pulmonary tuberculosis. The higher sensitivity observed among HIV positive patients may be due to a dissemination of the mycobacteria in the body stimulating the B cell lymphocytes. Also the higher antibody response observed among the HIV patients suggest that the B cell immune response toward the selected antigens is T-cell independent.

The sensitivity of the serological test is not significantly different between the smear positive, smear negative and only clinically confirm cases for both populations HIV negative/unknown and HIV positive. The added value of the serological test as a new diagnostic tool would be to improve the case detection in a setting where only SM is realized for TB diagnostic. Therefore we looked at the combination of SM and ELISA serological test (Figure 39 and 40). We noticed a major improvement regarding the diagnostic sensitivity among extra-pulmonary TB and also HIV population. There is less added value of the serological test among the HIV negative/unknown pulmonary TB.

These data indicate an interesting added value of the serological TB diagnostic where TB diagnostic is known to be difficult: HIV patients and extra-pulmonary TB. However, this improvement regarding the TB diagnostic sensitivity needs to be balanced with the weak specificity observed among non TB high risk population; this serodiagnostic test may not differentiate enough between active and latent TB. These data do not confirm the preliminary results which showed a clear cut between the non-active TB and active TB. We learned from this multicenter clinical evaluation, that the sample size and geographic distribution is very important to evaluate accurately diagnostic tools.

Interferon gamma release assay (IGRAs) diagnostic tests start to be widely used in developed countries, Cellestis and Oxford immunotech received the FDA approval for the US market. The main indication of these new diagnostic products is to better diagnose latent TB infection. In the past only tuberculin skin test (TST) was used for this purpose. TST is known to cross react with BCG vaccination and other mycobacteria from the environment.
The satellite studies conducted on IGRAs tests showed first a very good sensitivity among active TB-HIV negative/unknown patients and slightly lower in HIV positive patients. The percentage of indeterminate results in HIV positive was high (33.3%) which has made the test uninterpretable. Indeterminate results are usually associated to a low level of CD4; unfortunately, in our study the CD4 count was available for a very small amount of patients. TST is still widely used in India, therefore it was interesting to compare the QuantiFERON-TB Gold to the TST as well on active and non-active TB. On active TB-HIV negative patients, the agreement between both methods was 80%. The sample size was small, only 20 patients, the differences between both methods was not significantly different.
Surprisingly IGRA and TST had a comparable percentage of positivity among the non-active TB-HIV negative, respectively 64.7% and 58.8% and an overall agreement of 70.6%. Our

results did not confirm what was reported in the literature, indicating that TST is less specific than IGRA due to cross reactivity with BCG vaccination which is widely used in India and interferences with non-tuberculosis mycobacteria (NTM) (120). However, we need to consider that the number of patients tested in parallel was small, only 68 individuals.
The higher percentage of positive results with the IGRA test among the non TB-HIV positive patients indicates that the IGRA test has a better sensitivity than the TST for detection of LTBI among HIV population regardless the high percentage of indeterminate results with IGRA.

The objectives of satellite studies conducted with a new IGRA test using the RD1 selected peptides from the INMI were:

- to evaluate if these new tools could better differentiate between LTBI and active TB, 4 cytokines in response to RD1 peptide were tested (annex 4);
- to evaluate these 4 cytokines response in HIV-infected population (annex 5);
- to evaluate the usefulness of IP-10 as a biomarker to monitor tuberculosis therapy (Annex 6).

This study showed that the *in vitro* IFN-ϒ response to RD1 selected peptides was significantly associated with active TB and was more specific than the QFT-IT and the TST. Other markers such as IP-10, MCP-2 and IL-2 showed also a significantly higher response in patients with active TB. However no significant difference between patients with active TB and the household contacts was found with these cytokines.
TST, QFT-IT and RD1 selected peptides elicit *M. tuberculosis* immune responses by effectors memory T-cells. TST is a crude preparation of several mycobacteria antigens, QFT-IT is based on a pool of overlapping peptides spanning the whole length of CFP-10 and ESAT-6 proteins and an additional peptide from RD11. The RD1 selected peptides correspond to restricted epitopes of ESAT-6 and CFP-10; this selection was designed to reduce false positive among non-active TB. The better specificity observed with the RD1 selected peptides for active TB is compromised by a lower sensitivity among active TB. Whether it is more acceptable to have false positive tests results that may lead to overtreatment or false negative tests results that potentially lead to miss active TB cases is a matter of debate.
The results obtained with the HIV population showed a strong IP-10 response to RD1 selected peptides independently of the CD4 count. On the other hand, we also observed a

strong response among the HIV patients without active TB, leading this marker with a low potential for differentiation between active and latent TB. The study has some limits: the number of HIV subjects enrolled in that satellite study was not very high, the BCG status was unknown for 96.4% of the patients and also the CD4 T-cell counts were not performed on the total population. At the moment it is unclear whether higher detection of IP-10 was related to higher sensitivity or to the lower specificity of the assay. Further studies are needed to elucidate it in both endemic and low endemic TB countries.

The longitudinal study conducted on 17 patients with active TB who were followed until therapy completion demonstrated for the first time that IP-10 secreted response to selected RD1 peptides decreased during specific TB treatment. We showed that IP-10 might be a useful biomarker for monitoring therapy efficacy in patients with active TB. Additional studies performed on a larger number of individuals are needed to evaluate the consistency of these results.

VI-Conclusion

This large prospective multicenter tuberculosis diagnostic tool evaluation performed on more than 2000 patients across India's country allowed a comparison of the major TB diagnostic tools: smear microscopy (SM), culture (liquid and solid) strain identification and new markers like a serological prototype tests and interferon gamma release assay (IGRA) versus tuberculin skin test (TST).

The strong commitment of all partners involved in that study, sharing and following a unique clinical trial evaluation protocol was essential to accomplish this work. The study supervision by a TB scientist expert, dedicated full time on this project for 2 years was essential to achieve an outstanding clinical evaluation.

India, with the ninth largest economy in the world and the third largest by purchasing power parity, has a very strong inequitable healthcare system. TB affects mostly the poorest. TB prevalence is 5 time higher between the poorest and the richest in that country. For the poorest population, smear microscopy is often the unique diagnostic tool utilized in decentralized health center. Detection of acid fast bacilli (AFB) has therefore a crucial and epidemiological importance in the diagnostic algorithm for TB where there are limited resources.

In our study, the sensitivity of SM was within the expected range, detecting about 2/3 of active pulmonary TB in HIV-uninfected and half in HIV-infected. During the study the SM were realized in laboratories with strong expertise in the field of TB diagnostic; the sensitivity of SM is often lower in decentralized health care facilities due to less qualified technicians and lack of resources. SM using Zielh-Neelsen stain requires 20 minutes reading time per slide. The implementation of LED microscopy may contribute to increase significantly the quality of SM in decentralized health care facility. With LED microscopy, reading time is dividing by 4 and the sensitivity is higher (28). The Revised National TB Control Programme (RNTP) 2012 report, emphasis the implementation of LED microscopy in decentralized health care facilities. The introduction of LED microscopy needs training, quality management and monitoring of performance.

Evaluation of automated liquid culture system shows a higher sensitivity and a significant reduction of the time to detection (TTD) over the traditionally Lowenstein-Jensen (LJ) medium. Liquid culture brings the advantage to detect mycobacteria directly in the blood of the patient and therefore contribute to improve the difficult diagnostic of disseminated TB often encountered among the HIV-TB infected population. Unfortunately, the cost of

automated culture system does not allow its utilization in decentralized health care system. This kind of equipment is mostly encountered in the private sector, public medical college hospital and some national reference laboratories. Only patients with private insurance can afford to pay the cost of such diagnostic tests.

The study realized on the ELISA serological test is one of the most important which had ever been published in the literature. Many controversial reports have been written regarding the performances of TB serological diagnostic tests, most of the time the studies were carried out on a limited number of patients and often realized in non-endemic countries. ELISA test has the advantage to be affordable and can be performed in many laboratories in India.
We observed a higher sensitivity among HIV/TB co-infection with the ELISA test compared to TB/HIV negative, however the lack of specificity observed among non-active TB population makes that test difficult to be used as a marker for active TB. The introduction of this kind of new TB diagnostic tool may lead to over TB treatment.

The evaluation of the interferon gamma release assays showed that interferon gamma (IFN-ϒ) response to RD1 selected peptides was significantly higher in active TB patients than in household contacts and community controls; it is also associated with active TB with a higher specificity than the commercial Quantiferon-TB Gold in-Tube (QFT-IT) from Cellestis. The response of the 3 other cytokines IP-10, MCP-2 and IL-2 to RD1 selected peptides does not improve the differential diagnostic between active and latent TB. IP-10 response to RD1 selected peptides improves detection among the HIV population and is not affected by the level of CD4 count; this higher sensitivity may be due to the fact that IP-10 is mainly secreted by monocytes/macrophages while IFN-ϒ and IL-2 are mainly secreted by $CD4^+$ T-cells. However the percentage of positive results among the non-active TB was high and therefore this marker cannot differentiate between active and non-active TB; these results may be due to the fact that IP-10 is detected in contact. It is impossible in a country like India to rule out contact with *M. tuberculosis*.

Surprisingly, the comparative study between the commercial QFT-IT and TST did not show in that clinical evaluation any added value of the QFT-IT on the TST despite of the high BCG coverage in India. The commercial IGRA tests require laboratory infrastructure and are much more expensive (50$/patient with Quantiferron) than TST (2$/patient). The added value of IGRA tests seems very limited in a country like India.

From this tool box evaluation, we showed that TB diagnostic tests based on immune response in a high endemic country do not help to differentiate latent from active TB; only microbiological tests can confirm the active TB. SM alone detects 40 to 60% of the active TB, implementation of liquid culture would improve significantly the TB diagnostic. Introduction of new automated molecular tests detecting MTB and rifampicin resistance would also improve TB management in India.

In the India 2012 RNTP annual report, a chapter has been written concerning these new diagnostic tools (LED microscopy, liquid culture and Cartridge Based Nucleic Acid Amplification test (Genexpert)). Today these new tools are not introduced in decentralized health care facilities.

Bring these technologies at the level of primary and community health centre would require a strong financial support. Money could be found. The government spends more subsidizing fertilizer for farmers than on all public health care. Fuel subsidies, mostly helping the better off, are equally costly. Ways to raise funds exist. Taxing gold and diamond imports, for example, would bring in nearly $10 billion in annual revenue (59).

This study was the opportunity to evaluate a wide range of diagnostic tools. Actually, none of them can predict the evolution from latent TB to active TB before the installation of clinical symptoms in order to start a preventive therapy. This kind of new tool would be a great added value in the management of tuberculosis disease, mostly for HIV population who is 10 times more at risk to develop an active TB.

C O N C L U S I O N S

THESE SOUTENUE PAR : Marc LEPORTIER

Une évaluation prospective et multicentrique d'outils de diagnostic de la tuberculose a été réalisée sur plus de 2000 patients dans différentes régions de l'Inde et avait pour objectif de comparer les différentes techniques de diagnostic de cette infection : examen microscopique, culture (solide et liquide), identification des souches et de nouveaux marqueurs, test prototype sérologique et tests basés sur la production d'interféron gamma (IFN-γ) après stimulation des lymphocytes (Interferon Gamma Release Assay : IGRA).
Une forte implication de tous les partenaires engagés dans cette étude, partageant un protocole d'évaluation clinique unique a été obtenu pour la réalisation de ce travail et a nécessité la supervision d'un expert scientifique dédié à temps plein pendant deux ans.
L'Inde, qui est la neuvième puissance économique du monde et la troisième la plus importante en parité de pouvoir d'achat possède un système de santé très inéquitable. La prévalence de la tuberculose est 5 fois plus importante entre les plus pauvres et les plus riches. Pour la population la plus déshéritée, l'examen direct microscopique est souvent le seul outil de diagnostic de la tuberculose utilisé dans les centres de santé décentralisés. La détection des bacilles acido-alcoolo-résistants (BAAR) présente donc une importance épidémiologique cruciale dans l'algorithme du diagnostic de la tuberculose dans les zones à ressources limitées.
Dans notre étude, la sensibilité de l'examen microscopique correspondait aux normes attendues, détectant les deux tiers des tuberculoses pulmonaires actives chez les sujets non infectés par le virus de l'immunodéficience humaine (VIH) et la moitié chez les sujets infectés par le VIH. Au cours de l'étude, les examens microscopiques ont été réalisés dans des laboratoires ayant une très forte expérience dans le domaine du diagnostic de la tuberculose. La sensibilité de l'examen microscopique est souvent inférieure dans les centres de santé décentralisés en raison d'une moins bonne qualification des techniciens et du manque de ressources. L'examen microscopique utilisant la coloration de Zielh-Neelsen nécessite 20 minutes de lecture par lame. La mise en place de la microscopie à fluorescence utilisant une lampe LED pourrait contribuer à améliorer significativement la qualité de l'examen microscopique dans les centres de santé décentralisés. Avec ce système, le temps de lecture est divisé par 4 et la sensibilité est plus élevée (Cuevas *et al*, 2011). Le rapport 2012 du programme national de contrôle de la tuberculose de l'Inde (Revised National TB Control Programme : RNTP), met en évidence la nécessité de l'introduction des microscopes à lampe LED dans les centres de santé décentralisés. Cependant, ceci implique la formation, le management de la qualité et le suivi de la performance par le personnel utilisateur.
L'évaluation du système automatisé de la culture en milieu liquide a montré une meilleure sensibilité et une réduction du temps de détection des mycobactéries significativement supérieure au milieu de culture solide traditionnel de Lowenstein-Jensen. La culture en milieu liquide a aussi l'avantage de détecter les mycobactéries directement dans le sang des patients et de contribuer donc à améliorer le diagnostic difficile des tuberculoses disséminées souvent rencontrées parmi les sujets ayant une co-infection tuberculose et VIH. Malheureusement, le coût important d'un système de culture en milieu liquide automatisé ne permet pas son utilisation dans un système de santé décentralisé. Ce type d'équipement est surtout rencontré dans le secteur privé, les hôpitaux publics universitaires et quelques

laboratoires nationaux de référence. Seuls les patients ayant une assurance privée peuvent payer le coût de tels tests de diagnostic.
L'étude réalisée avec le test sérologique ELISA est l'une des plus importantes qui n'ait jamais été publiée dans la littérature. Beaucoup de rapports controversés ont été écrits concernant les performances des tests de diagnostic sérologiques de la tuberculose; la plupart du temps, les études ont été faites sur un nombre limité de patients, et souvent réalisées dans des pays non endémiques. Le test ELISA présente l'avantage d'être peu couteux et peut être réalisé dans beaucoup de laboratoires en Inde. On a observé une sensibilité plus élevée parmi les patients ayant une co-infection tuberculose-VIH par rapport aux patients non infectés par le VIH. Cependant le manque de spécificité obtenu parmi la population ne présentant pas de tuberculose active fait que ce test est difficile à être utilisé comme marqueur de la tuberculose active. L'introduction de ce genre de nouvel outil de diagnostic de la tuberculose risque de conduire vers des traitements antituberculeux en excès.
L'évaluation des tests IGRA a montré que la réponse de l'IFN-γ aux peptides RD1 sélectionnés était significativement supérieure chez les patients présentant une tuberculose active par rapport aux sujets contacts et communautaires ; elle présente aussi une meilleure spécificité vis-à-vis de la tuberculose active que le test commercial « QuantiFERON®-TB Gold IT ». La réponse des 3 autres cytokines IP-10, MCP-2 et IL-2 aux peptides sélectionnés RD1 n'améliore pas le diagnostic différentiel entre la tuberculose active et latente. La réponse de l'IP-10 aux peptides RD1 sélectionnés améliore la détection parmi la population VIH sans être affectée par le nombre de lymphocytes T CD4 ; cette sensibilité plus élevée peut être due au fait que l'IP-10 est principalement secrétée par les monocytes/macrophages tandis que l'IFN-γ et l'IL-2 sont principalement secrétés par les cellules T CD4. Cependant, le pourcentage de positifs parmi les cas de patients ne présentant pas de tuberculose active était élevé. Ce marqueur ne permet donc pas de différencier une tuberculose active et latente. Ces résultats peuvent être dus au fait que l'IP-10 est détecté chez les sujets contacts. Il est impossible, dans un pays comme l'Inde, d'exclure les contacts avec *M. tuberculosis*.
L'étude comparative entre le test commercial « QuantiFERON® -TB Gold IT » et le test cutané à la tuberculine n'a pas montré cependant dans cette évaluation clinique un avantage du test commercial sur le test cutané historique de Mantoux malgré l'importante couverture vaccinale du BCG en Inde.
Les tests commercialisés basés sur la réponse de l'interféron gamma exigent des infrastructures de laboratoires, et sont beaucoup plus onéreux : 50 US $ par patient avec QuantiFERON contre 2 US $ par patient avec le test tuberculinique. La valeur ajoutée des tests IGRA semblent donc très limitée dans un pays comme l'Inde.
Nous avons montré, à partir de cette évaluation de « boîte à outils » de diagnostic de la tuberculose, que les tests de diagnostic basés sur la réponse immunologique dans un pays endémique n'aident pas à différencier entre tuberculose latente et active ; seuls les tests microbiologiques peuvent confirmer une tuberculose active. L'examen direct détecte 40 à 60% des tuberculoses actives. L'introduction de la culture en milieu liquide améliorerait significativement le diagnostic de la tuberculose. L'introduction des nouveaux tests moléculaires automatisés détectant simultanément les mycobactéries et la résistance à la rifampicine améliorerait aussi significativement la prise en charge de la tuberculose en Inde. Dans le rapport 2012 du RNTP, un chapitre est consacré à ces nouveaux outils de diagnostic (microscope à lampe LED, culture en milieu liquide et tests d'amplification génique en cartouche (Genexpert)). Aujourd'hui, ces nouveaux outils ne sont pas encore introduits dans les centres de santé décentralisés du fait de contraintes économiques.
La santé n'apparait pas en effet constituer une priorité en Inde surtout pour les populations pauvres. Cependant le gouvernement indien dépense par exemple plus d'argent pour

subventionner les paysans en produits fertilisants que pour l'ensemble de la santé publique. Des subventions de carburant, aidant surtout les riches, sont également coûteuses. Des moyens pour soulever des fonds existent. Taxer l'importation d'or et de diamants, par exemple, apporterait près de 10 milliards de US $ de revenu par an (The Economist, Indian Development, 2013).
Cette étude a permis d'évaluer un large éventail d'outils de diagnostic de la tuberculose. En réalité, aucun d'eux ne peut prédire l'évolution d'une tuberculose latente vers une tuberculose active. Un nouvel outil qu'il reste à développer présenterait une grande valeur ajoutée pour la prise en charge de la tuberculose principalement pour la population infectée par le VIH qui a 10 fois plus de risque de développer une tuberculose active.

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Pour le Président de l'Université Claude Bernard Lyon 1,

Professeure C. VINCIGUERRA

P/La Directrice et par délégation
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Annexes

Annex 1: PGL-Tb1 production and purification/control

Annex 2: Study protocol

Annex 3: A Toolbox for Tuberculosis Diagnosis: An Indian Multicentric Study (2006-2008): Microbiological Results

Annex 4: IFN-ϒ, but not IP-10, MCP-2 or IL-2 response to RD1 selected peptides associates to active tuberculosis

Annex 5: Is IP-10 an Accurate Marker for Detecting *M. tuberculosis* Specific Response in HIV-Infected Persons?

Annex 6: IP-10 response to RD1 antigens might be a useful biomarker for monitoring tuberculosis therapy

ANNEX 1

PGL-Tb1 Production and Purification/Control

Mycobacterial culture

The culture was done with the *M. tuberculosis* Canetti strains obtained from Institut Pasteur (Canetti strain CIPT 140010059). Cultures were grown for 3 weeks on Sauton medium at 37°C. The mycobacteria cells were harvested from the surface of the growth medium by pouring the medium.

Glycolipid extraction

The harvested bacteria were suspended in chloroform/methanol (1:2, v/v) in a glass container. The flask were kept for 48h at room temperature. After these two days we can observe two phases inside the bottle, the inferior phase contains dead cells, mycolic acid, proteins, polysaccharides and 1 to 2% lipids. The upper phase contains only lipids including PGL-Tb1.
The supernatant crude extract are dried and dissolved in chloroform.
A second extraction is applied to the cells.
The crude extract in chloroform contains all kind of lipids.

Purification

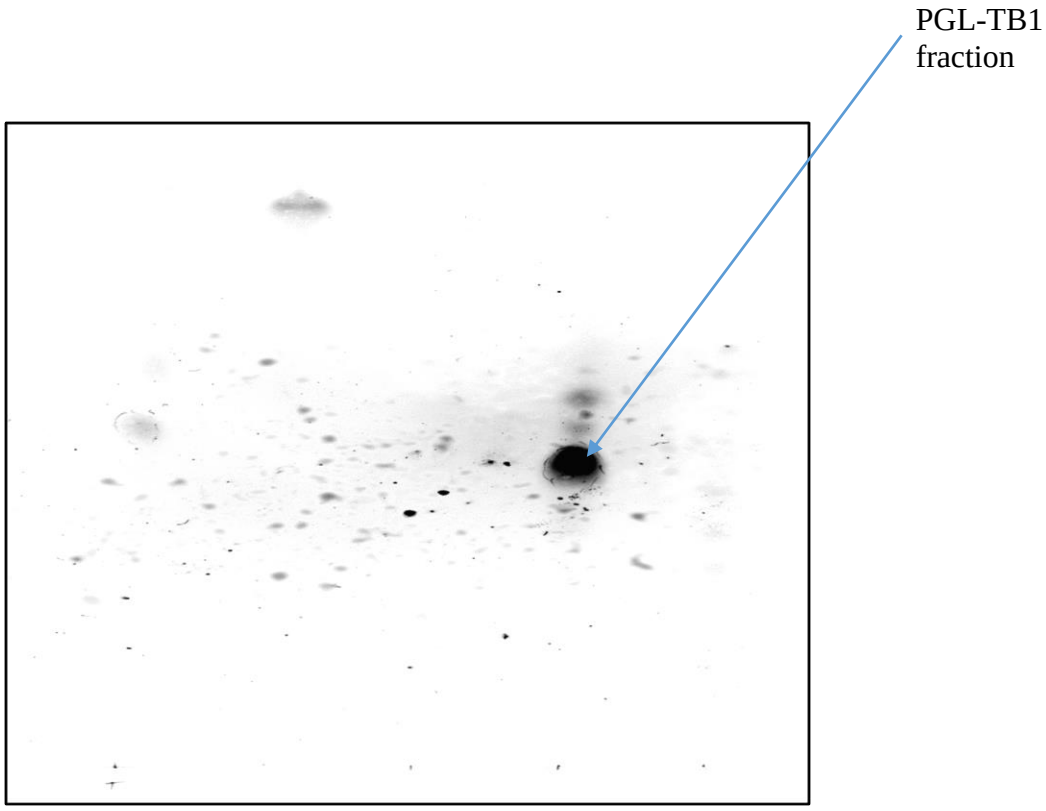
1. Glass column with the diameter of 5.5cm was used to prepare the silica column.
2. 2. Silica gel 60 (Merck, Germany) with 230-400 mesh was used in preparing the column. The column was added with silica gel 60 and eluted several times by a mixture of Chloroform and Methanol (CDH, Germany) at 95:5 proportions.
3. Thin Layer C was done with the culture extract of *M. tuberculosis* along with the reference PGL-Tb1 (purified molecule with the method defined by Daffé *et al*, 1987) to confirm the presence of PGL-Tb1 before beginning the purification process.
4. The dissolved extract was loaded slowly in the column using a clean Pasteur pipette. Extreme care was taken not to disturb the column.
5. Solvent mixture of Chloroform and Methanol (95:5) was used for the elution.
6. Eluates were collected in clean and dry test tubes.
7. Each elution was subjected to TLC along with the reference PGL-Tb1 molecule.

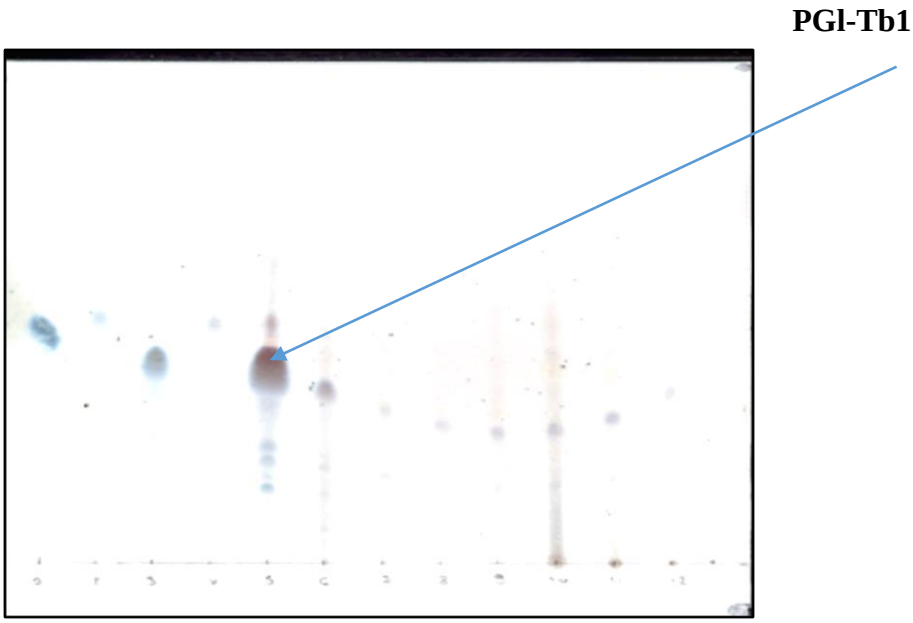
8. With the help of TLC, the presence of PGL-Tb1 molecule was observed from the elution 17th to 25th. Other molecules (impurities) were also noticed in TLC along with PGL-Tb1.
9. Solvents were evaporated from 17th to 25th elution tubes. Product in each tube was dissolved with a small volume of chloroform and pooled.
10. The recovered and pooled material was loaded in another newly prepared silica gel 60 column. Column preparation was done in the same way as mentioned in steps 1 and 2.
11. The column was first eluted with the solvent mixture of chloroform and methanol at 99:1 to remove the impurities without disturbing the PGL-Tb1. TLC was performed for this elution at regular interval in order to make sure that no PGL-Tb1 is lost.
12. We continued with 95:5 mixtures of chloroform and methanol to obtain the PGL-Tb1 fractions.
13. TLC was used to verify each fraction with reference to PGL-Tb1 molecule. PGL-Tb1 was observed from 1st to 30th elutions. TLC of these elutions have revealed that the presence of impurities were much lower than the elutions after 1st column run. Solvent was evaporated from each tube and the product from each tube was dissolved with a small volume of chloroform and all the tubes were pooled.
14. The third column for purification was performed from the pooled product obtained in the previous step. The elution was performed with the mixture of Petroleum ether and Ethyl acetate at 10:3 ratio and elutes were collected as before. TLC was performed for these elutions at regular interval in order to make sure that no PGL-Tb1 is lost during these elutions.
15. This was followed by the elution with 95:5 mixtures of chloroform and methanol in the same column to obtain the PGL-Tb1 and all the elutions were collected as before.
16. TLC was performed and revealed that the elute of 95:5 Chloroform and methanol contained PGL-Tb1 with minimal impurities. These elutes were pooled and preserved for further testing.

Thin Layer Chromotography (TLC)

1. Ready to use TLC paper (Silica gel particles coated as layer in aluminum sheet (E.Merck, Germany)) was used. TLC paper was cut to a suitable size.
2. A thin line was drawn in the bottom of the TLC paper with a fine tipped pencil. A dot mark was made on the line for each sample run. Adequate distance was maintained between the dots.

3. Using a 10µl capillary tube, the sample was applied on the spot on the TLC paper. Care was taken to avoid any chemical contamination. For every TLC run, known PGL-Tb1 at mg/ml concentration was used as reference.
4. The TLC paper was kept in a standing position in a TLC developing tank with a solvent mixture of Chloroform and Methanol at 95:5. The level of solvent was kept below the line of the pencil mark and where the specimen was applied.
5. Just before the solvent movement to reach half inch before the edge, the TLC paper was removed from the TLC tank and allowed to air dry.
6. TLC paper was immersed in a trough contains phosphomolibdic acid for few seconds and then allowed to air dry.
7. The TLC paper was exposed under hot (temp) light till the bands appeared.





Thin layer chromatography

NMR Analysis

NMR analysis was performed in NMR spectrophotometer 250MHZ, Bruker Avence 250 (Bruker, USA). The solvent used for the analysis was Chloroform-D (CDCl3) (Sigma-Aldrich). The NMR spectroscopy recording of the reference PGL-Tb1 (purified with old purification method described by Daffe et al, 1987) was used for the comparison. Also NMR spectroscopy published earlier (Daffe *et al*, 1987) was referred.

ELISA to check the sero-reactivity and background of the purified product.

1. Preparation of PGL-Tb1 antigen coated plates

- Polystyrene Microtitre plates (Polysorb, Nunc A/S, Denmark) were used for the coating.
- The product of new purification (here after referred as new PGL-Tb1) at a concentration of 25µg/ml in PBS was used for coating ELISA wells.
- The diluted antigen was added (100µl/ well) and incubated for 2 hours at 37ºC followed by overnight at RT.
- Excess antigen solution was removed and the plates were blocked with 0.5% of BSA (Sigma-Aldrich) for 30 minutes.
- Plates were washed for 3 times with PBS and dried before use.
- The reference PGL-Tb1 antigen was also coated simultaneously in the same way.

2. Materials for ELISA

2.1 Sera samples for testing

- Serum samples from 22 Healthy blood donors (Healthy population (HP)) were included for the test. The OD values of these sera will be used to make a comparison between new PGL-Tb1 and old PGL-Tb1 to know the level of background OD value with each antigen.
- Sera from 16 confirmed TB patients (TB) were included for the test. The O.D value obtained from these sera will be used to compare the sero-reactivity of both new and old PGL-Tb1. The new PGL-Tb1 molecule has to show at least similar or better seroreactivity with serum samples of TB patients compared to old PGL-Tb1.

2.2 Serum diluent buffer

PBS 0.150M + 0.5% BSA

2.3 Wash buffers

- PBS powder pH 7.2, 0.150M (bioMérieux SA, France) – reconstituted in 1 liter distilled water
- Distilled Water

2.4 Anti-Human IgG-HRP conjugate

Pre diluted anti-Human IgG Human provided by R&D immunoassay, bioMérieux SA, France.

2.5 Substrate

OPD Substrate tablets and OPD Substrate buffer were provided by R&D immunoassay,
bioMérieux SA, France. One OPD tablet was dissolved in 5ml of OPD buffer just prior to use.

2.6 Stopping reagent

1.8N H_2SO_4 (bioMérieux SA, France)

3. ELISA Procedure

3.1 Serum dilution

Each serum was diluted in serum diluent buffer at 1/250 dilution (4µl of serum in 1 ml of serum diluent buffer) and mixed properly.

3.2 Test Procedure

- ELISA strips coated with new PGL-Tb1 and old PGL-Tb1 were arranged adjacently in the Plate.
- 100µl of diluted serum was added into the appropriate micro wells of new PGL-Tb1 and old PGL-Tb1. Each serum was tested in duplicate for both antigens.
- Plate was covered with an adhesive sheet and incubated for one hour and 30 minutes at 37°C.
- Micro wells were washed 6 times with PBS. After the last wash, the excess liquid was removed.
- 100µl of conjugate was added into each well. Plate was covered with an adhesive sheet and incubated for 1 hour at 37°C.
- Micro wells were washed 6 times with PBS. After the last wash, the excess liquid was removed.
- 100µl of prepared OPD substrate was added to each well and incubated for 30 minutes at room temperature (18 - 25°C) in the dark.
- 100µl of stop reagent was added to each well.
- Plate was read for optical density (OD) value using primary wavelength of 490nm and reference wavelength of 620nm in the Flex-Tek instrument (bioMerieux SA, France).

CLINICAL EVALUATION OF TOOL BOX FOR TUBERCULOSIS DIAGNOSIS

A collaborative multi centre study of
Foundation Mérieux
and
Indian Council of Medical Research.

STUDY PROTOCOL

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1 Specimen copies of Form 1, 2 and 3 for Clinical Evaluation, Laboratory evaluation and Clinical follow-up

2 Format for Fortnightly report

3 Format for TB ELISA Sheet

4 Format of sera index sheet

I OBJECTIVE OF THE MAIN STUDY

The role of laboratory in the management of Tuberculosis is not fully established[1]. Laboratory plays a critical role not only in the diagnosis and management of the Tuberculosis disease, but also in control and elimination strategies[2]. Though it remains to be demonstrated, more sensitive, more rapid, and more patient-friendly diagnostic tools might have a significant impact on disease control by abbreviating diagnostic delay and reducing the period of transmission[3].

Laboratory can play its crucial role in the management and control of Tuberculosis effectively only if following goals are focused[4].

- Nearly 80-90 % case detection with the confirmation of bacterial infection of adult pulmonary cases.
- Maximum case detection in paediatric and non-pulmonary TB cases.
- Turnaround time of 24 hours for a provisional diagnosis of TB with the ability to detect at least 80% of culture positive TB cases.
- Confirmative diagnosis with identification susceptibility with thin 2 weeks of time for at least 80% TB cases.

Currently no single diagnostic tool can help to achieve these goals and it is evident that using carefully selected diagnostic tools (tool box) may come to help[5]. Moreover the combination of diagnostics tools in the toolbox may differ geographically due to facts such as population, availability of tools, cost, etc. One of the objectives of the TB control management group is to identify the right combination of tools for the toolbox by an extensive evaluation. The present study propose the multi centre evaluation selected diagnostic tools to understand which or which combination of diagnostic tools may serve better for the maximum case detection.

Following are the selected diagnostic tools included in the study.

1. Clinical based diagnosis that includes Clinical suspicion and Chest X ray.
2. Smear Microscopy,
3. Conventional culture method (LJ)
4. Automated culture with identification (BacT/ALERT + Accuprobe MTD)
5. Antibody response for the *Mycobacterium tuberculosis* complex specific antigens by using the ELISA based kit currently under development by bioMérieux.
6. Direct detection based on Molecular diagnosis (Gen-Probe MTD and PCR)

Foot Notes:

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II STUDY POPULATION

Main Study

Population	AIMS	Safdarjung	JALMA & S.N Medical college	P.D.Hinduja Hospital and JJ Hospital	NIMS	TRC	Total
Pulmonary Tuberculosis (Adult)	40	100	100	150	150	150	**640**
Non Pulmonary Tuberculosis	20	50		50	50		**220**
HIV/TB Co infected Case	30			100	20		**150**
Bacteriologically documented Pediatrics Tuberculosis			50		50		**100**
TB cases bact not documented but responded to ATD (Pediatric)			50				**50**
Control 1 Healthy Population - Blood bank samples	200			200	200		**600**
Control 2 -Lab staff and Health Care Workers	30		30	30	30	50	**170**
Control 3: Non-TB, other Pulmonary diseases		50	50	50	50		**200**
Control 4: Healthy population : Health Check up people				100			**100**
Total Number of samples							**2230**

III POPULATION DESCRIPTION

1. **Tuberculosis suspected Patients – Pulmonary**
   The patients who are suspected as high or very high possibility of having Tuberculosis after the clinical evaluation based on clinical symptoms and chest X ray. Also patients should meet inclusion and exclusion criteria as stated in the study protocol.

2. **Tuberculosis suspected Patients – Extra Pulmonary**
   The patients who are suspected as high or very high possibility of having Extra pulmonary Tuberculosis after the clinical evaluation based on clinical symptoms and chest X ray. These patients should be without the involvement of pulmonary tuberculosis. Also patients should meet inclusion and exclusion criteria as stated in the study protocol.

3. **HIV/TB Co infected patients**
   The patients who are confirmed as positive (seroreactive) for HIV infection and also suspected as high or very high possibility of having of pulmonary and / or Extra pulmonary Tuberculosis after the clinical evaluation based on clinical symptoms and X ray. Also patients should meet inclusion and exclusion criteria as stated in the study protocol.

4. **Control Population 1 – Healthy blood donors**
   Blood donors are routinely examined to qualify them as healthy before each donation. These donors are also routinely screened for VDRL, HIV, HBsAg, HCV, etc. So the qualified donors are considered as healthy individuals and these people can serve as ideal control population to validate the diseased. The leftover serum after the routine blood bank screening can be used as sample mainly for the serology test. Also consider the samples only from the donors who are fulfilling the inclusion and exclusion criteria.

5. **Control Population 2 - Patients suffered with other lung diseases**
   Lung diseases other than Pulmonary TB may also show the similar symptoms and chest X ray pattern. The laboratory diagnostic tool used for screening should be able to distinguish between other lung diseases and pulmonary TB to help the clinician in making the final diagnosis. This group is included in the study to understand the ability of serology-based test to distinguish between other lung diseases and pulmonary TB. Patients with pulmonary diseases only will be included in this group.

6. **Control Population 3 – Health care workers and Staff**

Laboratory staffs working in Mycobacteria section and health care staffs handling the confirmed TB patients are considered for this group. An estimated risk ratio of 0.6 to 2.0 among health care workers indicating only a modest increase in risk compare to general population[6]. Considering the fact of possible continues exposure to *Mycobacterium tuberculosis*, this population can act as an ideal control group for tests like antibody detection. The inclusion is subjected to the inclusion and exclusion criteria stated in the study protocol.

7. **Control Population 4 – Healthy adults coming to lab for health check up**

People who are coming to hospital for the routine health check up and reported as healthy based on the clinical and laboratory procedures should be considered for this group. This population can act as healthy group apart from blood donors in validating the diagnostic tools like antibody detection based tests.

Foot Notes:

6. Menzies D., A.Fanning, L.Yuan and M.Fitzgerald. Tuberculosis among health care workers. The new England Medical Journal. 1995: 332(2);92-98

IV PROCEDURAL NOTES

1) Project initiation

1. Project will be initiated at each center once the institute ethical committee clearance has been obtained. The copy of ethical committee approval letter for the study should be submitted to ICMR through study coordinator.
2. Engineers from bioMérieux India (Pvt) Ltd will install the instruments (MB/BacT and Genprobe).
3. The appointed student should start working from the time of project initiation.
4. The study protocol was designed by Prof.P.H.Lagrange and the steering committee of TB experts. Field coordinator will provide training in following the study protocol and also using BacT/ALERT and Genprobe instruments. The training period is for 5 working days.
5. The sample inclusion can start immediately after the project initiation.

2) Material supply

1. Please refer the table no 5a and 5b for the list of materials, which will be supplied for the project.
2. Laboratory consumables will be provided in a single lot at the time of project initiation.
3. The BacT/ALERT culture bottles, LJ and Genprobe reagents will be provided periodically in order to maintain the sufficient shelf life of these reagents at the user end. Please refer the material supply table for the schedule. In case of any need of change in the schedule, inform the study coordinator well in advance.

3) Patient inclusion

1. Follow the study inclusion and exclusion criteria mentioned in table 2 to include the subjects in the study.
2. Please take the concerned form from the patient or guardian before include the patient in the study. The concerned form should be designed by each center in the regional language. The copy of English version of concerned form should be submitted to ICMR through study coordinator.
3. Make sure that the patient will be accessible for the follow-up of at least 6 months. Avoid include any patient or controls if they are impossible to follow them up like they are from far away location, etc.
4. Fill the ‘Form 1- clinical evaluation’ based on the subject’s clinical evaluation. If any subject found to be unsuitable for the study with reference to the exclusion criteria, exclude them from the study.
5. Form 1- Clinical evaluation does not have a column for patient name. This is as per the ethical requirement of the European region since the form is designed in Europe and the final analysis of the data will be done in Europe. The centers are requested to attach a removable slip to keep a patient name and contact details and remove this slip before submitting the forms at the end of the study.

6. Collect necessary samples. In case of sputum, try to obtain 3 collections (on spot, early morning and on spot). Please refer the Table 3 for the sample collection guidelines.
7. Transport samples along with filled Form1 and signed patient concerned form to the laboratory. If the sample transportation to the lab is delayed, store the specimen at 4° C. When the duration of transportation is longer, make sure that the samples are shipped in a cold condition with the proper packing.
8. Allocate study number as per the instructions given in under chapter 4. The study ID number is unique for each patient. Without proper study number, do not proceed further.

4 Sample handling and processing.

1. "Form 2-Laboratory Evaluation" has to be opened before performing the sample analysis. Attach Form 2 with Form 1 for further follow-up.

Samples for Mycobacteria and routine culture

2. Sputum specimen collected at three intervals should be processed and cultured separately. Do not mix the samples and process.
3. Use BacT/ALERT MB bottles for blood culture of Mycobacteria. Use a set of 3 bottles collected at three intervals for the same patient.
4. The sample for the culture should be processed for culture inoculation on the same day of collection.
5. Perform smear Microscopy as per your routine procedure. It is preferred to perform smear microscopy examination from the deposit after sputum digestion and decontamination.
6. All the sputum and other non-sterile specimens should be subjected to digestion and decontamination protocol. The recommended protocol is NALC-NaOH. For the detail procedure, refer "Manual for test procedures".
7. Store 0.5 ml of sediment at –70°C or –20°C for each sample processed for culture. This sediment can be used for re-inoculation incase the primary culture is contaminated. Refer the "Manual for test procedures" for the detailed protocol for the re-inoculation. If the primary BacT/ALERT and / or LJ are not shown any contamination in 2 weeks of incubation, this sediment can be discarded.
8. The positive growth indication has to be confirmed by AFB smear. The confirmed AFB growth has to be identified by Accuprobe to know whether it is *Mycobacterium tuberculosis* or MOTTs. If growth is observed in more than one BacT/ALERT or LJ culture bottles of the same patient, use any one of them for the Accuprobe Test. Refer the "Manual for test procedures" for the detailed protocol for AFB growth confirmation and Accuprobe identification.
9. Record all the results.

Serum samples

10. Separate serum samples from the clotted blood after centrifuging. Make sure that the sample is not hemolysed.
11. Divide the sera in 3 vials at equal volume. Label the vials with study ID number. Store the sera at –20 or –70 in a 3 separate box.

12. Each box should contain a sera index sheet. Please fill the study ID number and volume of sera for each serum. Please note that same box is used for different population serum samples (TB patients, controls, etc).
13. Sera in the first box are for testing. Sera in the 2nd box are to be reserved for the purpose of future testing in the laboratory. Sera in the 3rd box are to be sent to the study coordinator.
14. Once the number of serum collected is reached 50, the third box should be sent to coordinator along with sera index sheet.
15. Perform PGL-TB1 and ESAT6-CFP10 ELISA for every 21 samples in a single plate. For the ELISA run, use only the ELISA sheet provided for the study. Make a photocopy of every ELISA sheet filled with study ID numbers and the ELISA reading printout should be sent to the study coordinator along with fortnightly report.
16. Record all the results.

5) Clinical follow-up

1. Once any patient included in the study is confirmed as Tuberculosis, follow the patient based on the 'Form 3- Clinical follow up'. Attach "Form 3-Clinical Evaluation" along with filled Form 1 and Form2 of the patient for the follow-up.
2. Fill the Form 3 periodically.
3. Once 6 months follow up is completed, close the forms by completing the sum-up in the Form 3.
4. All the forms (Form 1, Form 2 and Form 3) are provided with two sheets for each patient. The second sheet is the carbon copy.
5. Send the compiled forms (Form 1, 2 and 3 for TB patients and contacts and Form 1 and 2 for all other subjects) to the field co-coordinator. The original forms should be sent to the field coordinator and the carbon copy should be kept with the evaluators.

6) Data entry and record maintenance.

1. For the TB patients, Form 1, 2 and Form 3 should be filled completely. For all other subjects, Form 1 and Form 2 should be filled.
2. All the laboratory results should be maintained in the laboratory register. It must be possible at any given point of time any results of any patient should be traceable. Do not discard any record or any printouts, etc.
3. The center will also be given the EPI Info software and a DBS file which is the data collection sheet of EPI info. These forms should be filled for each subject. At the end of the study, the DBS file of EPI info should be submitted to the field coordinator.

7) Reporting

Center

1. Each center should send a fortnightly report to field coordinator. This report is to monitor the study progress in terms of subject inclusion etc. And also this report helps in managing the logistics of materials.
2. Copy of every ELISA run sheet along with the results printout should be submitted to study coordinator.

3. The Forms should be submitted along with patient concerned form in original to the field coordinator once all the necessary entries in the forms are made. The center should keep a copy of all.

Field coordinator.

1. After collecting the fortnight report from every center, the field coordinator will compile and prepare a fortnight report about all centers. This report will be submitted scientific coordinators and chief scientific coordinator.
2. After verification, all the original patient entry forms will be submitted to the chief scientific coordinator and the copies will be maintained with field coordinator till further instruction.
3. The DBS file of each center will be verified for the completeness in entries and will be submitted to chief scientific coordinator.

8) Quality assurance

1. Field coordinator will organize an external quality control program for all the study center. Based on the request from Prof.Lagrange, the samples will be sent to each center. These samples should be analyzed as a clinical samples and report should be submitted back to the field coordinator. The field coordinator will collect all the reports and submit to Prof.Lagrange for the verification.
2. There will be two runs and quality assurance during the 6 months of study period.

9) Study coordination

1. The field coordinator in India is authorized to coordinate to all the centers on all the scientific and other operation.
2. The field coordinator will help in establishing the study protocol, establishing the bench work involved for the study and also in operating the systems for the study.
3. The field coordinator will be in regular touch with each center in order. The field coordinator also visits every center at least once in 2 months. If any center needs an immediate attention, the study coordinator should visit immediately.

V INCLUSION AND EXCLUSION CRITERIA

Table 2

	Inclusion criteria	Exclusion criteria
TB Population (Adult and paediatrics): Tuberculosis suspected patients	• Strong clinical evidence of Tuberculosis. Based on the provided clinical evaluation form 1, the patient has to be evaluated as **high or very high suspicion of TB**. • **Fresh cases:** Not having the history of previous treatment (> 1 month) or failure. • Patients should be **traceable for 6 months follow up**	• Recently treated (in last 1 year) or patients who are under anti-TB treatment at the time of sample collection for bacteriology (> 1 month) (Affects the culture). • Patients who are under fluoroquinolones **at the time of sample collection** for bacteriology (Affects the culture).
Control Population 1: Healthy people (Blood Donors)	• Donors who are enrolled in the blood bank (of the same hospital or the blood bank which is located in the same area) • Examined and certified as healthy by the Blood bank clinician	• History of Tuberculosis • History of close contact with confirmed TB cases at the time of blood donation. • Any infectious diseases at the time of inclusion.
Control Population 2: Patients suffered with other lung diseases but not with tuberculosis	• Patients who are diagnosed for other illness like lung carcinoma, pneumonia, etc. • Clinically and bacteriologically should be ruled out for *Mycobacterium tuberculosis* infection. • Patients should be traceable up to 1 month. • HIV status to be known	• Patients who are not confirmed of type of lung diseases. • Patients who are immunosuppressed or immunodeficient since they are prone to TB disease • Patients with the report of close contact with confirmed TB cases since it is difficult to confirm that they do not have tuberculosis at the point of collection..
Control Population 3: Health care workers and laboratory staff	• Working for more than one year in the hospital or laboratory facility. • Examined and certified as healthy by the clinician. • Follow up for at least 6 months	• Any Tuberculosis history or history of recent family contact with confirmed TB patient.
Control Population 4: Healthy adults coming to lab for health check-up	• Examined and certified as healthy by the clinician • Mantoux test results	• Should not have any Tuberculosis history or recent contact history with confirmed TB patient.

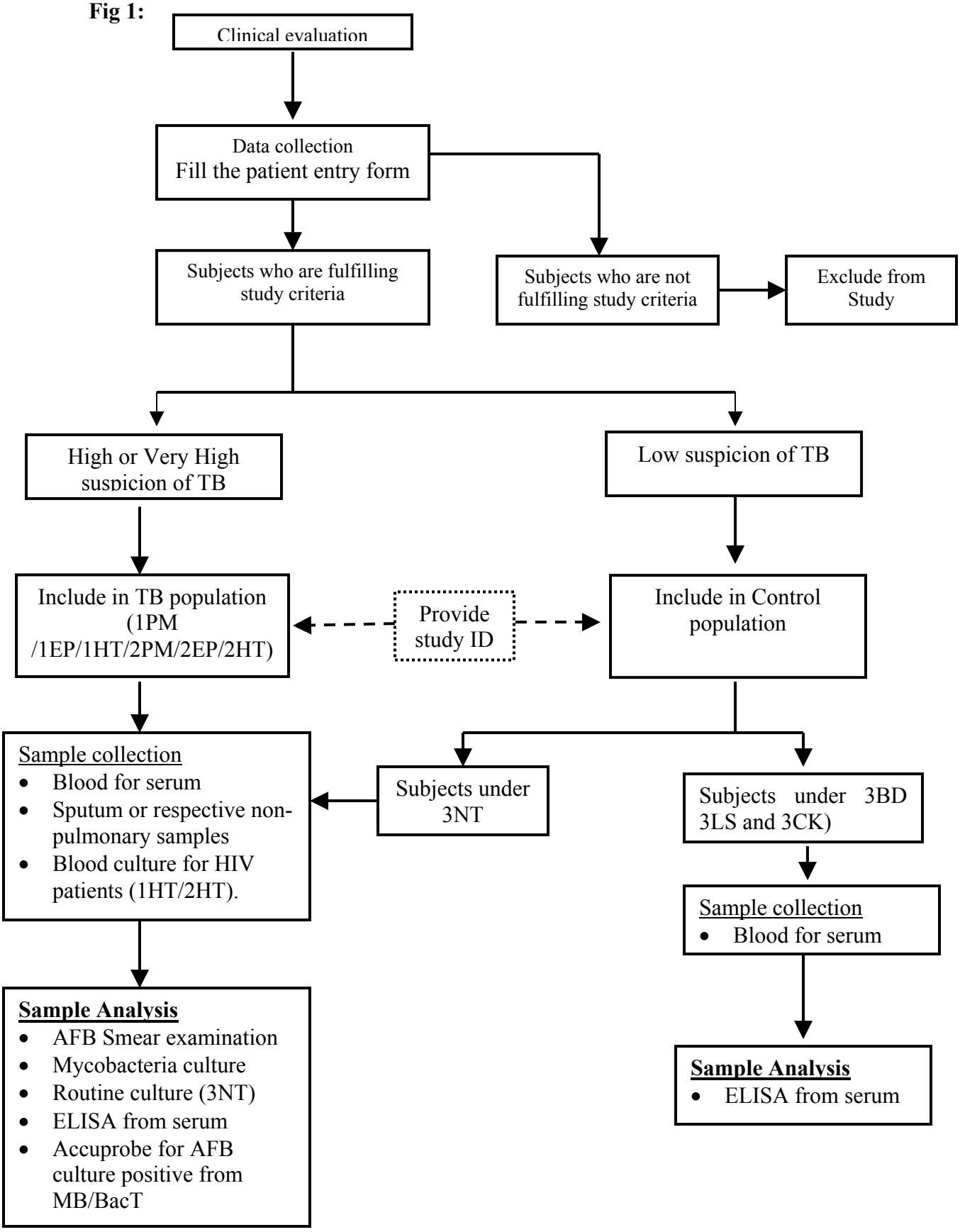
VI GUIDELINES FOR SAMPLE COLLECTION

Table 3

Study Population	Sample from each patient / Subject	Containers
Pulmonary TB Population: Tuberculosis suspected patients	Sputum: 3 Samples (Spot, Early morning and Spot) in sterile containers for culture.	Sterile, wide mouth container
	Non sputum: One sample in a sterile container for culture	Sterile, wide mouth container or sterile syringe or any other sterile container,
	Minimum of 5 ml of blood for serum separation.	Vaccutainer Tube (Red Cap)
Extra - Pulmonary TB Population: Tuberculosis suspected patients	One sample in a suitable sterile container for culture	Sterile, wide mouth container or sterile syringe or any other sterile container,
	Minimum of 5 ml of blood for serum separation.	Vaccutainer Tube (Red Cap)
HIV/ TB Population: Tuberculosis suspected patients	Sputum: 3 Samples (Spot, Early morning and Spot) for smear and culture.	Sterile, wide mouth container
	Non sputum: One sample in a sterile container for culture	Sterile, wide mouth container or sterile syringe or any other sterile container,
	Minimum of 5 ml of blood for serum separation.	Vaccutainer Tube (Red Cap)
	3 x 5 ml of blood in Heparin tube for Blood culture.	Vaccutainer Tube (Green Cap)
Control Population 1 Blood donors	Minimum of 3 ml separated serum from the collection of blood bank.	Serum vials
Control Population 2: Patients suffered with other lung diseases but not with tuberculosis	One sample in a sterile container for culture	Sterile, wide mouth container or sterile syringe or any other sterile container,
	Minimum of 3 ml of blood for serum separation	Vaccutainer Tube (Red Cap)
Control Population 3: Health care workers and laboratory staff	Minimum of 3 ml of blood for serum separation	Vaccutainer Tube (Red Cap)
Control Population 4 Health check up people	Minimum of 3 ml separated serum from the collection of Laboratory.	Serum vials

VII FLOW CHART FOR STUDY

Fig 1:



VIII GUIDELINES FOR STUDY ID NUMBER

Center Code

First Digit of the study ID	All India Institute of Medical Sciences	1
	Safdarjung Hospital, New Delhi	2
	Jalma Institute, Agra	3
	S.N.Medical College Hospital, Agra	4
	P.D.Hinduja Hospital, Mumbai	5
	J.J,Hospital, Mumbai	6
	Nizams Institute of Medical Sciences	7
	Tuberculosis Research Center, Chennai	8

Group code

2nd Digit of the study ID	Adult - TB Suspected cases	1
	Pediatric – TB Suspected cases	2
	Control Group	3
	Satellite study sample	4

Category of Samples

3rd Digit of the study ID	Pulmonary TB Cases	PM
	Extra Pulmonary TB Cases	EP
	HIV/TB Cases	HT
	Blood donors	BD
	Lab staffs / Health care workers	LS
	Non-TB pulmonary diseases	NT
	Health check-up People	CK
	Close contacts of TB	CN
	Skin Tuberculosis	SK

Serial Number

4^{th}, 5^{th} and 6^{th} Digits	Serial Numbers for each samples	001 to . . .

Example

AIMS	1
TB Suspected	1
Pulmonary TB Cases	PM
Sample serial Number	001
So the Study ID Number is	11PM001

Note :

- Study ID number is unique for each subjects included in the study. The centers should follow their own pattern for numbering samples. The study ID number will not differ for different sample collected for same subject.
- For the same patient, if the sera sample is collected for more than one time (Ex-follow up for the contacts), use a,b,c,d…. after the last digit of study ID. This is applicable only for serum samples not for samples which are

  Example 34CN001 (at the time inclusion), 34CN001a (follow-up First month sample), 34CN001b (follow-up second month sample) so on.
- No separate Study ID is needed for the satellite studies in case the subjects are also included for the main study. Use the same Study ID for the Satellite studies too. Use satellite study ID only when the subject is included exclusively for the satellite study (Ex. Skin Tuberculosis and Close contacts).

IX RESULT ANALYSIS

The results will be analysed using sophisticated statistical system in Saint Louis Hospital, Paris and R & D Laboratory of bioMérieux, Marcy l'Etoile.

The final statistical analysis would be done based on the following criteria.

1. **Confirmed TB:** Following mentioned criteria would be used to include the patient under confirmed TB case. Patients who do not fall in any one of these categories will be considered statistically as biased and removed for the analysis.

	Clinical suspicion score for TB	AFB Smear	Culture	Clinical Follow-up for 6 months	Lab follow-up
1	Very high / High	Positive	*M.tuberculosis* complex growth	Responding to ATD treatment	-
2	Very high / High	Negative	*M.tuberculosis* complex growth	Responding to ATD treatment	-
3	Very high / High	Positive / Negative	*M.tuberculosis* complex growth	Not responding to ATD treatment (Suspicion of MDR-TB Infection)	Confirm the susceptibility using BacT/ALERT SIRE kit.
3	Very high / High	Positive	No growth	Responding to ATD treatment	-
4	Very high / High	Positive / Negative	No growth	Not responding to ATD treatment (Suspicion of MDR-TB Infection)	Gen-probe MTD and/ or PCR should be positive for *M.tuberculosis* complex
5	Very high / High	Negative	No growth	Responding to ATD treatment.	

2. **Control group 2 : Other lung diseases :** Following mentioned criteria will be used to include the people under the control group 3. People who do not fall in any one of these categories will be considered statistically as biased and removed for the analysis.

	Clinical suspicion score for TB	AFB Smear	Culture	Clinical Follow-up for 6 months	Other clinical aetiology	Lab follow-up
1	Very Low	Negative	No growth	No sign of developing Tuberculosis.	Should have a confirmed evidence of any other pulmonary diseases.	Routine culture for bacteria or fungal in case of pulmonary infection.

3. **Control 3 : Health care workers:** Following mentioned criteria will be used to include the people under the control group 2. People who do not fall in any one of these categories will be considered statistically as biased and removed for the analysis.

	Clinical suspicion score for TB	AFB Smear	Culture	Clinical Follow-up for 6 months	Lab follow-up
1	Very Low	-	-	No sign of developing Tuberculosis.	-

4. **Control group 4: Healthy adults for Health check up:** Following mentioned criteria will be used to include the people under the control group 4. People who do not fall in any one of these categories will be considered statistically as biased and removed for the analysis.

	Clinical suspicion score for TB	AFB Smear	Culture	Clinical Follow-up for 6 months	Other clinical aetiology	Lab follow-up
1	Very Low	-	-	No sign of developing Tuberculosis.	Should not have any other pulmonary diseases and non-pulmonary diseases.	-

X STUDY MANAGEMENT

- The study would be controlled and monitored by Chief Scientific coordinator, **Prof. P.H.Lagrange,** St'Louis Hospital, Paris, France (Principle Investigator), Scientific Coordinators **Prof. N.K.Ganguly**, Indian Council of Medical Research (ICMR), New Delhi, India and **Dr.D.Tanna**, Director General, Foundation Merieux, Lyon France.
- Mr.Satheesh Kumar THANGARAJ, Field coordinator, India will co-ordinate on behalf of Foundation Mérieux in India.
- Evaluators and Evaluator assistant of each centre will control centre activity. Evaluator assistant, is a student in each centre, will be involved in the data collection, sample collection, sample processing performing tests and sample storage.
- Chest physician, clinician and Paediatrician of each centre will be closely associated in the study.

XI SATELLITE STUDIES

Population	AIMS	Safdarjung	JALMA & S.N Medical college	P.D.Hinduja Hospital and JJ Hospital	NIMS	TRC	Total
TMA vs Home made PCR	40		100	75			215
TB- Leishmania co-infection for Antibody response	100						100
Comparison of fresh and Frozen samples for ELISA		50					50
Antibody response on patients of skin tuberculosis		50					50
Evaluation of RD1 Selected peptide				400		400	800
TB Cases treated and followed up to 5yrs						100	100
Healthy Family contacts			100	50		100	250
Leprosy cases			100				100
TB - Filoriousis co-infection						25	25
USP vs NALC-NaOH method for sample processing for culture and TMA				50	50		100
DBS vs Serum					100		100
Total Number of samples							**1915**

XII MATERIALS PROVIDED

Table 5a

	Materials	AIMS	Safdarjung	JALMA	PD	NIMS	TRC	Total Qty
1	BacT/ALERT MP	3	5	8	9	9	5	**39**
2	MAS Tests	3	5	8	9	9	5	**39**
3	BacT/ALERT MB	4			14	3		**21**
4	Mb/Bact Enrichment fluid	4			14	3		**21**
5	bioMérieux LJ Slants	22	30	45	71	60	32	**260**
6	Blood agar	14	16	18	32	31	21	**132**
7	Gen-Probe Amplified MTD KITS	1		3	4	4		**12**
8	Mycobacteria Pos and Neg Controls for Amplified MTD kit	2		2	2	2		**8**
9	Accuprobe MTBC	4	7	9	10	10	8	**48**
10	Accuprobe Culture ID Kits	2	2	2	2	2	2	**12**
11	Genprobe Detection Kit	2	2	2	2	2	2	**12**
12	bioMérieux TB ELISA Plates (21 samples + 3 controls in duplicate	5	8	16	11	10	6	**56**
13	LDR Cq	2	2	2	2	2	2	**12**
14	Poly sterine tubes	4	4	4	4	4	4	**24**
15	Polypropylene tubes	2	2	2	3	3	2	**14**
16	Tubes stoppers	100		200	300	300		**900**
17	100ul tips (Gen-probe)	300	300	500	600	600	300	**2600**
18	1250ul Tips (Gen-Probe)	300	300	500	600	600	300	**2600**

Table 5b

	Materials	AIMS	Safdarjung	JALMA	PD	NIMS	TRC	Total Qty
19	Syringe and needles - 2 ml	300	600	900	1500	1500	600	**5400**
	Syringe and needles - 5 ml	400	400	1000	900	900	800	**4400**
20	Syringe and needles - 10 ml	100	100	100	200	200	200	**900**
21	Screw cap vials for storage	2000	2200	5000	3000	3000	2000	**17200**
22	Screw cap vials box	40	44	100	60	60	40	**344**
23	Plastic test tubes 5 ml	500	800	1600	1100	1100	600	**5700**
24	Pipette tips 1ml	1000	1000	1000	1000	1000	1000	**6000**
25	Pipette tips 0.1ml	1000	1000	2000	1000	1000	1000	**7000**
26	Pipette tips 0.01ml	1000	1000	2000	1000	1000	1000	**7000**
27	Pipette tips box autoclavable 1ml	2	2	2	2	2	2	**12**
28	Pipette tips box autoclavable 0.1ml	2	2	2	2	2	2	**12**
29	Pipette tips box autoclavabl 0.01ml	2	2	2	2	2	2	**12**
30	Tips for Eppendorf multi stepper		50	50		50		**150**
31	Sterile sample collection container	500	500	1000	1200	1200	700	**5100**
32	Gloves (3 pair / Day for 12 months)	1200	1200	1200	1200	1200	1200	**7200**
33	Masks	1200	1200	1200	1200	1200	1200	**7200**
34	Vaccutainers	200	300	1500	700	500	600	**3800**
35	Sterile centrifuge tubes (50 ml)	300	600	900	1000	1000	1000	**4800**
36	Glass slides for smear Microscopy	500	500	900	1000	1000	1000	**4900**
37	NALC (Sigma) 25 g	1	1	2	2	2	1	**9**
38	NaOH 500 g	1	1	1	1	1	1	**6**
39	Tri-Sodium Citrate 500 g	1	1	1	1	1	1	**6**
40	Na2HPO4 500g	1	1	1	1	1	1	**6**
41	KH2PO4 500g	1	1	1	1	1	1	**6**
42	Whatman filter paper no3 100 sheets / box					1		**1**
43	Heparin Vacutainers				400			**400**
44	Patient entry form 1	500	400	900	1000	800	700	**4300**
45	Patient entry form 2	400	300	800	900	700	600	**3700**
46	Patient entry form 3	100	100	200	200	200	200	**1000**

XIII ADENDUMS

FORM 1 - Clinical Evaluation

LAB ID NO	Study ID NO	Study Centre	
		Date of Enrolment	

1) Specimens Collected (*Please mark ✓against the appropriate check box)* Date :DD/MM/YY

❒ Sputum	❒ BAL	❒ Urine
❒ Pleural fluid	❒ Pericardial fluid	❒ Peritoneal fluid
❒ Blood culture *(for HIV/TB cases)*	❒ CSF	❒ Pus
❒ Tissue / Biopsy for culture	❒ Blood in Heparin tubes	❒ Blood for Serology
❒ Synovial fluid	❒ *Others, Please specify*	❒ *Others, Please specify*

Please collect only appropriate sample for each study group

In case sputum is the sample, how many samples have been collected? 1 2 3

Patient Study Group

❒ Symptomatic Pulmonary TB Suspect (1PM)	❒ Control Group 1 (*Healthy blood donors)* (3BD)
❒ Symptomatic Extra-Pulmonary TB Suspect (1EP)	❒ Control Group 2 *(Lab staffs & Health care workers)*(3LS)
❒ HIV/TB Cases (1HT)	❒ Control Group 3 *(Non TB - other pulmonary diseases)* (3NT)
❒ Only for Satellite study	❒ Control Group 4 *(Health check up people)* (3CK)

2) Patient Details

Age / Sex		M	F	
IP / OP Number				
Address				

3) TB Risk information

Close contact with TB Patient	❒ No	❒ Yes	❒ Unknown
History of Tuberculosis in the family	❒ No	❒ Yes	❒ Unknown
Personal history of TB	❒ No	❒ Yes	❒ Unknown
If yes, when	❒ In last 2 years	❒ 2 to 10 Years	❒ Beyond 10 Years
If yes, treatment completed	❒ Yes	❒ No	❒ Unknown

FORM 1 - Clinical Evaluation

4) Immunologic debilitating condition at the time of examination? ❒ No ❒ Yes ❒ Unknown

If yes, check all the following that apply?

❒ HIV Infection	❒ Diabetes mellitus	❒ Lung CA / Neck CA
❒ End stage renal disease	❒ Transplant	❒ Under immunosuppressant drug
❒ Leukemia / Lymphoma	❒ Silicosis	❒ Poor nutrition

5) PPD during this evaluation ❒ No ❒ Yes ❒ Not examined

If yes, enter diameter of induration in mm ☐

6 Clinical History (*Please mark ✓against the appropriate check box)*

General	❒ Fever	❒ Night Sweats
	❒ Weight loss	❒ Anorexia
	❒ Fatigue	
Pulmonary	❒ Cough for more than 2 weeks	❒ Blood with expectoration
	❒ Pleuritic pain	❒ Chest pain
	❒ Productive cough	❒ Dry cough
	❒ Dyspnea	
Lymphatic	❒ Enlarged lymph nodes	❒ Sinus tract lymphadenopathy associated
Abdominal	❒ Hepatomegaly	❒ Splenomegaly
	❒ Abdominal pain	❒ Abdominal tenderness
	❒ Abdominal mass	❒ Anal fissures / Fistulae / Perirectal abscesses
	❒ Ascites	❒ Malaena / Frank blood stool
Genito -Urinal	❒ Dysuria	❒ Hematuria
	❒ Frequent Urination	❒ Pyuria
	❒ Scrotal Mass	❒ Pelvic pain
	❒ Menstrual irregularities / Infertility	❒
Skeletal	❒ Joint swelling / Pain	❒ Difficulty or limitation of motion
	❒ Cold Abscess	
CNS	❒ Headache	❒ Increased intracranial pressure
	❒ Decreased level of consciousness	❒ Neck stiffness
	❒ Cranial Nerve Palsies	❒ Cold Abscess
	❒ Convulsions	
Pericardial	❒ Dyspnea	❒ Chest pain
	❒ Orthopnea	❒ Pedal edema
Others, Please specify,		
	❒	❒
	❒	❒
	❒	❒
	❒	❒

FORM 1 - Clinical Evaluation

7 Chest X ray

Right Lung		Left Lung
❒	Mass lesion	❒
❒	Infiltrate	❒
❒	Cavities	❒
❒	Fibrosis	❒
❒	Consolidation	❒
❒	Miliary Pattern	❒
❒	Bronchiectasis	❒
❒	Atelectasis	❒
❒	Tracheal deviation	❒
❒	Pleural thickening	❒
❒	Pleural effusion	❒
❒	Pericardial effusion	❒
❒	Increased Heart size	❒
❒	Hilar / Mediastinal adenopathy	❒
❒	Bony lesions	❒
❒	Raised dome of diaphragm	❒

8 Clinical suspicion

Based on the assessment of the evidence presently available, what is the likelihood that this patient / subject has active tuberculosis at the time of enrolment?

Low ❒

High ❒

Very High ❒

9 Broad-spectrum Antibiotics

❒ Yes ❒ No

Please mention antibiotic here

Patient improved ❒

Patient not improved ❒

10 Final Diagnosis

Pulmonary TB ❒

Lymphatic TB ❒

Abdominal TB ❒

Genito-Urinal TB ❒

Skeletal TB ❒

CNS TB ❒

Pericardial TB ❒

Others, Please specify here, ❒

Others, Please specify here, ❒

Non-TB Case (Control group) ❒

11) Anti – TB Drug therapy regimen

Date of therapy initiation DD / MM / YYYY

❒ Rifampicin	❒ Streptomycin	❒ Ethambutol
❒ INH	❒ Pyrazinamide	❒ *Others, Please specify here,*
❒ *Others, Please specify here,*	❒ *Others, Please specify here,*	❒ *Others, Please specify here,*
❒ *Others, Please specify here,*	❒ *Others, Please specify here,*	❒ *Others, Please specify here,*

Name of the Physician

Signature

Date : DD / MM / YYYY

FORM 1 - Clinical Evaluation

JJ / SN ID No	LAB ID No	Study ID No	Study Centre	
			Date of Enrolment	

1) Specimens Collected (*Please mark ✓against the appropriate check box)* Date :DD/MM/YY

- ❒ Sputum
- ❒ Pleural fluid
- ❒ Blood culture *(for HIV/TB cases)*
- ❒ Tissue / Biopsy for culture
- ❒ Synovial fluid
- ❒ BAL
- ❒ Pericardial fluid
- ❒ CSF
- ❒ Blood in Heparin tubes
- ❒ *Others, Please specify*
- ❒ Urine
- ❒ Peritoneal fluid
- ❒ Pus
- ❒ Blood for Serology
- ❒ *Others, Please specify*

Please collect only appropriate sample for each study group

In case sputum is the sample, how many samples have been collected? 1 2 3

Patient Study Group

- ❒ Symptomatic Pulmonary TB Suspect (1PM)
- ❒ Symptomatic Extra-Pulmonary TB Suspect (1EP)
- ❒ HIV/TB Cases (1HT)
- ❒ Only for Satellite studies
- ❒ Control Group 1 (*Healthy blood donors)* (3BD)
- ❒ Control Group 2 *(Lab staffs & Health care workers)* (3LS)
- ❒ Control Group 3 *(Non TB - other pulmonary diseases)* (3NT)
- ❒ Control Group 4 *(Health check up people)* (3CK)

2) Patient Details

Age / Sex		M	F	
IP / OP Number				
Address				

3) TB Risk information

Close contact with TB Patient	❒ No	❒ Yes	❒ Unknown
History of Tuberculosis in the family	❒ No	❒ Yes	❒ Unknown
Personal history of TB	❒ No	❒ Yes	❒ Unknown
If yes, when	❒ In last 2 years	❒ 2 to 10 Years	❒ Beyond 10 Years
If yes, treatment completed	❒ Yes	❒ No	❒ Unknown

4) Immunologic debilitating condition at the time of examination? ❒ No ❒ Yes ❒ Unknown

If yes, check all the following that apply?

❒ Positive for HIV Infection ❒ Diabetes mellitus ❒ Lung CA / Neck CA

❒ End stage renal disease ❒ Transplant ❒ Under immunosuppressant drug

❒ Leukemia / Lymphoma ❒ Silicosis ❒ Poor nutrition

If HIV infection is positive

Kits used for test 1 2

CD4 / CD8 count *(if the information is available)* CD4 CD8

5) PPD during this evaluation ❒ No ❒ Yes ❒ Not examined

If yes, enter diameter of induration in mm

6 Clinical History (*Please mark ✓against the appropriate check box)*

General
❒ Fever ❒ Night Sweats
❒ Weight loss ❒ Anorexia
❒ Fatigue

Pulmonary
❒ Cough for more than 2 weeks ❒ Blood with expectoration
❒ Pleuritic pain ❒ Chest pain
❒ Productive cough ❒ Dry cough
❒ Dyspnea

Lymphatic
❒ Enlarged lymph nodes ❒ Sinus tract lymphadenopathy associated

Abdominal
❒ Hepatomegaly ❒ Splenomegaly
❒ Abdominal pain ❒ Abdominal tenderness
❒ Abdominal mass ❒ Anal fissures / Fistulae / Perirectal abscesses
❒ Ascites ❒ Malaena / Frank blood stool

Genito -Urinal
❒ Dysuria ❒ Hematuria
❒ Frequent Urination ❒ Pyuria
❒ Scrotal Mass ❒ Pelvic pain
❒ Menstrual irregularities / Infertility ❒

Skeletal
❒ Joint swelling / Pain ❒ Difficulty or limitation of motion
❒ Cold Abscess

CNS
❒ Headache ❒ Increased intracranial pressure
❒ Decreased level of consciousness ❒ Neck stiffness
❒ Cranial Nerve Palsies ❒ Cold Abscess
❒ Convulsions

Pericardial
❒ Dyspnea ❒ Chest pain
❒ Orthopnea ❒ Pedal edema

Others, Please specify,
❒ ❒
❒ ❒
❒ ❒

FORM 1 - Clinical Evaluation

7 Chest X ray

Right Lung		Left Lung
❒	Mass lesion	❒
❒	Infiltrate	❒
❒	Cavities	❒
❒	Fibrosis	❒
❒	Consolidation	❒
❒	Miliary Pattern	❒
❒	Bronchiectasis	❒
❒	Atelectasis	❒
❒	Tracheal deviation	❒
❒	Pleural thickening	❒
❒	Pleural effusion	❒
❒	Pericardial effusion	❒
❒	Increased Heart size	❒
❒	Hilar / Mediastinal adenopathy	❒
❒	Bony lesions	❒
❒	Raised dome of diaphragm	❒

8 Clinical suspicion

Based on the assessment of the evidence presently available, what is the likelihood that this patient / subject has active tuberculosis at the time of enrolment?

Low ❒

High ❒

Very High ❒

9 Broad-spectrum Antibiotics

❒ Yes ❒ No

Please mention antibiotic here

Patient improved ❒

Patient not improved ❒

10 Final Diagnosis

Pulmonary TB ❒

Lymphatic TB ❒

Abdominal TB ❒

Genito-Urinal TB ❒

Skeletal TB ❒

CNS TB ❒

Pericardial TB ❒

Others, Please specify here, ❒

Others, Please specify here, ❒

Non-TB Case (Control group) ❒

11) Anti – TB Drug therapy regimen

Date of therapy initiation DD / MM / YYYY

❒ Rifampicin	❒ Streptomycin	❒ Ethambutol
❒ INH	❒ Pyrazinamide	❒ *Others, Please specify here,*
❒ *Others, Please specify here,*	❒ *Others, Please specify here,*	❒ *Others, Please specify here,*
❒ *Others, Please specify here,*	❒ *Others, Please specify here,*	❒ *Others, Please specify here,*

Name of the Physician

Signature

Date : DD / MM / YYYY

FORM 2 – Laboratory Evaluation

Lab ID: | **Study ID**

AFB Smear Report

	Date	Result (*Please mention the smear gradation code*)	Smear Gradation code
First Sample			**1.** No AFB Seen in 100 Oil Immersion Field (OIF) **2.** Scanty AFB Seen (1 to 9 AFB / 100 OIF) **3.** 1+ AFB Seen (10 to 99 AFB / 100 OIF) **4.** 2 + AFB Seen (1 to 10 AFB / OIF) **5.** 3 + AFB Seen (More than 10 AFB / OIF)
Second Sample			
Third Samples			

Mycobacteria culture report

		First Sample	Second Sample	Third Samples
Primary culture	**Date of inoculation**			
	BacT/ALERT MP			
	LJ			
Blood Culture – *(Only for HIV/TB cases)*	**BacT/ALERT MB**			
Re inoculation	**Date of inoculation**			
	BacT/ALERT MP			
	LJ			

Notes :
- *For positive, please mention the time taken to become positive*
- *Please enter the re-inoculation row only if the primary culture was contaminate*
- *Please fill in one column only if single sample is collected*

Accuprobe MTBC Identification

Accuprobe MTBC reading | ❒ *M.tuberculosis* Complex | ❒ MOTT

Identification of MOTTs, (*if the further speciation is done*).

Routine culture

Growth of Pathogenic Bacteria / Fungal observed — Yes ❒ No ❒

Identification of isolated organisms

FORM 2 – Laboratory Evaluation

Molecular Tests

TB PCR ❒ Positive ❒ Negative ❒ Equivocal

Please mention the PCR method ❒ Homebrew ❒ Roche ❒ Others

Amplified MTD results ❒ Positive ❒ Negative ❒ Equivocal

RLU reading for MTD _____

Repeat MTD Result (Only in case of Equivocal result in the primary Test) ❒ Positive ❒ Negative ❒ Equivocal

RLU reading for Repeat MTD _____

HIV Status of the Patient Positive ❒ Negative ❒

Kits used _____ _____

CD4 / CD8 count *(if the information is available)* CD4 _____ CD8 _____

TB Serology report Date : DD / MM / YYYY

PGL-TB1 & ESAT6-CFP10 ELISA kit	Negative control	Positive control	Standard	Patient sera	Index
Mean OD Value in PGL-TB1 wells					
Mean OD Value in ESAT6-CFP10 wells					

Specimen / Culture Storage Details

Specimen	Storage		Transferred to study coordinator	
	Date	Volume	Date	Volume / No of vials
Serum				
Culture Specimen			NA	NA
Culture				

Signature of Microbiologist
Dr.

Date : DD / MM / YYYY

FORM 2 – Laboratory Evaluation

Lab ID: | **Study ID**

AFB Smear Report

	Date	Result (*Please mention the smear gradation code*)	Smear Gradation code
First Sample			**1.** No AFB Seen in 100 Oil Immersion Field (OIF) **2.** Scanty AFB Seen (1 to 9 AFB / 100 OIF) **3.** 1+ AFB Seen (10 to 99 AFB / 100 OIF) **4.** 2 + AFB Seen (1 to 10 AFB / OIF) **5.** 3 + AFB Seen (More than 10 AFB / OIF)
Second Sample			
Third Samples			

Mycobacteria culture report

		First Sample	Second Sample	Third Samples
Primary culture	**Date of inoculation**			
	BacT/ALERT MP			
	LJ			
Blood Culture – *(Only for HIV/TB cases)*	**BacT/ALERT MB**			
Re inoculation	**Date of inoculation**			
	BacT/ALERT MP			
	LJ			

Notes :
- *For positive, please mention the time taken to become positive*
- *Please enter the re-inoculation row only if the primary culture was contaminate*
- *Please fill in one column only if single sample is collected*

Accuprobe MTBC Identification

Accuprobe MTBC reading ❒ *M.tuberculosis* Complex ❒ MOTT

Identification of MOTTs, (*if the further speciation is done*).

Routine culture

Growth of Pathogenic Bacteria / Fungal observed Yes ❒ No ❒

Identification of isolated organisms

Molecular Tests

TB PCR ❒ Positive ❒ Negative ❒ Equivocal

Please mention the PCR method ❒ Homebrew ❒ Roche ❒ Others

Amplified MTD results ❒ Positive ❒ Negative ❒ Equivocal

RLU reading for MTD

Repeat MTD Result (Only in case of Equivocal result in the primary Test) ❒ Positive ❒ Negative ❒ Equivocal

RLU reading for Repeat MTD

FORM 2 – Laboratory Evaluation

HIV Status of the Patient Positive ❒ Negative ❒

Kits used

CD4 / CD8 count
(if the information is available) CD4 CD8

TB Serology report Date : DD / MM / YYYY

PGL-TB1 & ESAT6-CFP10 ELISA kit	Negative control	Positive control	Standard	Patient sera	Index
Mean OD Value in PGL-TB1 wells					
Mean OD Value in ESAT6-CFP10 wells					

RD1 –Selected peptide results

(*Only for the centres perform RD1 satellite study)* Date : DD / MM / YYYY

RD1 Selected peptide Assay	CTR	DMSO	PHA	ESAT-6 Pool	CFP 10 Pool
OD Value of IFN-α ELISA					
Assay interpretation					

Date : DD / MM / YYYY

Quantiferron –TB Gold	Nil Control	ESAT-6	Mitogen
IFN-α in IU/mL ELISA			
QFT TB Gold Assay interpretation	❒ Positive		❒ Negative

Specimen / Culture Storage Details

Specimen	Storage		Transferred to study coordinator	
	Date	Volume	Date	Volume / No of vials
Serum				
Culture Specimen			NA	NA
Culture				

Signature of Microbiologist
Dr.

Date : DD / MM / YYYY

FORM 3 - Clinical follow up

Patient response to Anti-TB Treatment: (*Clinical follow up)*

JJ / SN ID No ☐ **Study ID number** ☐

(*Please mark ✓against the appropriate check box)*

1) Symptoms

Time from initiation of Anti-TB Drugs treatment

	1 Month	2 Months	3 Months	4 Months	5 Months	6 Months
Not cleared	❒	❒	❒	❒	❒	❒
Improved	❒	❒	❒	❒	❒	❒
Cleared	❒	❒	❒	❒	❒	❒

2) CXR

Time from initiation of Anti-TB Drugs treatment

	1 Month	2 Months	3 Months	4 Months	5 Months	6 Months
Not cleared	❒	❒	❒	❒	❒	❒
Improved	❒	❒	❒	❒	❒	❒
Cleared	❒	❒	❒	❒	❒	❒

3) Microbiology conversion

Time from initiation of Anti-TB Drugs treatment

	1 Month	2 Months	3 Months	4 Months	5 Months	6 Months
Not cleared	❒	❒	❒	❒	❒	❒
Improved	❒	❒	❒	❒	❒	❒
Cleared	❒	❒	❒	❒	❒	❒

4) Clinical follow-up for Close contacts

If the subject is included in the study as close contact, fill the following information

During 6 months clinical follow-up, whether the subject has developed any symptoms for TB

❒ No ❒ Yes

If yes, mention the month ☐

If yes, open a new file with new Study ID number. Mention the new Study ID number ☐

Patient responded to Anti Tuberculosis treatment

Yes ❒ No ❒ Incomplete Follow-up ❒ Non compliance ❒

Physician	Signature	
	Name	Dr.
Microbiologist	Signature	
	Name	Dr.
Student	Signature	
	Name	
	File completed on Date	: DD / MM / YYYY

(*Please mark file completed once the all entries including clinical follow-up information have been filled)*

FORM 3 - Clinical follow up

Patient response to Anti-TB Treatment: (*Clinical follow up)*

JJ ID No	
Study ID No	

(*Please mark ✓against the appropriate check box)*

1) Symptoms *Time from initiation of Anti-TB Drugs treatment*

	1 Month	2 Months	3 Months	4 Months	5 Months	6 Months
Not cleared	❒	❒	❒	❒	❒	❒
Improved	❒	❒	❒	❒	❒	❒
Cleared	❒	❒	❒	❒	❒	❒

2) CXR *Time from initiation of Anti-TB Drugs treatment*

	1 Month	2 Months	3 Months	4 Months	5 Months	6 Months
Not cleared	❒	❒	❒	❒	❒	❒
Improved	❒	❒	❒	❒	❒	❒
Cleared	❒	❒	❒	❒	❒	❒

Patient responded to Anti Tuberculosis treatment

Yes ❒ No ❒ Incomplete Follow-up ❒ Non compliance ❒

Physician	Signature	
	Name	Dr.
Microbiologist	Signature	
	Name	Dr.
Student	Signature	
	Name	
	File completed on Date	: DD / MM / YYYY

(*Please mark file completed once all the entries including clinical follow-up information have been filled)*

TB Tool box study - Fortnightly report

Center Name _____ **Center code** _____

Reporting period _____

	Group	Study number sequence	No of patients / Subjects included in the study	No of sera sample sets collected	No of samples collected for culture	No of smear set done	No of smear positives	No of culture done	No culture Positive in the reporting period	No of *M.tuberculosis* identified from the positive culture	No of samples analyzed for PGL-TB1 and ESAT6-CFP10 ELISA
TB Suspected cases	Pulmonary TB Cases (PM)										
	Extra Pulmonary TB Cases (EP)										
	HIV/TB Cases (HT)										
Control group	Blood donors (BD)										
	Lab staffs / Health care workers (LS)										
	Non-TB pulmonary diseases (NT)										
	Health check-up People (CK)										
Satellite study	Satellite study 1										
	Satellite study 2										
	Satellite study 3										
	Satellite study 4										
	All subjects included in the study										

bioMérieux TB ELISA

Kit Lot n° _____ Date of run _____

Study Center _____

	1	2	3	4	5	6	7	8	9	10	11	12
	PGL-TB1	ESAT6-CFP10	PGL-TB1	ESAT6-CFP10	PGL-TB1	ESAT6-CFP10	PGL-TB1	ESAT6-CFP10	PGL-TB1	ESAT6-CFP10	PGL-TB1	ESAT6-CFP10
A												
B												
C												
D												
E												
F												
G												
H												

Instructions

Run 21 sera samples in each ELISA run. Always run duplicate for every diluted serum.

Include controls(positive, negative and standard) in duplicate provided in the ELISA Kit.

Avoid using any sera which are contaminated for ELISA.

Controls are pre diluted and no needed to dilute before run

Always run complete plate

Do not interchange any reagents with anyother ELISA Kit. REAGENTS FROM OTHER ELISA KITS DO NOT WORK

Make sure all reagents and sera are at room temperature before sera dilution and ELISA run.

Repeat the sample, if any sample OD value is not matching among duplicates (Standard Deviation among duplicates > 0.030)

Remarks

LEPORTIER Marc

TB Tool box study - Sera index sheet for storage and transport

From Date:

Till Date:

Box Number

S.NO	Study ID No	Volume	S.NO	Study ID No	Volume	S.NO	Study ID No	Volume
1			28			55		
2			29			56		
3			30			57		
4			31			58		
5			32			59		
6			33			60		
7			34			61		
8			35			62		
9			36			63		
10			37			64		
11			38			65		
12			39			66		
13			40			67		
14			41			68		
15			42			69		
16			43			70		
17			44			71		
18			45			72		
19			46			73		
20			47			74		
21			48			75		
22			49			76		
23			50			77		
24			51			78		
25			52			79		
26			53			80		
27			54			81		

TB Tool box study - Sera index sheet for storage and transport

Instructions for Box number

First Digit	Center code	Each center code
Second digit	Collection lot	First 81 samples in the collection are in lot number 1
		Second 81 samples in the collection are in lot number 2
		Third 81 samples in the collection are in lot number 3
		And so on…
Third digit	Serial number of collection	1 for the sera for tests
		2 for the sera for reserve
		3 for the sera to be sent to coordinator

Examples

Example1: Box number 121 is for center AIMS, Second collection and the sera meant for performing ELISA test.

Example2: Box number 842 is for center TRC, fourth collection and the sera box meant for reserve at TRC.

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PLOS ONE

A Toolbox for Tuberculosis Diagnosis: An Indian Multicentric Study (2006-2008): Microbiological Results

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Abstract

Background: The aim of this multicentric prospective study in India was to assess the value of several microbiological tools that contribute to the diagnosis of tuberculosis (TB) according to HIV status.

Methods: Standard microbiological tools on individual specimens were analyzed.

Results: Among the 807 patients with active TB, 131 were HIV-infected, 316 HIV-uninfected and 360 had HIV-unknown status. Among the 980 non-active TB subjects, 559 were at low risk and 421 were at high risk of *M. tuberculosis* (Mtb) exposure. Sensitivity of smear microscopy (SM) was significantly lower in HIV-infected (42.2%) than HIV-uninfected (75.9%) ($p = 0.0001$) and HIV-unknown pulmonary TB patients (61.4%) ($p = 0.004$). Specificity was 94.5% in non-TB patients and 100% in health care workers (HCW) and healthy family contacts. Automated liquid culture has significantly higher diagnostic performances than solid culture, measured by sensitivity (74.7% *vs.* 55.9%) ($p = 0.0001$) and shorter median time to detection (TTD) (12.0 vs. 34.0 days) ($p = 0.0001$). Specificity was 100% in HCW and cured-TB patients, but was lower in non-TB patients (89%) due to isolation of Mycobacteria other than tuberculosis (MOTT). TTD by both methods was related to AFB score. Contamination rate was low (1.4%). AccuProbe hybridization technique detected *Mtb* in almost all culture-positive specimens, but MOTT were found in 4.7% with a significantly higher frequency in HIV-infected (15%) than HIV-uninfected TB patients (0.5%) ($p = 0.0007$). Pre-test classification significantly increased the diagnostic value of all microbiological tests in pulmonary TB patients ($p < 0.0001$) but to a lesser degree in extrapulmonary TB patients.

Conclusions: Conventional microbiological tools led to results similar to those already described in India special features for HIV-infected TB patients included lower detection by SM and culture. New microbiological assays, such as the automated liquid culture system, showed increased accuracy and speed of detection.

Citation: Lagrange PH, Thangaraj SK, Dayal R, Despande A, Ganguly NK, et al. (2012) A Toolbox for Tuberculosis Diagnosis: An Indian Multicentric Study (2006-2008): Microbiological Results. PLoS ONE 7(8): e43739. doi:10.1371/journal.pone.0043739

Editor: Petros C. Karakousis, Johns Hopkins University School of Medicine, United States of America

Received May 2, 2012; **Accepted** July 23, 2012; **Published** August 24, 2012

Funding: This work was supported by Fondation Merieux. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: Delia Goletti is an Academic Editor for PLoS ONE. Satheesh K. Thangaraj and Marc Leportier are employees of BioMérieux. There are no patents, products in development or marketed products to declare. This does not alter the authors' adherence to all the PLoS ONE policies on sharing data and materials, as detailed online in the guide for authors.

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Introduction

Tuberculosis (TB) is a major global health problem. In 2010, 8.8 million incident cases of TB and 1.1 million deaths were reported by the World Health Organization (WHO) [1]. In India 2.3 million new TB cases are estimated to have occurred in 2010, accounting for one-quarter of the world's new TB cases [1]. This makes India the country with the highest TB burden in the world. It has been estimated that in 2010, only 41% of the 1,522,147 million notified were smear positive, and that two out of five Indians were infected with *M.tuberculosis* [2]. There are an estimated 2.5 million Indians living with HIV infection. Regarding smear-positive TB, HIV infection was 1.4 times more likely to occur among smear-negative patients and 1.3 times more likely among extrapulmonary patients [3].

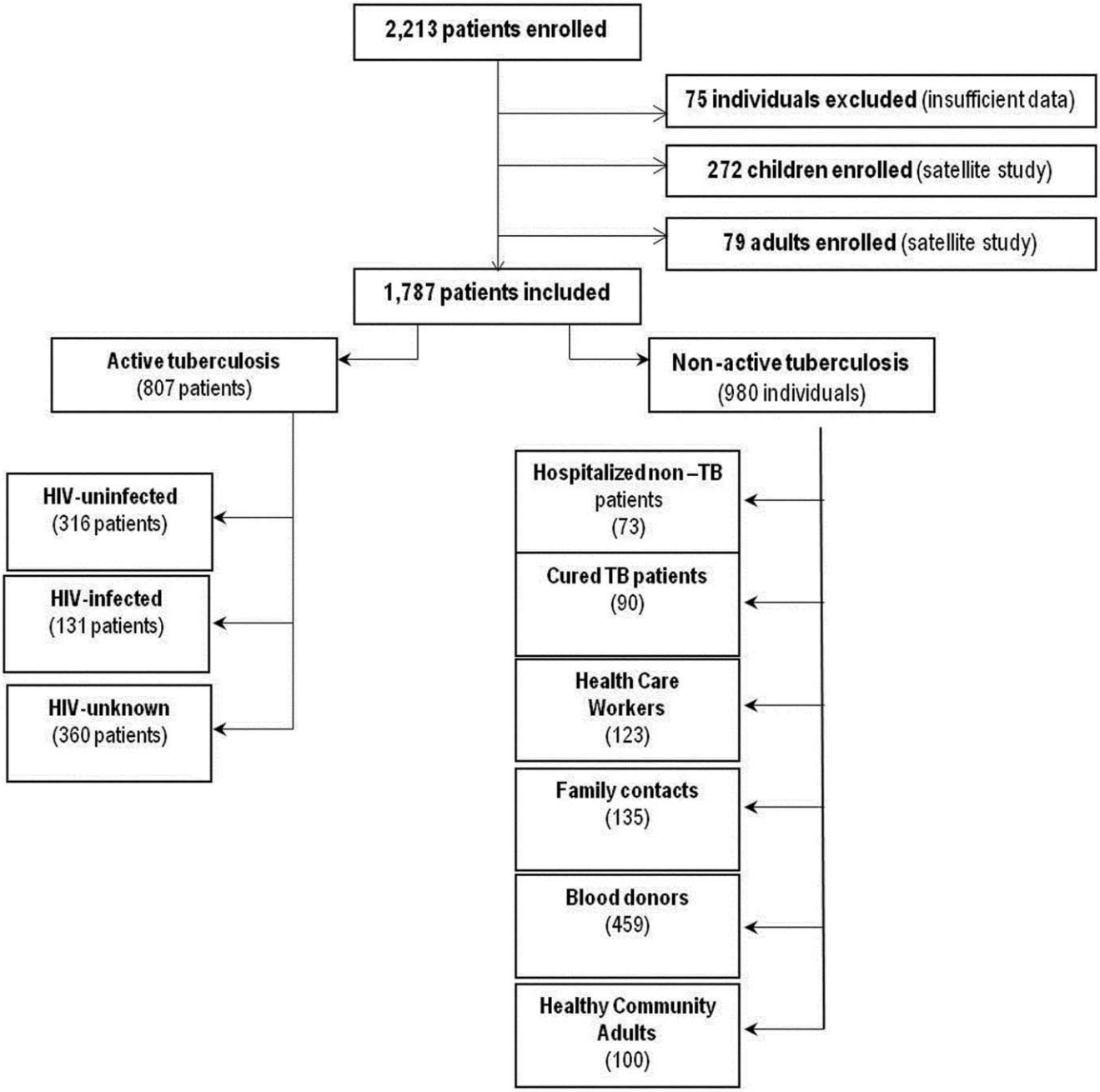

Figure 1. Flow chart of patients recruited to the multicentric study stratified by patient subgroups.
doi:10.1371/journal.pone.0043739.g001

The basis of TB control programs consists of timely diagnosis and correct treatment of patients with active TB: early diagnosis is essential for controlling the spread of this disease.

Diagnosis of TB relies mostly on the detection of acid-fast bacilli by microscopy (smear) and less frequently by culture. Microscopy is rapid, usually specific, and inexpensive but has low sensitivity [4–5] that may be improved using new sputum processing and fluorescence microscopy [6]. Culture is more sensitive, but it can take several weeks to obtain results. In addition, solid culture may be falsely negative in 10–30% of cases [7]. Faster and more accurate diagnostic tests are needed to better control TB [8]. Nucleic acid amplification tests, which can give results in a few hours, have been developed to address these issues [9–11].

The development of real-time PCR testing platforms has significantly improved PCR technologies, the advantages of which are shortened turnaround time, automation of the procedure that reduces hands-on time and decreased risk of cross-contamination [12]. Recently, Cepheid, (Sunnyvale, CA, USA) developed a real-time PCR test (Xpert MTB/RIF) on the GeneXpert System that simultaneously detects both MTB and Rifampin resistance [13]. Recent studies reported high sensitivity and specificity of the test in respiratory specimens collected from patients living in countries with high and low prevalence of TB [14,15].

Therefore the aim of the first part of the multicentric prospective study in India was to assess the value of several microbiological tools that contribute to TB diagnosis according to

Table 1. Demographic and clinical characteristics of the patients with active tuberculosis.

	Patients with active tuberculosis (n = 807)					
	Pulmonary tuberculosis (n = 656)			Extrapulmonary tuberculosis (n = 151)		
	HIV-infected	HIV-uninfected	HIV-unknown	HIV-infected	HIV-uninfected	HIV-unknown
	(n = 93)	(n = 270)	(n = 293)	(n = 38)	(n = 46)	(n = 67)
	Percentage					
Disease Localization	71.0	85.4	81.4	29.0	14.6	18.6
Median Age (IQR)	35.0	34.0	30.0	36.0	30.0	28.0
Ratio M/F	4.8	2.3	2.3	5.3	1.7	1.0
Male prevalence	82.8	66.3	70.0	84.2	63.0	50.8
Diabetes	0.0	11.1	2.4	0.0	6.5	1.5
Poor Nutrition	0.0	1.1	10.9	0.0	0.0	4.5
Past TB	28.4[a]	15.2	21.4	26.5[c]	10.9	5.9
TB <2 years	13.4	6.0	9.2	11.8	4.4	4.4
TB 2 to10 years	14.9	7.1	9.2	14.7	4.4	1.5
TB>10 years	0.0	2.2	3.1	0.0	2.2	0.0
Contact with TB	12.9[b]	8.5	8.2	12.5[d]	19.6	6.0

[a]26/93 patients have no record;
[b]23/93 patients have no record;
[c]4/38 patients have no record;
[d]6/38 patients have no record.
doi:10.1371/journal.pone.0043739.t001

HIV-status. The second part will involve the immunological assays (manuscript in preparation).

Materials and Methods

Study Populations

Adults and children with suspected active TB were prospectively enrolled from January 2006 to July 2008 with a real capability of a six month follow-up with antituberculous therapy, as a strict inclusion criterion. Nine centers were involved: four tertiary care centers: 3 public (AIIMS and Safdarjung Hospital, New Delhi; Nizam's Institute of Medical Sciences, Hyderabad) and 1 private (P D Hinduja Hospital and medical Research Centre, Mumbai), two referral centers (JALMA, Agra; Tuberculosis Research Centre-TRC, Chennai), one pediatric hospital (SN Medical College, Agra) and two centers caring for HIV-infected patients (JJ hospital, Mumbai; Government Hospital for Thoracic Medicine, Chennai).

Non-active TB controls were also enrolled: blood donors from three centers (AIIMS, Hinduja and Nizam's), healthy community adults (HCA-check up individuals) from Hinduja outpatients,

Table 2. Extrapulmonary TB localizations according to HIV status.

	HIV-infected	HIV-uninfected	HIV-unknown	Total
	(n = 38)	(n = 46)	(n = 67)	(n = 151)
	Percentage (95% CI)			
Cervical lymphadenopathy	5.3 (1.4−17.6)	30.4 (18.9–45.1)	32.8 (22.6–45.0)	25.2 (18.8–32.8)
Mediastinal lymphadenopathy	7.9 (2.7−21;1)	0.0 (0.0–8.0)	0.0 (0.0–5.6)	2.0 (0.7–5.8)
Pleural	27.8 (13.7−44.3)	26.1 (15.4–40.6)	19.4 (11.6–30.7)	23.2 (17.1–30.7)
Abdominal	29.0 (16.8−45.1)	17.4 (9.0–31.0)	7.5 (3.2–16.5)	15.9 (10.8–22.7)
CNS	15.8 (7.3−30.8)	15.2 (7.5–28.5)	0.0 (0.0–5.6)	8.6 (5.1–14.3)
Disseminated	10.5 (4.1−27.5)	2.2 (0.4–11.6)	0.0 (0.0–5.6)	3.3 (1.4–7.6)
Skeletal	5.3 (1.4−17.6)	2.2 (0.4–11.6)	38.8 (27.9–51.0)	19.2 (13.6–26.4)
Genito–urinary	0.0 (0.0–9.5)	2.2 (0.4–11.6)	0.0 (0.0–5.6)	0.7 (0.1–3.8)
Skin	0.0 (0.0–9.5)	0.0 (0.0–8.0)	2.2 (0.4–11.6)	0.7 (0.1–3.8)
Rectal	0.0 (0.0–9.5)	2.2 (0.4–11.6)	0.0 (0.0–5.6)	0.7 (0.1–3.8)
Pericarditis	0.0 (0.0–9.5)	2.2 (0.4–11.6)	0.0 (0.0–5.6)	0.7 (0.1–3.8)

TB: tuberculosis, CNS: central nervous system; TB: HIV: Human Immunodeficiency Virus; CI: confidence interval.
doi:10.1371/journal.pone.0043739.t002

Table 3. Respective proportion of patients classified as microbiologically documented and clinical tuberculosis according to disease localization and HIV-status.

	Pulmonary TB				Extrapulmonary TB			
	HIV-infected	HIV-uninfected	HIV-unknown	Total	HIV-infected	HIV-uninfected	HIV-unknown	Total
	(n = 93)	(n = 270)	(n = 293)	(n = 656)	(n = 38)	(n = 46)	(n = 67)	(n = 151)
	Percentage (95% CI)				Percentage (95% CI)			
Documented	61.3 (50.6–71.2)	84.1 (79.2–88.2)	83.6 (78.8–87.7)	80.5 (77.3–83.5)	47.4 (31.0–64.2)	63.0 (47.6–76.8)	41.8 (29.9–54.5)	49.7 (41.1–57.9)
Clinical	**38.7**[(a)](28.8–49.4)	15.9 (11.8–20.9)	16.4 (12.3–21.1)	19.5 (16.5–22.7)	52.6 (35.8–69.0)	**37.0**[(b)](23.2–52.5)	58.2 (45.5–70.2)	**50.3**[(a)](42.1–58.6)

TB: tuberculosis; HIV: Human Immunodeficiency Virus; CI: confidence interval.
[(a)] $p<0.0001$;
[(b)] $p = 0.03$; TB: tuberculosis.
doi:10.1371/journal.pone.0043739.t003

hospitalized non-active-TB patients from three centers (Hinduja, AIIMS and Safdarjung), leprosy patients from one center (JALMA), healthy family contacts (HFC) from 2 centers (JALMA, TRC), health care workers (HCW-laboratory staff and nurses) from three centers (Hinduja, AIIMS and TRC), and cured TB patients from one center (TRC).

All enrolled individuals were stratified by risk for TB (defined as previous exposure to active TB cases, past TB, diabetes, poor nutritional conditions, immunological or other debilitating conditions) and therefore non-active TB controls were divided into "low TB risk" (blood donors and healthy community adults) and "high TB risk" (HCW, healthy family contacts, cured TB and no TB patients). Subjects at risk of TB were included with one condition: a real capability of a six month follow-up to exclude occurrence of active TB. The HIV-infected subjects were not undergoing antiretroviral therapy.

Clinical and Chest X-Ray Examinations

Clinical symptoms and radiological findings were first assessed independently by each clinician taking part in the enrollment. Each individual's data were initially recorded at each site using a standardized questionnaire involving 3 files (clinical/radiological evaluation, clinical/radiological follow-up and laboratory analysis) for patients with active TB and control groups (except for blood donors). The clinical TB suspicion (CSTB) score was done by the clinician in charge of the patient (3 categories were selected: very high, high, low), as previously reported [16].

Table 4. Demographic and clinical characteristics of the non-active TB individuals.

	Individuals without active TB (n = 980)					
	Low risk of TB exposure		High risk of TB exposure			
	(n = 559)		(n = 421)			
	Blood donors	Healthy Community Adults	Non -TB Patients [(a)]	Health Care Workers	Healthy Family Contacts	Cured TB
	(n = 459)	(n = 100)	(n = 73)	(n = 123)	(n = 135)	(n = 90)
	Percentage					
Pulmonary disease	NA	NA	58.9	NA	NA	100.0
Median Age (IQR)	NA	44.0	40.0	30.0	32.0	34.0
Ratio M/F	NA	1.7	2.5	1.0	1.0	3.1
Male prevalence	NA	63.0	71.2	49.6	51.1	75.6
Diabetes	0.0	0.0	20.9	0.8	0.0	0.0
Poor Nutrition	0.0	0.0	0.0	0.0	0.0	0.0
Past TB	NA	0.0	10.9	0.0	0.7	100.0
TB <2 years	NA	0.0	1.4	0.0	0.0	28.1
TB 2 to10 years	NA	0.0	5.5	0.0	0.0	71.9
TB>10 years	NA	0.0	4.1	0.0	0.7	0.0
Contact with TB	0.0	0.0	4.7	0.0	100	0.0

[(a)]The hospitalized non-active TB control group presented a pulmonary disease in 58.9% of the 73 enrolled individuals with a definitive diagnosis excluding active TB: bacterial or atypical pneumonia (34.9%); Bronchitis (11.6%), MOTT infection (11.6%), lung cancer (7.0%), COPD (7.0%), complicated Asthma (7.0%), Lung abscess (4.7%), post-TB Lung fibrosis (2. 3%), Mediastinal lymphadenopathy (2.3%) and Sarcoïdosis (2.3%);
doi:10.1371/journal.pone.0043739.t004

Table 5. Frequency of smear microscopy positive results of patients with active-TB according to their disease localization and HIV-status.

	Pulmonary TB		Extrapulmonary-TB		Pulmonary vs Extrapulmonary
	AFB-positive/total number of patients tested per group (Percentage)	Comparison by HIV Status *p* value	AFB-positive/total number of patients tested per group (Percentage)	Comparison by HIV Status *p* value	*p* value
HIV-infected	38/90* **(42.2%)**	0.0001 vs HIV-uninfected	3/31** **(9.7%)**	0.34 vs HIV-uninfected	0.0004
		0.0047 vs HIV-unknown		0.37 vs HIV-unknown	
HIV-uninfected	205/270 **(75.9%)**	0.0002 vs HIV- unknown	9/46 **(19.6%)**	1.00 vs HIV-uninfected	0.002
HIV-unknown	180/293 **(61.4%)**	–	13/67 **(19.4%)**	–	0.0001
Total	423/653* **(64.8%)**		25/144** **(17.4%)**		0.0001

3 patients had no AFB records;
**7 patients had no AFB records.
doi:10.1371/journal.pone.0043739.t005

Written informed consent was obtained from all patients before enrolment. Parents signed the written consent for the children population. The study was approved by the local ethical committees: the Institutional Ethical Committee of Tuberculosis Research Centre in Chennai (TRC-IEC No: 2006005), the Institutional Review Board at Hinduja Hospital, Mumbai (No: 316-05-CR), the Institutional Ethical Committee of AIIMS, New Delhi (AIIMS-IEC No: A-35:05/10/2005), the Institutional Ethical Committee of the Safdarjung Hospital, New Delhi (N° Sur./1/2007), the NIMS Institutional Ethics Committee (No.EC/264 (A) /2005) and the Ethical Committee of National JALMA Institute for Leprosy and others Mycobacterial Diseases, Agra (minutes: 27/04/2006).

Microbiological Tests

Conventional assays (SM and solid-culture). Most patients with suspected pulmonary TB had 3 sputum samples submitted for Smear Microscopy (SM) and culture. Extrapulmonary samples were harvested on at least one occasion (CSF, pericardial, pleural or ascitis fluid, urines). Respiratory specimens were collected in aseptic tubes and processed by the N-acetyl-L-cysteine (NALC)-NaOH method before SM and culture.

•SM: Smears were stained using the hot Ziehl-Neelsen (ZN) method according to standard guidelines and read using light microscopy. The semi-quantitative yield of AFB was recorded according to WHO recommendations [17]. Positive results were defined by an AFB number above the threshold for positivity (i.e., 10 AFB/100 HPF on the IUATLD/WHO scale); and negative results by a smear with no AFB/100 HPF (high power field). Moreover, among positive scores, scanty results were included and defined as 1–9 AFB per 100 HPF. The SM results were analyzed by sputum samples at their highest level [18,19]. Diagnostic smears were those performed at enrollment and follow-up smears were those performed during treatment.

•Mycobacteria growth detection:

1. Two culture techniques were used:

   ○ *Solid media*: 0.2 mL of each decontaminated and reconstituted sample were inoculated in one tube of Lowenstein-Jensen (LJ) (bioMérieux India Inc.) and incubated at 37°C. Tubes were periodically observed for microbial growth. The enumeration of numbers of colonies per tube and time to detection (TTD) were recorded.

   ○ *Automated liquid media*: 0.5 mL of reconstituted BacT/ALERT® MAS fluid was added to BacT/ALERT® MP bottle (bioMérieux India Inc.) and 0.5 mL of each decontaminated and reconstituted sample were inoculated to the prepared BacT/ALERT® MP bottle. The inoculated bottles were loaded in the BacT/ALERT® 3D or MB/BacT® system. Detection of mycobacterial growth was monitored automatically and the time of first positivity of the vials during the culture was recorded for each inoculated bottle. The Time to Detection (TTD) of mycobacterial cultures was defined as the day that the bottles gave a positive Growth Index (GI) reading and ZN staining demonstrated AFB bacilli.

2. The AFB growth was confirmed by both the LJ medium and BacT/ALERT® MP flagged positive bottle using smear ZN staining.
3. Identification of the bacilli as *M.tuberculosis* complex was performed using a rapid DNA hybridization test specific for *M.tuberculosis* complex (Accuprobe MTBC; GenProbe

Table 6. Frequency of smear microscopy positive results of adult high-risk controls studied.

AFB-positive/total number of subjects tested per group (Percentage)			
No TB patients	**Health Care Workers**	**Healthy Family contacts**	**Cured TB patients**
4/73 **(5.5%)**[a)]	0/123 (0.0%)	0/135 (0.0%)	0/90 (0.0%)

TB: tuberculosis; HIV: Human Immunodeficiency Virus; a) For comparison of the ''no-TB group'' with the ''Health care group'', or with ''healthy family contacts'' or ''cured-TB patients'' the p value were at least = 0.03; AFB: acid fast bacilli.
doi:10.1371/journal.pone.0043739.t006

Table 7. Comparative proportions of the semi-quantitative smear microscopy results (AFB score) obtained in groups of patients with active pulmonary TB enrolled at the different centers independent of their HIV-status.

	Smear microscopy AFB score							
	Positive over total (%)							
Center	0	Scanty	(+)	(++)	(+++)	(++) and (+++)	*p* value*	Total AFB(+)
HINDUJA	51/162 (31.5)	12/162 (7.4)	33/162 (20.4)	23/162 (14.2)	43/162 (26.5)	66/162 (40.7)	1	111/162 (68.5)
AIIMS	50/90 (55.6)	6/90 (6.7)	15/90 (16.7)	8/90 (8.9)	11/90 (11.8)	19/90 (21.1)	**0.0003**	**40/90 (44.4)**
SAFDARJUNG	51/95 (53.7)	1/95 (1.1)	14/95 (14.7)	16/95 (16.8)	13/95 (13.7)	29/95 (30.5)	0.07	**44/95 (46.3)**
JALMA	31/89 (34.8)	6/89 (6.7)	13/89 (14.6)	10/89 (11.2)	29/89 (32.6)	39/89 (43.8)	0.6	58/89 (65.2)
NIZAM	7/49 (14.3)	7/49 (14.3)	8/49 (16.3)	9/49 (18.4)	18/49 (36.7)	27/49 (55.1)	**0.05**	42/49 (85.7)
TRC	40/168 (23.8)	13/168 (7.7)	29/168 (17.3)	38/168 (22.6)	48/168 (28.0)	86/168 (51.2)	**0.01**	128/168 (76.2)
TOTAL	230/653 (35.3)	45/653 (6.9)	112/653(17.2)	104/653 (15.9)	162/653 (24.8)	266/653 (40.7)		423/653 (64.8)

AFB: acid fast bacilli.
*p value of the cumulative group (++ and +++) *versus* the mean of total enrolled patients with the same AFB score.
doi:10.1371/journal.pone.0043739.t007

Inc., San Diego, CA, USA). Two ml were drawn from positive liquid media and centrifuged for 20 min at 3,500×*g*, and the pellet was used in the hybridization test. Samples were read in a Leader 50 luminometer (Gen-Probe Inc. San-Diego, CA, USA). Samples producing signals greater than 30,000 RLUs (Leader 50 luminometer) were considered positive. Cultures were probed on the first working day following the MB/BacT instrument positive flag. Starting from the evidence of a very high number of AFB in the MB/BacT medium from the first signal, this procedure was adopted to shorten the identification time. Extended species Identification of the MOTTs (mycobacteria other than *M.tuberculosis)* was not performed.

4. All positive cultures were stored and kept at −20°C for future studies.

New Tests

Blood culture: around 3–5 mL of blood from HIV-positive patients with suspected TB were either collected in a sterile SPS or heparinized tube, or directly inoculated into a BacT/ALERT® MB blood culture bottle at the patient's bedside. The inoculated bottles were immediately transferred to the microbiological laboratory for loading in the BacT/ALERT® 3D or MB/BacT® system for incubating and monitoring mycobacterial growth. Identical procedures for the TTD and mycobacterial identification were performed as above.

NAAT assays: Amplified *Mycobacterium tuberculosis* Direct (AMTD) test (GenProbe Inc., San Diego, CA, USA): GenProbe MTD analysis was performed from the processed specimen in case of sputum and other non- sterile specimens using the required volume as indicated by the manufacturer.

Immunological Tests

HIV-infection was diagnosed by two ELISA (Retroquic Comb Aids-RS, Span Diagnostics, India and HIV TRI-DOT, J. Mitra & Co, India) in serum. The person was considered HIV-positive when the serum was reactive in both tests if a serum was reactive in only one ELISA, HIV-Western Blot was done as a confirmatory test to rule out a false ELISA test result [3].

Data Collection

These data were subsequently transferred to EPIINFO files by one of the authors (ST). Each file included the patient's characteristics (serial number, study center, date of enrollment, nature of specimen collected, patient study group, age, sex, permanent address), risk of TB, clinical symptoms (cough for more than 2 weeks, persistent low-grade fever –higher than 37.5C for

Table 8. Comparative proportions of the semi-quantitative smear microscopy results (AFB score) obtained in groups of patients with active pulmonary TB according to their HIV status.

	Smear microscopy AFB score							
	Positive over total (%)							
HIV status	0	Scanty	(+)	(++)	(+++)	(++) and (+++)	*p* value*	Total AFB(+)
HIV-infected	52/90 (57.8)	6/90 (6.7)	11/90 (12.2)	14/90 (15.6)	7/90 (7.8)	21/90 (23.3)	0.0017	38/90 (42.2)
HIV-uninfected	65/270 (24.1)	25/270 (9.3)	49/270 (18.1)	51/270 (18.9)	80/270 (29.6)	131/270 (48.5)	0.034	205/270 (75.9)
HIV-unknown	113/293 (38.6)	14/293 (4.8)	52/290 (17.7)	39/293 (13.3)	75/293 (25.6)	114/293 (38.9)	0.616	180/293 (61.4)
TOTAL	230/653 (35.2)	45/653 (6.9)	112/653 (17.2)	104/653(15.9)	162/653 (24.8)	266/653 (40.7)		423/653 (64.8)

AFB: acid fast bacilli; HIV: Human Immunodeficiency Virus;
*p value of the cumulative group (++ and +++) in HIV-infected *versus* the mean of total enrolled patients with same AFB score.
doi:10.1371/journal.pone.0043739.t008

Table 9. Impact of the physicians' clinical suspicion of TB (CSTB) on the sensitivity of smear microscopy in tuberculosis patients according to disease localization and HIV status.

	Pulmonary TB					Extrapulmonary TB				
	TB diagnosis*	CSTB Low	CSTB High	CSTB Very High	*p* value "TB diagnosis vs very high"	TB diagnosis High	CSTB Low	CSTB High	CSTB Very	*p* value "TB diagnosis vs very high"
	N of AFB-positive/N of patients within the group					N of AFB-positive/N of patients within the group				
			(%)				(%)			
HIV-infected	38/90	0/1	21/56	18/32	0.2	3/31	0/2	2/19	1/10	1
	(42.2)	(0)	(37.5)	(56.2)		(9.7%)	(0)	(10.5)	(10.0)	
HIV-infected	205/270	0/1	39/83	167/186	0.0002	9/46 (	-	7/37	2/9	1
	(75.9)	(0)	(47.0)	(89.8)		19.6%)		(18.9)	(22.2)	
HIV-unknown	180/293	0/5	48/134	133/154	0.0001	13/67	0/2	11/58	2/7	0.6
	(61.4)	(0)	(26.1)	(74.8)		(19.4%)	(0)	(19.0)	(28.6)	
Total	423/653	0/7	107/273	318/372	0.0001	25/144	0/4	20/114	5/26	0.7
	(64.8)	(0)	(39.2)	(85.5)		(16.6)	(0)	(17.5)	(19.2)	

TB: tuberculosis; AFB: acid fast bacilli. *TB diagnosis by SM within the group considered; CSTB: clinical suspicion of TB
doi:10.1371/journal.pone.0043739.t009

Table 10. Comparative frequency of positive results of liquid versus solid culture in pulmonary and extrapulmonary active tuberculosis patients according to HIV status.

	Pulmonary TB				Extrapulmonary-TB			
	Liquid culture	Solid culture	Total	*p value of* liquid vs solid cultures*	Liquid culture	Solid culture	Total	*p value of* liquid vs solid cultures*
	Positive over total (%)				Positive over total (%)			
HIV-infected	55/86**	38/87**	56/87**	0.3	14/30	1/33	15/33	0.2
	(64.0)	(43.7)	(64.4)		(46.7)	(30.3)	(45.5)	
HIV-uninfected	218/269**	184/268**	225/270	0.0001	27/46	20/45	29/46	0.2
	(81.0)	(68.7)	(83.3)		(58.7)	(44.4)	(63.0)	
HIV-unknown	238/293	164/293	245/293	0.0001	39/67	27/67	39/67	0.03
	(81.2)	(56.0)	(83.6)		(58.2)	(40.3)	(48.2)	
Total	511/648	386/648	526/650	0.0001	80/143	57/145	83/146	0.004
	(78.9)	(59.6)	(80.9)		(55.9)	(39.3)	(56.8)	

TB: tuberculosis; HIV: Human Immunodeficiency Virus; CI: confidence interval. **p* value of Comparison. **missing recorded culture;
doi:10.1371/journal.pone.0043739.t010

Table 11. Comparative frequency of positive results of liquid versus solid culture in the adult high-risk controls studied.

	High risk controls			
	Liquid culture	Solid culture	Total	*p value of* liquid vs solid cultures
	Positive over total (%)			
No TB patients	7/73* (**9.6**)	4/73* (5.5)	8/73* (11.0)	**0.5**
Health Care Workers	0/2 (0)	0/2 (0)	0/2 (0)	NA
Healthy Family Contacts	1/3* (**33.3**)	0/3 (0)	1/3* (33.3)	NA
Cured TB patients	0/90 (0)	0/90 (0)	0/90 (0)	NA

NA: not available; TB: tuberculosis; HIV: Human Immunodeficiency Virus.
*Cultures were positive with Mycobacteria other than Tuberculosis (MOTT).
doi:10.1371/journal.pone.0043739.t011

2 weeks-, weight loss -more than 10% of the usual weight within the last 3 months-, night sweats, anorexia, fatigue, dyspnea, chest pain and haemoptysis), whether TST was performed or not (if performed, the diameter of induration was recorded), chest X-ray findings, the effect of a 10-day antibiotic trial, the final clinical diagnosis, the CSTB, the final therapeutic intervention with the therapy initiation date and the anti-TB drugs prescribed. The last part of the file consisted of treatment outcome obtained during the 6 to 9 month follow-up of each individual with TB (clinical symptoms relief, chest X-rays and microbiological conversion) and the absence of clinical symptoms in the control groups. Patients with both pulmonary and extrapulmonary localizations (infiltrate and pleural effusion, for instance) were classified as pulmonary TB.

Active TB was defined "microbiologically confirmed" after identification of *M.tuberculosis* in LJ and/or in BacT/ALERT MP culture, molecular tests or histology. Conversely, patients were classified as having "clinical TB" if the diagnosis was based on clinical and radiologic criteria (after excluding other diseases) including appropriate responses to anti-tuberculous therapy.

Statistical Analysis

Median and interquartile (IQR) ranges were calculated. For continuous variables the Mann-Whitney U test or Wilcoxon for pair-wise comparisons were used. For categorical variables Chi square was used. Analysis was carried out with SPSS v 14 for Windows (SPSS Italia SRL, Bologna, Italy) and EPIINFO (CDC, Atlanta, USA).

Results

Characteristics of the Enrolled Population

Two thousand two hundred and thirteen (2213) subjects were enrolled in the 2-year period. Seventy-five adults were not included because their files were incomplete (**Figure 1).** Additionally, 272 children were evaluated separately [20], while 79 adults were included in a satellite study [21–23]. The remaining files correspond to 807 patients with active TB: 131 HIV-infected, 316 HIV-uninfected and 360 with unknown HIV-status. Among the 980 adults without active TB (controls), we identified 6 groups according to their characteristics. The "non active TB group" was necessary to evaluate the specificity and the predictive values of the different tests used in the present manuscript and in those in preparation.

The demographic characteristics of the enrolled population and their risk factors for TB are shown in **Table 1–4.** Among the enrolled 807 active-TB patients, 81% had pulmonary-TB and 19% extrapulmonary-TB. Clinical diagnosis was retained in 24%, mostly in extrapulmonary-TB (43%) patients and in those HIV-infected (43%). Extrapulmonary TB was significantly more frequent in the HIV-infected (29%) patients compared to HIV-uninfected (14.6%; $p = 0.0008$) and HIV-unknown patients (18.6%; $p = 0.018$), with no significant difference between the latter two groups ($p = 0.18$) (**Table 1**). The localization of extrapulmonary TB is reported in **Table 2.** The proportion of patients classified as having microbiologically documented TB and clinical TB according to disease localization and HIV status is shown in **Table 3.** The frequency of clinical TB was significantly higher in extrapulmonary-TB patients than pulmonary-TB patients ($p<0.0001$) and in HIV-infected compared to HIV-uninfected and HIV-unknown pulmonary TB patients ($p<0.0001$). The final diagnosis of the hospitalized non–active TB control group is reported in **Table 4.**

Regarding the symptoms, in pulmonary-TB the prevalence of persisting cough was significantly lower in HIV-infected (73.1%) compared to HIV-uninfected (90.3%) or HIV-unknown status (93.0%) patients ($p = 0.0001$). Regarding the radiological signs the prevalence of infiltrates was significantly lower in HIV-infected (17.7%) compared to HIV-uninfected (53.5%) and HIV-unknown pulmonary-TB patients (61.4%)($p<0.0001$). In extrapulmonary-TB, pleural effusion was observed in half of the patients independent of the HIV-status. Finally, the rate of major clinical symptoms and radiological findings was not significantly different in confirmed TB compared to possible-clinical TB and was independent of their HIV-status.

Microbiological Results

All specimens were tested by SM and solid and liquid cultures; only a portion of them were evaluated by PCR (manuscript in preparation). Disseminated TB was evaluated using liquid culture from blood and performed only in HIV-infected patients.

Smear microscopy (SM). All the enrolled individuals had an SM examination for at least one specimen (if positive): 49% had two specimens and 23% had three, however no records were found in the 3 with pulmonary TB or the 7 with extrapulmonary TB. Twenty four SM-positive patients were culture-negative (3.0%-24/790), with no significant difference between pulmonary (3.1%) and extrapulmonary TB patients (2.8%). About half of them were classified as scanty SM.

The overall sensitivity was significantly higher in pulmonary TB patients than in extrapulmonary TB patients ($p<0.0001$) (**Table 5**). The sensitivity varied according to their HIV-status, being significantly lower in HIV-infected than in HIV-uninfected and HIV-unknown patients. The sensitivity was significantly higher in HIV-uninfected compared to HIV-unknown pulmonary

Table 12. Influence of the physicians' clinical suspicion score of tuberculosis on the frequency of *Mycobacterium tuberculosis* culture -positive results in active pulmonary and extrapulmonary TB patients according to their HIV status.

	Pulmonary TB					Extrapulmonary TB				
	TB diagnosis*	CSTB Low	CSTB High	CSTB Very High	*p* value "TB diagnosis vs very high"	TB diagnosis*	CSTB Low	CSTB High	CSTB Very High	*p* value "TB diagnosis vs very high"
	(%)					(%)				
HIV-infected	56/87*	0/1	36/55	20/31	1	15/33	0/2	7/21	8/10	0.07
	(64.4)	(0)	(65.5)	(64.5)		(45.5)	(0)	(33.3)	(80.0)	
HIV-uninfected	225/270	0/1	41/83	164/186	0.1	29/46	NA	23/37	6/9	1
	(83.3)	(0)	(61.4)	(89.3)		(63.0)		(62.2)	(66.7)	
HIV-unknown	245/293	4/5	96/134	145/154	0.001	39/67	1/2	33/58	5/7	0.6
	(83.6)	(80.0)	(71.6)	(94.2)		(48.2)	(50)	(56.9)	(71.4)	
Total	526/650	4/7	183/272	339/371	0.0001	83/146	1/4	63/116	19/26	0.1
	(80.9)	(57.1)	(67.3)	(91.4)		(56.8)	(25.0)	(54.3)	(73.1)	

NA: not available; AFB: acid fast bacilli; TB: tuberculosis; HIV: Human Immunodeficiency Virus. *TB diagnosis by culture within the group considered. CSTB: clinical suspicion of TB.

doi:10.1371/journal.pone.0043739.t012

TB patients. Similarly, the sensitivity was lower in HIV-infected compared to HIV-uninfected and HIV-unknown extrapulmonary TB patients but the difference was not significant.

No specimens were obtained in the low-risk control groups. Specificity of SM was calculated for each high-risk control group: in the HCW, HFC and cured TB patients it was 100%, whereas in the hospitalized non-active TB patients it was 94.5% (**Table 6**). A significant difference in specificity was found between the hospitalized non-active TB patients and the other groups. In the hospitalized non-active TB patients, AFB-positivity, detected by culture, was associated with the presence of MOTT.

The Positive Predictive value (PPV) and the Negative Predictive value (NPV) of the SM were calculated with the specificity observed in non-active TB patients. In the pulmonary and extrapulmonary TB patients, the PPV was 0.991 and 0.862, the NPV was 0.233 and 0.367 and the likelihood ratio was 11.90 and 3.17, respectively.

SM sensitivity and the degree of infectivity may vary according to the populations of active TB patients enrolled at the different centers: the bacillary yield in sputum being directly related to the degree of infectivity and severity of the pulmonary disease [24]. AFB scores of the pulmonary TB patients enrolled at different centers are shown in **Table 7**. By combining the "++" and "+++" results, a gradient of infectivity was observed among the different centers: it was significantly higher in patients enrolled at Nizam's and TRC, significantly lower at AIIMS and not different at Hinduja, Safdarjung and JALMA compared to the mean infectivity observed in the total enrolled population. AFB scores of the pulmonary TB patients varied also according to their HIV status as shown in **Table 8.** A significant lower infectivity was observed in HIV-infected than in HIV-uninfected TB patients ($p<0.0001$). No significant difference was observed between HIV-uninfected and HIV-unknown TB patients ($p = 0.04$).

The effect of the physician's clinical suspicion of TB (CSTB) on SM sensitivity was evaluated according to TB localization and HIV status (**Table 9**). Compared to the global SM sensitivity observed in all pulmonary TB patients, a significantly higher sensitivity was observed in patients with a "very high" CSTB, ($p<0.0001$). However, the higher sensitivity observed in the HIV-infected patients with a "very high" CSTB was not significant ($p = 0.2$). No significant differences were found among those with extrapulmonary TB. The pre-test classification significantly increased the likelihood ratio in pulmonary TB patients (to 15.6; $p<0.0001$) but not in extrapulmonary TB patients (to 3.51, $p = 0.8$).

Culture of specimens. Since solid medium is recommended for culture in India, it was relevant to compare the performance of a solid and an automated liquid culture system using the same specimen from each enrolled patient. Their respective performances were evaluated using sensitivity, TTD and contamination rate.

Diagnostic Values: Sensitivity, Specificity, NPV and PPV

The overall sensitivity of the liquid culture was significantly higher than the solid culture, both for pulmonary and extrapulmonary specimens, independent of the HIV status (**Table 10**). Among patients with pulmonary TB, the sensitivity of liquid and solid cultures was significantly lower in HIV-infected patients than the other groups ($p<0.05$); no significant difference was found in HIV-uninfected and HIV-unknown patients ($p>0.05$). Among patients with extrapulmonary TB, the sensitivity of the liquid and solid cultures was different only in HIV-unknown patients ($p = 0.03$). The cumulative sensitivity for the 2 culture methods

Table 13. Comparative frequency of Mycobacteria other than Tuberculosis Mycobacteria (MOTTs) isolated in culture-positive specimens of pulmonary and extrapulmonary TB patients according to their HIV status.

	Pulmonary TB		Extrapulmonary TB		Total	
	Positive MOTTs over total culture-positive (%)		Positive MOTTs over total culture-positive (%)		Positive MOTTs over total culture-positive (%)	
		p value for		*p* value for		*p* value for
HIV-infected	7/56 (12.5)	HIV-infected vs HIV-uninfected 0.06	4/15 (26.7)	HIV-infected vs HIV-uninfected 0.1	11/71 (15.5)	HIV-infected vs HIV-uninfected 0.007
HIV-uninfected	11/224* (4.9)	HIV-infected vs HIV-unknown 0.002	2/29 (6.9)	HIV-infected vs HIV-unknown 0.004	13/253 (5.1)	HIV-infected vs HIV-unknown 0.0001
HIV-unknown	5/245 (2.0)	HIV-uninfected vs HIV-unknown 0.1	0/39 (0)	HIV-uninfected vs HIV-unknown 0.1	5/284 (1.8)	HIV-uninfected vs HIV-unknown 0.05
Total	23/525 (5.4)		6/83 (7.2)	Total pulmonary vs extrapulmonary 0.2	29/608 (4.8)	

HIV: Human Immunodeficiency Virus; TB: tuberculosis; MOTTs: Mycobacteria other than Tuberculosis.
*missing recorded identification; TB: tuberculosis.
doi:10.1371/journal.pone.0043739.t013

was higher than that calculated for each methodology, although the difference was not significant ($p = 0.7$).

No specimens were harvested in the control low-risk groups (blood donors and HCA). The overall specificity (cumulative results of liquid and solid cultures) was calculated in each high-risk control group (**Table 11**). In the hospitalized non-TB patients, the specificity was 89% and was significantly lower than in cured TB patients (100%) ($p = 0.01$). Culture-positive results were associated with the isolation of MOTTs in the pulmonary specimens. The specificity in the other groups varied from 100% for the HCW group to 66.7% for the HFC group. The positive results were associated again with isolation of MOTTs in pulmonary specimens from the last group. All of these infected individuals were successfully treated, mostly with Clarithromycin.

The PPV and the NPV of the culture were calculated with the specificity observed in non-active TB patients. Respectively, in the pulmonary and extrapulmonary patients, the PPV was 0.985 and 0.922, the NPV was 0.344 and 0.512 and the likelihood ratio was 7.38 and 5.93.

The effect of the CSTB on the cumulative sensitivity of the culture (solid plus liquid) of the TB patients (**Table 12**) was further analyzed according to TB localization and their HIV status. Compared to the overall sensitivity of the culture observed in all pulmonary TB patients, a significantly higher sensitivity was observed in patients with a "very high" CSTB, ($p<0.0001$).

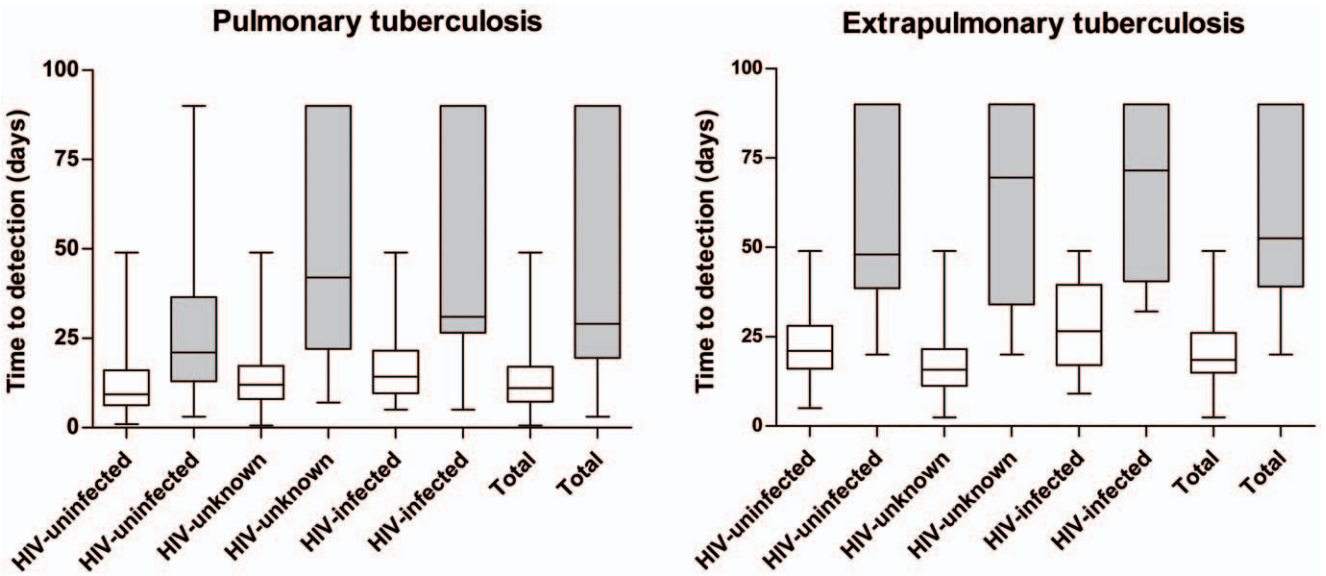

Figure 2. Comparative Time to Detection (TTD) of culture-positive specimens on solid (LJ) medium (grey bar) or with automated liquid (BacT/alert) (white bars) medium in active pulmonary and extrapulmonary TB patients according to their HIV status. TTD in days: median (IQR).
doi:10.1371/journal.pone.0043739.g002

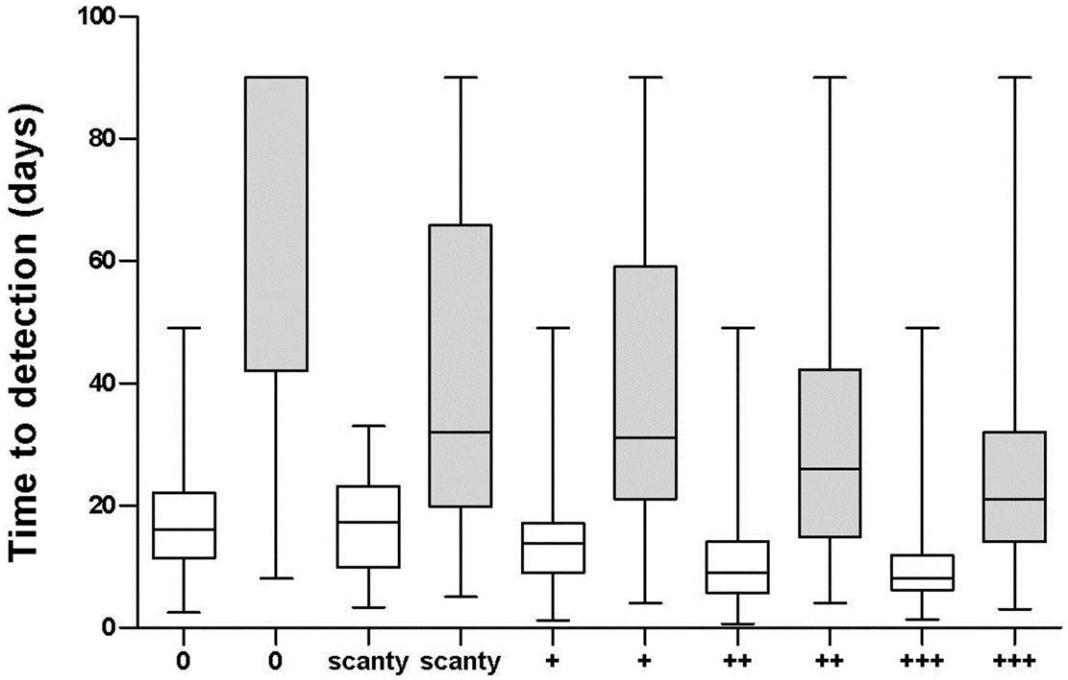

Figure 3. Time to Detection (TTD) of respiratory tract specimens cultured with the automated liquid culture BacT/ALERT MP (white bar) and with the Löwenstein-Jensen medium (grey bar) in relation to AFB scoring of TB patients. TTD in days: median (IQR).
doi:10.1371/journal.pone.0043739.g003

However, the higher sensitivity observed in the HIV-uninfected patients with a "very high" CSTB was not significant ($p = 0.1$). No significant differences were found between HIV-infected patients and all subgroups of those with extrapulmonary TB. The pre-test classification increased the likelihood ratio in both pulmonary and extrapulmonary-TB patients (to 8.34 and 6.67, respectively) but the difference was only significant for pulmonary TB patients ($p<0.0001$).

Species Identification

Identification of *M.tuberculosis* was performed using the Accuprobe assay in all culture-positive specimens. *M.tuberculosis* was found in almost all specimens (577/608, 94.9%) and MOTTs in 29 (4.8%) positive cultures (**Table 13**). The rate of MOTTs differed according to HIV status but not TB localization, which was significantly higher in the HIV-infected group compared to the HIV-uninfected and HIV-unknown groups. All the 29 active TB patients with MOTT isolation were successfully treated with anti-tuberculous therapy.

Time to Detection (TTD)

The TTD was calculated for the positive culture in each group of active-TB patients according to their TB localization and HIV status. The median TTD (in days) with the solid culture (34.0; IQR: 21.0–0.012.0; IQR: 7.8–18.5) was significantly higher than that of the automated liquid culture (12.0; IQR: 7.8–18.5) and was independent of the TB localization and HIV status ($p<0.0001$) (**Figure 2**). The TTD of MOTTs was also significantly shorter with the liquid culture (median and IQR, 12.8 and 8–23 days) than with the solid culture (63.0 and 27–90 days) ($p<0.0001$).

It has been demonstrated that the TTD with the automated liquid culture is inversely proportional to the sputum bacterial yield [25]. We further evaluated the relationship between the AFB score and the TTD of the two culture techniques (**Figure 3**). By regression analysis a highly significant inverse relationship was found between the AFB score and the mean TTD of each culture system (BacT/ALERT MP: $R^2 = 0.924$; LJ: $R2 = 0.885$) ($p<0.0001$) and a significant difference was observed for all the categories of the mycobacterial yield ($p<0.0001$) (**Figure 3**).

Contamination Rate

The overall contamination rate in the TB culture was very low for both the liquid (1.9%) and the solid cultures (1.4%) and the difference between the two systems was not significant ($p = 0.5$). There was no statistical difference between the different groups (**Table 14**). No contamination was observed in the 421 specimens from controls. The 26 patients with contaminated culture were treated successfully with anti-TB drugs.

Blood Culture

Among the HIV-infected TB patients, a high frequency of dissemination was reported, evidenced by multi-organ infection and the presence of *M.tuberculosis* in the peripheral blood [26]. In this study (**Table 15**) the overall sensitivity of the blood culture was low with no statistically significant difference between pulmonary TB and extrapulmonary TB ($p = 0.7$). The TTD of the blood culture was not significantly different in pulmonary-TB compared to extrapulmonary-TB. The median number of circulating CD4+ cells/μL was lower in patients with positive blood culture than in those with a negative blood culture, but the difference was not significant ($p = 0.4$). Among the 11 HIV-infected patients with positive blood cultures, 2 were initially classified as "disseminated TB", 2 as TB meningitis, 1 as pleural effusion, and the remaining 6 as pulmonary TB. Among the 11 HIV-infected patients being positive for *M.tuberculosis* in the blood culture, in 4 cases the positive blood culture was the only microbiological confirmatory test available. The 7 other patients also had culture-positive pulmonary or extrapulmonary specimens.

Table 14. Comparative contamination rate of liquid versus solid culture in pulmonary and extrapulmonary TB patients according to their HIV status.

Group	Pulmonary TB			Extrapulmonary TB			Total	
	Liquid culture	Solid culture	p value of liquid vs solid culture	Liquid culture	Solid culture	p value of liquid vs solid culture	Liquid culture	Solid culture
	Positive over total (%)			Positive over total (%)			Positive over total (%)	
HIV-infected	0/86 (0)	1/86 (1.2)	1	0/30 (0)	0/30 (0)	NA	0/116 (0)	1/116 (0.8)
HIV-uninfected	9/267 (3.4)	3/267 (1.1)	0.1	0/45 (0)	0/45 (0)	NA	9/312 (2.9)	3/312 (0.9)
HIV-unknown	5/293 (1.7)	7/293 (2.4)	0.7	1/67 (1.5)	0/67 (0)	NA	6/360 (1.6)	7/360 (1.8)
Total	14/646 (2.2)	11/646 (1.7)	0.6	1/151 (0.7)	0/151 (0)	NA	15/797 (1.9)	11/797 (1.4)

HIV: Human Immunodeficiency Virus; TB: tuberculosis.
doi:10.1371/journal.pone.0043739.t014

A particular point to be noted was the significantly higher contamination rate of the blood culture compared to those obtained with the liquid culture on other specimens ($p<0.0001$).

Discussion

We present the results of a prospective multicenter study that was designed to investigate the diagnostic values of microbiological tools for active TB in India, a highly endemic country.

Several microbiological tools were systematically applied to each patient with suspected active TB: 2 conventional tools (SM and solid culture) and 2 relatively new tools, the automated liquid culture system (BacT/ALERT MP) and molecular identification on positive culture. Two other new tools were applied on a portion of patients: direct nucleic acid amplification on specimens and a blood culture on HIV-infected patients.

Detection of AFB in specimens has a crucial clinical and epidemiological importance in the diagnostic algorithm for TB in both low-incidence countries [5] and high-burden countries with limited resources [7]. AFB detection in pulmonary secretions identifies patients with the greatest potential for *M.tuberculosis* transmission [27,28]. Sputum SM offers the triple advantage of speed, simplicity and low cost. In many countries with a high TB burden, it is the most appropriate and most accessible diagnostic tool [29].

The sensitivity of SM in our study was within the expected range, detecting about 2/3 of the active pulmonary TB cases in HIV-uninfected and only half of the HIV-infected patients. This confirms data generated in high-burden countries with limited resources [1] and in India where the 0.630 million new cases notified in 2010 were SM-positive pulmonary TB [1,2]. Our study showed slightly higher global results for pulmonary TB with 65.2% SM-positive compared to 51.0% reported in India for the year 2010 [1]. However, the sensitivity of SM varied according to HIV status, TB localization and the site of enrolled patients, which might depend upon the type of population recruited.

As shown here, less than 50% of HIV-infected pulmonary TB patients and around 10% of extrapulmonary TB patients were SM-positive, confirming the concern about its low sensitivity in HIV-infected TB individuals [30,31]. The main issue with HIV-infected TB patients is the inability of the host response to control *M.tuberculosis* replication with low degree of lung lesions and a substantial diagnostic delay that increase of disease severity and a higher death rate [32–35]. Moreover, the magnitude of SM-negative TB in HIV-infected patients is underestimated: many of these patients die before TB is diagnosed, as shown by autopsy studies in Mumbai (India) where TB is the leading cause of death in these patients [36].

Paucibacillary TB is also an important diagnostic issue and we showed that SM detected only 17.3% of extrapulmonary-TB cases, and, it was even less efficient in the HIV-infected cases. This low detection rate might be associated with TB diagnosis delay, poorer treatment outcomes and greater mortality. In our study, the mortality rate was significantly higher ($p = 0.02$) in HIV-infected patients with extrapulmonary TB (13.2%) compared to those with pulmonary TB (2.2%). The CNS-TB in HIV-infected patients was frequently associated with death (80%).

In our study, the SM specificity was very high when evaluated in two high-risk populations: 100% in both the HCW and HFC groups. However, specificity was 94.5% when evaluated in the hospitalized non-active-TB patients; all SM-positive patients had MOTT infection. In India where TB is highly endemic, the frequency of MOTTs should not be ignored and we observed several mixed infections in HIV-infected and HIV-uninfected

Table 15. Performances of the blood culture using the BacT/alert automated culture in the HIV-infected TB patients according to their TB localization.

Tuberculosis patients	Pulmonary TB	Extrapulmonary TB	Total
Positive over total (%)	**7/55 (12.7)**	**4/32 (12.5)**	**11/87 (12.6)**
Median Time of positivity (IQR)(Days)	23.0 (20.0–28.0)	27.0 (19.0–27.0)	23.0 (16.0–27.0)
Median CD4+ cell counts/μL(IQR)			
Negative culture	186 (95–232)	185 (83–250)	188 (79–223
Positive culture	121 (70–199)	154 (97–211)	121 (42–186)
Contamination rate (%)	9/55 (16.4)	7/32 (21.9)	16/87 (18.4)

HIV: Human Immunodeficiency Virus; TB: tuberculosis.
doi:10.1371/journal.pone.0043739.t015

subjects, mostly in those with pulmonary TB. A recent study performed in India has shown such mixed infections (TB plus MOTTs) in TB patients in whom specimens were tested with PCR techniques using specific probes [37].

The pre-test probability using the CSTB score showed a significantly higher sensitivity of SM in groups of patients classified with the highest CSTB score ($p = 0.001$) with a significantly higher likelihood ratio only for pulmonary TB patients.

LJ medium has traditionally been the gold standard for TB diagnosis in India and in other resource-limited settings, although liquid culture is the gold standard of care in industrialized countries [38]. As demonstrated in our study, the BacT/ALERT MP liquid culture system was able to speed up the final diagnosis of both pulmonary and extrapulmonary TB, significantly reducing the TTD compared to the solid LJ culture ($p<0.0001$). Moreover, the sensitivity was found to be significantly higher using the BacT/ALERT MP system compared to the solid culture in pulmonary TB patients, independent of their HIV status ($p<0.0001$). These results confirm the data obtained in several published studies [39–42]. Our study results also showed that using both solid and liquid cultures resulted in incremented sensitivity detecting 83.3% in HIV-uninfected patients and 64.4% in HIV-infected pulmonary TB patients.

In our study, the specificity was 100% in cured TB patients, but was significantly lower (89%) in the hospitalized non-active TB patients (0.01); all patients had MOTT infection.

The pre-test probability using the CSTB score showed a significantly higher sensitivity of the culture in groups of patients classified with the highest CSTB score ($p = 0.001$) with a significantly higher likelihood ratio only for pulmonary TB patients.

The frequency of MOTTs in pulmonary and extrapulmonary TB specimens was higher in HIV-infected than in HIV-uninfected and HIV-unknown TB patients. MOTTs were detected earlier and more frequently using the liquid culture than the solid culture. The presence of MOTTs in these active TB patients might be considered as a bystander of mixed infection or bystander contaminants. Because all these patients were treated successfully with conventional anti-tuberculosis drug regimens for 6 months; the second hypothesis seems to be more likely. As discussed above, MOTTs were isolated in around 10% of hospitalized non-active TB patients. These patients were cured using second-line macrolides. Such a high prevalence of MOTTs in non-active-TB patients has not been previously reported in India and only few reports reported isolation of MOTTs in HIV-infected [43] and HIV-uninfected patients [44]. In our study, MOTTs was identified using the molecular technique (AccuProbe) and no strain speciation was performed. The use of a combination of genus-specific PCR primers might be useful [37].

Automated non-radiometric liquid culture systems are able to continuously monitor the positive results and data suggested that the TTD of *M.tuberculosis* using the MGIT960 system was a viable alternative to colony counting [25]. We established that both the BacT/ALERT MP system and the LJ medium showed an inverse relationship between TTD and semi- quantitative AFB score of sputum: an increase in TTD was correlated with a decreased yield (Figure 3). Thus, besides the already described advantages of the liquid culture system, the TTD might be considered as an additional internal quality control system for the smear microscopy tool.

The use of an automated blood culture system provided a novel opportunity to diagnose mycobacteremia in HIV-infected patients [45]. In a recent Indian prospective study on 52 HIV-infected patients with suspected active TB, mycobacteria were isolated in sputa or fecal samples from 20 patients and in 9 of these patients mycobacteria were also isolated from their blood specimens [46]: the calculated overall sensitivity in that study was 17.1%, not significantly different ($p = 0.6$) from our results (**Table 15**). *M. tuberculosis* bacteraemia was detected in HIV-infected patients when disseminated disease was not suspected, but we also showed that blood culture was not necessary for the final diagnosis of TB in 2/3 of the HIV-infected patients. Similar results were obtained in a South African study, where 22% of 71 HIV-infected TB patients were positive for *M.tuberculosis* on blood culture, but 75% of them had AFB+ pulmonary isolates [47].

One of the issues of the liquid culture system is a higher contamination rate compared to those observed with the solid medium [48]. Our study showed a very low overall contamination rate, both with the MB BacT/Alert (1.9%) and with the LJ medium (1.4%) for both the respiratory and extrapulmonary samples. On the other hand, it was almost 10 times higher with the blood cultures using the MB BacT/Alert. There was no clear explanation apart from possible technical failures during the process of inoculation of the vials.

In conclusion, conventional microbiological tools lead to results similar to those already described in India; special features being that the HIV-infected TB patients were less detected by SM and culture. Also, due to high endemicity of TB in India, the detection of MOTT is of high significance: mixed infections of both TB and MOTT found in this study were found in most of the HIV-infected and HIV- uninfected patients with pulmonary TB. This is also supported by some other recent Indian studies. New microbiological assays like the automated liquid culture system

showed an increase of accuracy and speed of identification without increasing the contamination rate.

Acknowledgments

We thank Andrea Baker for the editing and Dr Fabrizio Palmieri for the critical reading of the manuscript. We thank Dr Elisa Petruccioli for the help in performing the figures.

Author Contributions

Conceived and designed the experiments: PHL DM NKG. Performed the experiments: BSAK A. Sodha BJ VK MK VL DN PS A. Singh AD SVSM CR AR BR. Analyzed the data: DG EG PHL. Contributed reagents/materials/analysis tools: ML GV CL. Wrote the paper: PHL DG. Indian supervisor of the study: ST. Performed all the quality controls of the data: ST. Enrolled patients: AR CR A Sodha RD BJ KK VK VL DN AD SMVS SS. Supervised the experiments: AR CR.

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Is IP-10 an Accurate Marker for Detecting *M. tuberculosis*-Specific Response in HIV-Infected Persons?

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Abstract

Background: The suboptimal sensitivity of Interferon (IFN)-γ-based in-vitro assays, especially in immunocompromised individuals, emphasizes the need for alternative markers for diagnosing tuberculosis (TB). The objective of this study was to evaluate whether interferon-inducible protein (IP)-10, monocyte chemotactic protein (MCP)-2 and interleukin (IL)-2 can be useful biomarkers for evaluating a specific response to RD1 antigens associated to active TB disease in HIV-infected individuals.

Methodology/Principal Findings: The study was carried out in India, the country with the highest TB burden in the world. Sixty-six HIV-infected individuals were prospectively enrolled, 28 with active-pulmonary-TB and 38 without. The whole blood assay based on RD1-selected peptides (experimental test) and QuantiFERON-TB Gold In tube (QFT-IT) was performed. Plasma was harvested at day-1-post-culture and soluble factors were evaluated by ELISA. The results indicate that by detecting IP-10, the sensitivity of the experimental test and QFT-antigen (75% and 85.7% respectively) for active TB was higher compared to the same assays based on IFN-γ (42.9% and 60.7% respectively) and was not influenced by the ability to respond to the mitogen. By detecting IP-10, the specificity of the experimental test and QFT-antigen (57.9% and 13.2% respectively) for active TB was lower than what was reported for the same assays using IFN-γ-detection (78.9% and 68.4% respectively). On the other side, *in vitro* IL-2 and MCP-2 responses were not significantly associated with active TB.

Conclusions: HIV infection does not impair RD1-specific response detected by IP-10, while it significantly decreases IFN-γ-mediated responses. At the moment it is unclear whether higher detection is related to higher sensitivity or lower specificity of the assay. Further studies in high and low TB endemic countries are needed to elucidate this.

Citation: Goletti D, Raja A, Syed Ahamed Kabeer B, Rodrigues C, Sodha A, et al. (2010) Is IP-10 an Accurate Marker for Detecting *M. tuberculosis*-Specific Response in HIV-Infected Persons? PLoS ONE 5(9): e12577. doi:10.1371/journal.pone.0012577

Editor: Madhukar Pai, McGill University, Canada

Received June 28, 2010; **Accepted** August 6, 2010; **Published** September 7, 2010

Funding: The study was supported by grants from Fondation Mérieux and from the Italian Ministry of Health and Ricerca Finalizzata 06.76.1 and 07.103. ML and ST are employees of Biomerieux. Biomeriueux through their employees' involvement established the scientific, laboratory and medical network that allowed the accomplishment of a main project on "tool box for tuberculosis diagnosis" and consequently of the satellite studies. The study described in this manuscript was a satellite study of the main project. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: DG, SC and EG have a European patent N. 1723426 on T-cell assay based on selected RD1 peptides. ML and ST are employees of Biomerieux. This does not alter the authors' adherence to all the PLoS ONE policies on sharing data and materials.

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Introduction

Tuberculosis (TB) is the most frequent opportunistic infection in persons with HIV infection. In 2008, there were an estimated 1.4 million new cases of TB among persons with HIV infection, and TB accounted for 26% of AIDS-related deaths [1]. Innovative diagnostic tools for TB, new and enhanced treatment strategies, and validated markers of treatment efficacy are needed to reduce the burden of TB-HIV epidemic. This needs to be shown as being useful in TB-endemic settings [2].

A recent breakthrough in the diagnosis of *Mycobacterium tuberculosis* infection has been the development of T-cell-based Interferon-γ Release Assays (IGRAs) that use antigens belonging to *Mycobacterium tuberculosis* region of difference (RD1), including early secreted antigenic target-6 (ESAT-6) and culture filtrate protein 10 (CFP-10). Two commercial IGRAs, based on overlapping peptides from CFP-10 and ESAT-6 are now available. Evidence reviewed elsewhere [3,4] suggests that they are more specific, correlate better with *M. tuberculosis* exposure in low incidence settings and are less affected by bacillus Calmette-Guérin (BCG) vaccination than the tuberculin skin test (TST). However, although better than the TST, their accuracy in persons with HIV is still limited, particularly in "mitogen- unresponsive"[5–11] individuals and in those with low $CD4^+$ T-cell counts.

We developed an *in vitro* IFN-γ immune diagnostic assay for active TB, the novelty of which consists of the use of multiepitopic

RD1 peptides that are selected by computational analysis [12,13]. The response to these peptides can be detected in individuals with ongoing *M. tuberculosis* replication (such as during active TB disease and/or recent infection) and decreases during TB therapy [14,15]. Sensitivity of the assay to detect active TB is around 70% and thus needs to be improved [16–18]. Moreover, as for commercial IGRAs, sensitivity can be reduced in HIV-positive patients [5].

It has been recently shown that the accuracy of IGRAs may be enhanced by the addition of other *M. tuberculosis*-specific antigens [19,20] by improving the incubation step [21–23] or by measuring biomarkers other than IFN-γ. In particular, it has been shown that IFN-γ-inducible protein 10 (IP-10), monocytes chemotactic protein 2 (MCP-2) and interleukin (IL)-2 may be additional biomarkers for LTBI detection after RD1-specific stimulation in both adults [24–28] and children [29]. IP-10 is involved in trafficking monocytes and activated Th1 cells to inflamed foci [30]. Serum and pleural fluid IP-10 levels have been evaluated as biomarkers for diagnosis, prognosis, and monitoring of treatment efficacy in inflammatory and infectious diseases including TB [31]. *M. tuberculosis* antigen-dependent IL-2 production has been demonstrated in patients with active TB [25] and its serum concentrations (that are elevated in patients with active TB) return to normal with treatment [32].

The objective of this study was to evaluate whether IP-10, MCP-2 and IL-2 can be useful biomarkers for evaluating a specific response to RD1 antigens associated to active TB disease in HIV-infected individuals. To this end, we enrolled HIV-infected patients in India, the country with the highest TB burden in the world. India accounts for one fifth of the global incidence with an estimated 1.9 million cases annually, 5% of which are HIV-co-infected [33].

Results

Characteristics of the enrolled individuals

We enrolled 66 HIV-infected individuals; 28 of them had active pulmonary TB (19 microbiologically confirmed and 9 clinically diagnosed). All patients with active TB were treated as a single group in the analysis. Among those without active TB, only 3/38 presented symptoms related either to oral candidiasis (2/3) or chronic liver failure with ascitis (1/3) whereas the others (35/38) were free of clinical symptoms. $CD4^+$ T-cell counts and the demographic and clinical features of the enrolled participants are described in **Table 1**.

IP-10 response to RD1 selected peptides was associated with active TB: quantitative results

The median IP-10 response to the mitogen in those with active TB (3,986 pg/ml; IQR: 2517-6097) was not significantly different from what was recorded in individuals without active TB (4,565 pg/ml; IQR: 2368-7201). On the other hand, the median response to ESAT-6 selected peptides in patients with active TB (137.4 pg/ml; IQR: 0-580.7) was significantly different than in those without active TB (6.22 pg/ml; IQR: 0-187) ($p = 0.04$) (**Figure 1**). Similarly, the median response to CFP-10 selected peptides in those with active TB (268 pg/ml; IQR: 0-2055) was significantly different ($p<0.001$) from what was recorded in those without active TB (100 pg/ml; IQR: 0.00-353). The median responses to the QFT-antigen in those with active TB (3579 pg/ml; IQR: 2270-5519) was significantly higher than in those without active TB (2682 pg/ml; IQR: 868-79-5507) ($p = 0.04$) (**Figure 1**). No correlation between the response to IP-10 and CD4+ T-cell counts was found (data not shown).

No significant differences in MCP-2 and IL-2 responses were found between those with active TB and those without for all the stimuli tested (**Figure 2A–B**).

IP-10 response to RD1 selected peptides and QFT-antigen: qualitative data

Based on the significant difference found in the quantitative analysis of the response to IP-10, we performed a receiver-operator characteristic (ROC) analysis for the IP-10 response to

Table 1. Demographic and clinical characteristics of the HIV-infected individuals enrolled in the study.

	No active TB N. 38	Active TB N. 28
Median Age (IQR)	31.5 (27.7–40.0)	35.0 (30.5–38.0)
Female gender (%)	14 (36.8)	4 (14.3)
BMI (IQR)	19.8 (18.3–24.5)	20.0 (16.9–22.7)
Pulmonary TB microbiological diagnosis	–	19
Pulmonary TB clinical diagnosis	–	9
TST median (IQR)	0 (0–0)	5.0 (0–20.0)
TST negative*	30 (78.9)	13 (46.4)
TST positive*	8 (21.1)	12 (42.9)
TST unknown*	0	3 (10.7)
CD4+ T-cell/mm^3 median (IQR) **N. analyzed**	189 (93–455) 28	139 (42–206) 22
CD4+ T-cell/mm^3 (0–100) Number of individuals (%)**	8 (21.1)	9 (32.1)
CD4+ T-cell/mm^3 (101–200) Number of individuals (%)**	7 (18.4)	8 (28.8)
CD4+ T-cell/mm^3 (>200) Number of individuals (%)**	13 (34.2)	5 (17.9)
CD4+ T-cell/mm^3 Unknown	10 (26.3)	6 (21.4)

Footnotes: TB: tuberculosis; IQR: interquartile range; TST: tuberculin skin test; TST*: analyzed in 63/66; $CD4^+$ T-cell counts: analyzed in 50/66 individuals.**

doi:10.1371/journal.pone.0012577.t001

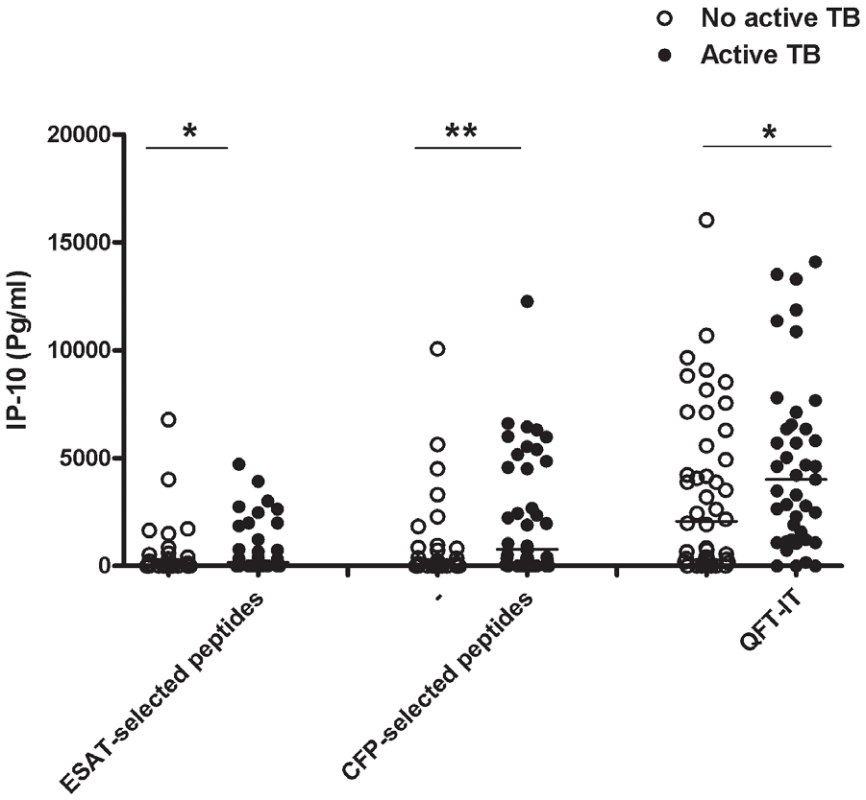

Figure 1. IP-10 production in response to RD1 selected peptides and QFT-antigen in HIV-infected individuals. IP-10 release in response to the RD1 selected peptides and QFT-antigen was evaluated at day 1 in the whole blood of patients with or without active TB. Horizontal lines indicate the median production. The data are presented as pg/mL. P values are reported, *: $p<0.05$; **:$p<0.005$. White circles indicate the individuals without active TB, black circles indicate the patients with active TB. IP-10 release in response to the RD1 selected peptides and to QFT-antigen was significantly higher in patients with active TB compared to those without.
doi:10.1371/journal.pone.0012577.g001

ESAT-6 and CFP-10 selected peptides in order to evaluate its potential use in discriminating the different stages of TB. In this analysis, we used the HIV-uninfected individuals with and without active TB as comparator groups that were enrolled in parallel, as reported in a previous study [34]. We constructed the curve using the highest response value found in response to RD1 selected peptides, either to CFP-10 or the ESAT-6 peptide pool.

Significant AUC analysis results were obtained (AUC, 0.72; 95% CI, 0.61–0.83, $p<0.0003$) for IP-10 response to RD1 selected peptides (**Figure 3A**). For scoring purposes we chose a cut-off point to maximize the sum of sensitivity and specificity. We found that a cut-off point of 350 pg/ml predicted active TB in the HIV-uninfected individuals with 68.29% sensitivity (95% CI, 51.91%–81.91%) and 71.11% specificity (95% CI, 55.69%–83.63%).

Significant AUC analysis results were also obtained (AUC, 0.63; 95% CI, 0.51–0.75, $p<0.02$). for IP-10 response to the TB antigen of the QFT format (**Figure 3B**). For scoring purposes we chose a cut-off point to maximize the sum of sensitivity and specificity. We found that in HIV-uninfected individuals, a cut-off point of 698 pg/ml predicted active TB with 90.24% sensitivity (95% CI, 76.87%–97.28%) and 42.22% specificity (95% CI, 27.66%–57.85%).

By using the cut-off point identified in the ROC analysis, we found that the sensitivity and specificity of the IP-10-response to RD1 selected peptides for active TB among the HIV-infected individuals was 75.0% and 57.9% respectively (Table 2). The proportion of IP-10-positive responses to RD1 selected peptides was significantly higher in patients with active TB (21/28, 75.0%) compared to those without (16/38, 42.1) ($p = 0.01$).

Similarly, by using the cut-off point identified in the ROC analysis, we found that the sensitivity and specificity of the IP-10-response to the TB antigen of the QFT format for active TB among the HIV-infected individuals was 85.7% and 13.2% respectively (Table 2). The proportion of IP-10-positive responses to the TB antigen of the QFT format was not significantly higher in patients with active TB than in those without ($p = 0.06$) (Table 2).

IFN-γ responses to RD1 selected peptides and QFT-antigen: qualitative data

In vitro IFN-γ response to the mitogen was absent in 11/28 (39.2%) of patients with active TB and in 6/38 (15.8%) of individuals without active TB, and this difference was significant ($p = 0.04$). Based on a cut-off point identified in the HIV-uninfected individuals [34], we found that the sensitivity and specificity of the IFN-γ assay based on RD1 selected peptides among the HIV-infected individuals were 42.9% and 78.9% respectively if evaluated on the whole population (Table 2). The sensitivity and the specificity of QFT-IT were 60.7% and 68.4% respectively.

Comparison of IP-10 and IFN-γ responses

There was a significantly higher proportion of positive response to IP-10 (21/28) than to IFN-γ (12/28) after RD1 selected peptides stimulation ($p = 0.03$) among the HIV-infected individuals with active TB. However, no significant difference was found in those without active TB (16/38 vs. 8/38 respectively) ($p = 0.08$)

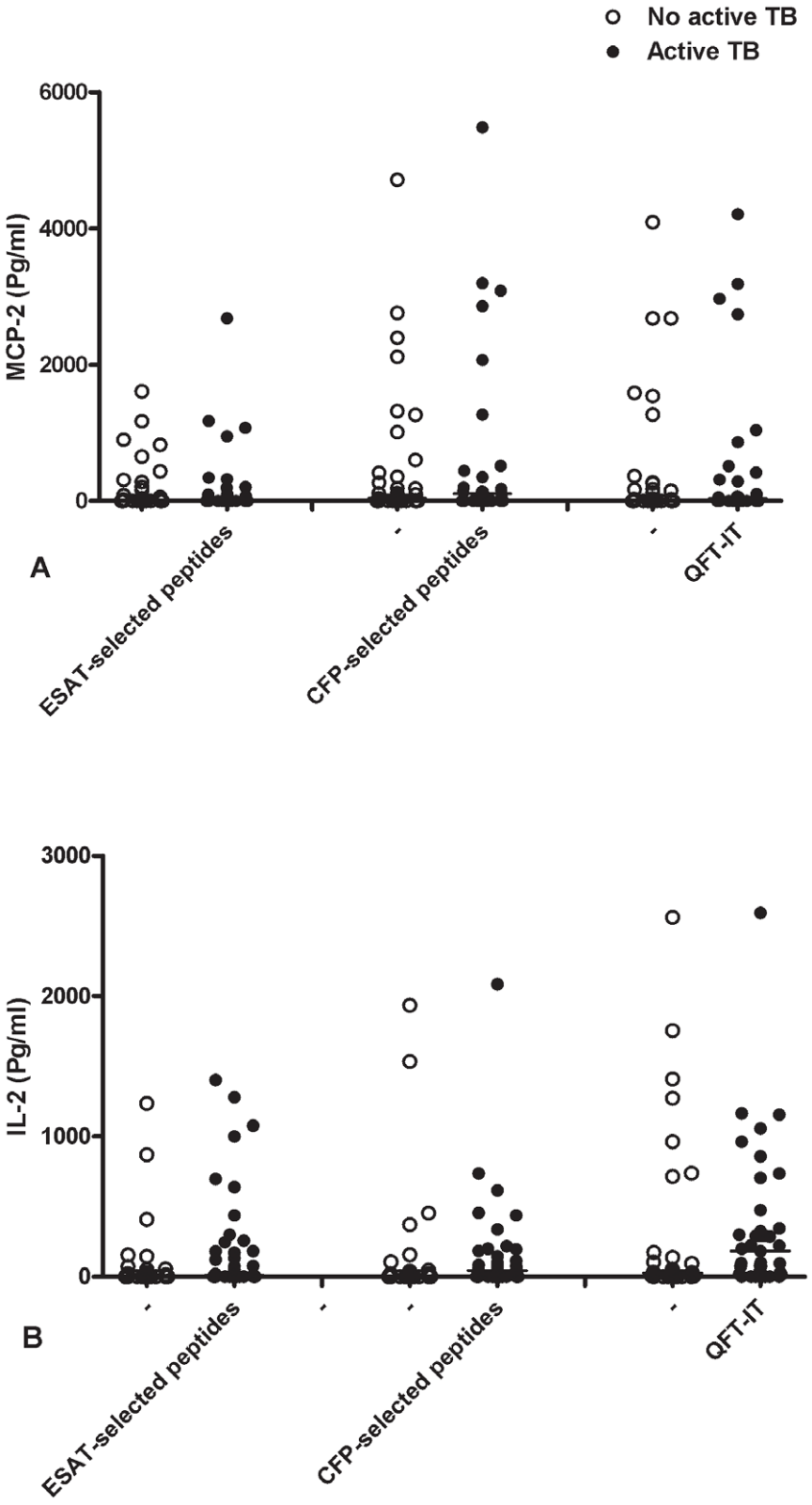

Figure 2. IL-2 and MCP-2 production in response to RD1 selected peptides and QFT-antigen in HIV-infected individuals. IL-2 (**A**) and MCP-2 (**B**) release in response to the RD1 selected peptides and QFT-antigen was evaluated at day 1 in the whole blood of patients with or without active TB. Horizontal lines indicate the median production. White circles indicate the individuals without active TB, black circles indicate the patients with active TB. No significant differences were found among the different comparisons performed.
doi:10.1371/journal.pone.0012577.g002

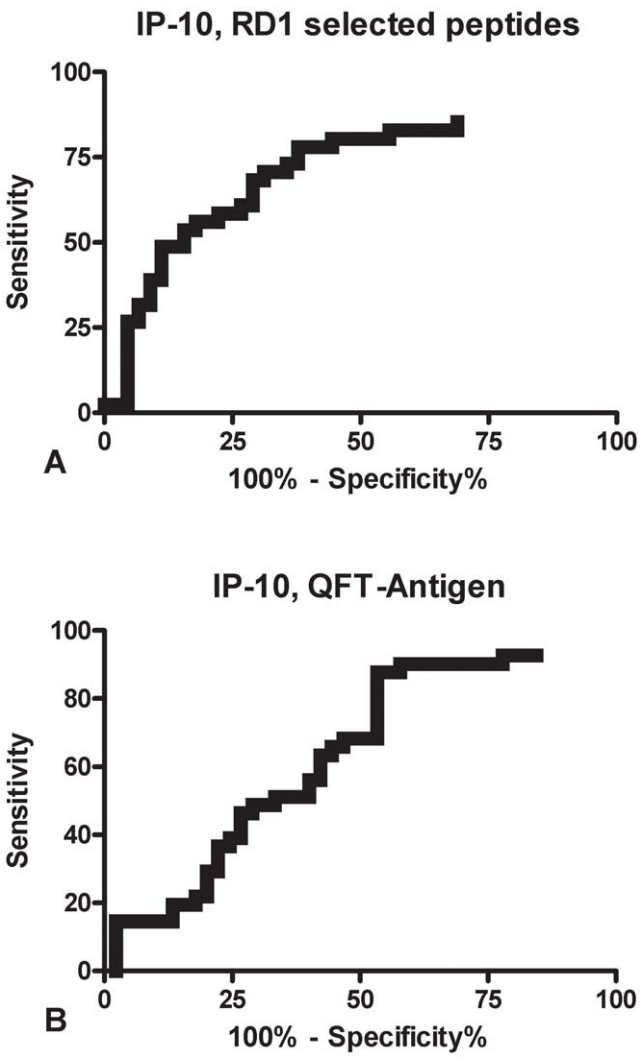

Figure 3. IP-10 release in response to RD1 selected peptides and TB antigen is associated with active TB. An ROC analysis was performed among the HIV-uninfected individuals using the active TB patients and the community controls as comparator groups. **A)** IP-10 release in response to the RD1 selected peptides is significantly associated with active TB. **B)** IP-10 release in response to the QFT-antigen is associated with active TB.
doi:10.1371/journal.pone.0012577.g003

(Table 2). Regarding the responses to the TB antigen of the QFT format, there was a higher proportion of positive response to IP-10 (24/28) than to IFN-γ (17/28) among those with active TB, although it was not significant (p = 0.06). Interestingly, there was a significantly higher proportion of positive response to IP-10 (33/38) than to IFN-γ (12/38) among those without active TB (p = 0.0001).

Impact of the response to the mitogen on the score of the assays based on IFN-γ and IP-10: correlation with the $CD4^+$ T-cell counts

We evaluated the impact of the ability to respond to the mitogen on the scoring of IFN-γ- or IP-10-based response to either the RD1 selected peptides or TB antigen of the QFT format. Among the 11 subjects with active TB classified as "mitogen-unresponsive", only 18.2% (2/11) responded to the assays based on IFN-γ in response to RD1 selected peptides compared to 58.8% (10/17) of the "mitogen-responsive", and this difference was significant (p = 0.02). Similar results were found for QFT-IT, with 36.4% (4/11) of those classified as "mitogen-unresponsive" vs. 76.5% (13/17) of those defined as "mitogen-responsive" and this difference was close to significance (p = 0.052). Differently, regarding the responses to IP-10 among those classified as "mitogen-responsive", 76.5% (13/17) responded to RD1 selected peptides, similar to the 72.7% (8/11) defined as "mitogen-unresponsive" (p = 1). Similar results were found for the response to the TB-Antigen of the QFT-IT format, 76.5% (13/17) of those defined as "mitogen-responsive" vs.100% (11/11) of those defined as "mitogen-unresponsive" (p = 0.13).

Among the patients without active TB, 15.6% (5/32) of the subjects classified as "mitogen-responsive" responded to the assays based on IFN-γ in response to RD1 selected peptides compared to 50.0% (3/6) of the "mitogen-unresponsive" (p = 0.09). Similar data were obtained in response to QFT-IT, 28.1% (9/32) vs. 50% (3/6) (p = 0.35), respectively. Regarding the responses to IP-10 among those defined as "mitogen-responsive", 37.5% (12/32) responded to RD1 selected peptides vs. 66.7% (4/6) of those defined as "mitogen-unresponsive". Similar results were found for the response to the TB-Antigen of the QFT-IT format, 84.4% (27/32) vs. 100% (6/6) (p = 0.57).

Finally, we evaluated whether there was a difference between the $CD4^+$T-cell counts of those defined as "mitogen- responsive" and "mitogen-unresponsive". We found that among all the patients tested, the $CD4^+$T-cell counts were significantly lower in those defined as "mitogen-unresponsive" (median: 41; IQR: 23-

Table 2. Response to the IP-10-based and IFN-γ-based assays in those with or without active TB in HIV-infected individuals.

	Active TB			**No active TB**		
Stimulus	**Marker detected**			**Marker detected**		
	IP-10	**IFN-γ**	***p* value**	**IP-10**	**IFN-γ**	***P* value**
	Positive over total (%)			**Positive over total (%)**		
RD1-selected peptide test						
	21/28 (75.0)	12/28 (42.9)	**0.028**	16/38 (42.1)	8/38 (21.1)	0.08
QFT-IT						
	24/28 (85.7)	17/28 (60.7)	0.06	33/38 (86.8)	12/38 (31.6)	**0.0001**

Footnotes: TB: tuberculosis; QFT-IT: QuantiFERON TB Gold In tube; IP: inducible protein; IFN: interferon; RD: region of difference.
doi:10.1371/journal.pone.0012577.t002

184) than in the "mitogen-responsive" subjects (median: 177; IQR: 95-366) (p = 0.03).

Evaluation of test response in relation to the CD4$^+$ T-cell count

To evaluate the impact of the CD4$^+$ T-cell count on the response to the TB tests in those with HIV infection in detail, we stratified the CD4$^+$ T-cell count into 3 categories: i) between 0–100/μl, ii) 101–200/μl, and iii) above 200/μl (**Table 1**). Among those with active TB, there was a significant difference by χ^2 for the trend among the categories of the CD4$^+$ T-cell counts in the proportion of responders to the IFN-γ based experimental test (p = 0.03), IP-10-based experimental test (p = 0.048) and for the IFN-γ-response to the TB antigen of the QFT-IT. Conversely, no significant difference was found among those without active TB (data not shown) in any of the comparisons performed (**Figure 4**).

Discussion

We are presenting the results of a prospective study conducted in HIV-infected individuals in India (where TB is highly endemic) that was designed to investigate if factors other than IFN-γ, such as IP-10, MCP-2 and IL-2 may improve detection of the response to RD1 antigens for the immunodiagnosis of active TB.

The results of this study demonstrate that besides IFN-γ, the IP-10 response to RD1 selected peptides is associated with active TB in HIV-infected subjects. There was a significantly higher proportion of responders to IP-10 than to IFN-γ. Response to RD1 selected peptides detected by either IP-10 or IFN-γ was dependent on the CD4$^+$ T-cell counts and IFN-γ mitogen response.

Regarding the response to the TB Antigen of the QFT-IT format, we found that the sensitivity of IP-10 for detecting active TB was similar to what was reported for the QFT-IT response (evaluated by definition by IFN-γ).However, differently from QFT-IT, the IP-10-mediated response was not dependent on the CD4$^+$ T-cell counts and IFN-γ mitogen response. Moreover, there was a significantly higher proportion of responders to IP-10 than to QFT-IT in those without active TB, leading to a low specificity for detecting active disease.

Finally, MCP-2 and IL-2 release was not significantly associated with active TB in response either to the RD1 selected peptides or to the TB Antigen of the QFT-IT format.

In this study, we confirmed the data generated in Italy and Africa; that the *in vitro* IFN-γ response to RD1 selected peptides is

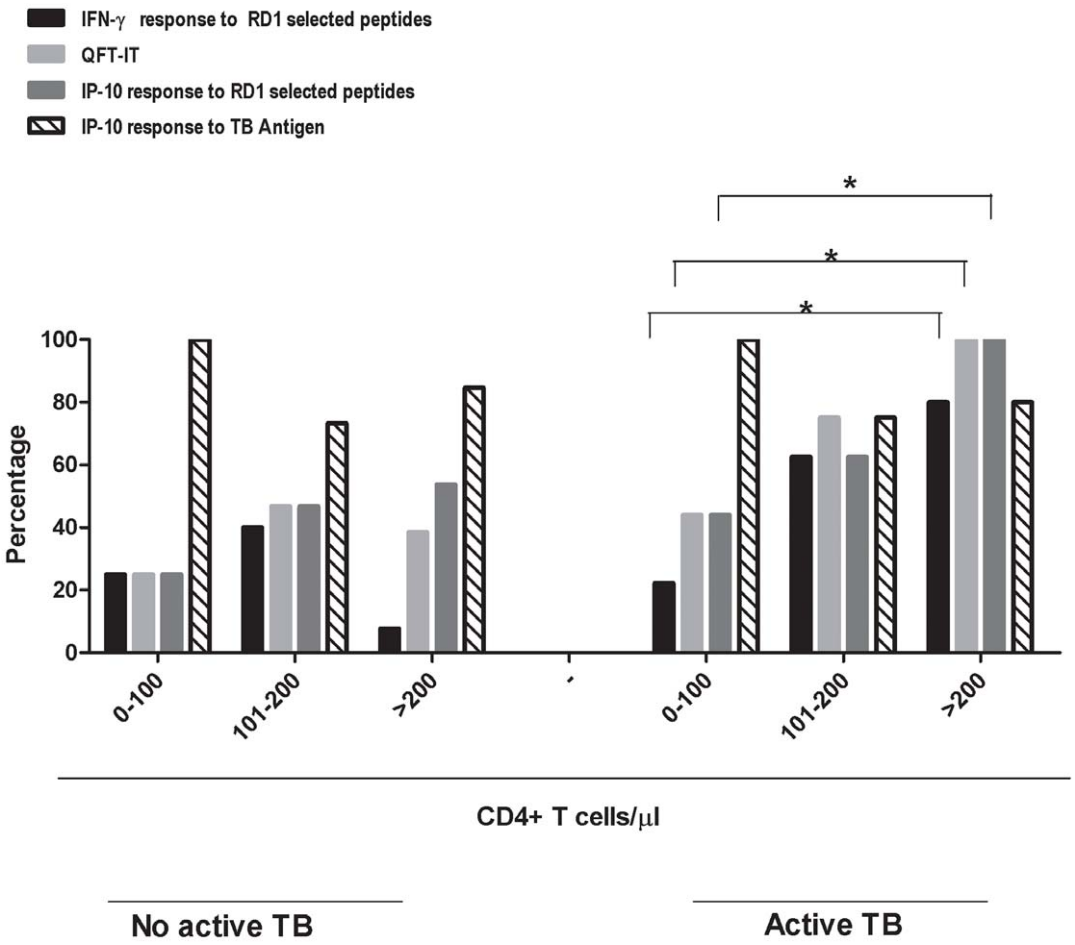

Figure 4. Percentage of positive response to IFN-γ and IP-10-based tests in response to RD1 selected peptides and QFT-antigen in HIV-infected individuals stratified in 3 CD4$^+$ T-cells categories: i) between 0–100/μl, ii) 101–200/μl, and iii) above 200/μl. P values are reported, *: p<0.05. Among those with active TB, there was a significant difference by χ^2 for the trend between the categories of the CD4$^+$ T-cell counts and the proportion of responders to the IFN-γ-based experimental test (p = 0.032), IP-10-based experimental test (p = 0.048) and for the IFN-γ-response to the TB antigen of the QFT-IT.
doi:10.1371/journal.pone.0012577.g004

associated with active HIV-TB and it is more specific than QFT-IT and/or the response to RD1 intact proteins [5,15]. However, here we showed that the accuracy (in terms of sensitivity for active TB detection) of this test is poor, especially in those defined as "mitogen-unresponsive" and in those with low $CD4^+$ T-cell counts. In respect to the previous studies, this diversity may be related to the use of the antiretroviral therapy in the Italian cohort (36% were receiving antiretrovirals) [5] that may have generated a higher immunological ability to respond, and/or to the use of the ELISPOT as a read-out test, which is characterized by a greater response detection performance than the ELISA [12].

The proportion of responders to QFT-IT in HIV-infected individuals is similar to what has already been reported in the literature [6–8,11] and was significantly impaired in those defined as "mitogen-unresponsive" compared to the "mitogen-responsive", as previously shown [reviewed in 11].

IP-10 detection improves the sensitivity for active TB in the IFN-γ-based tests in response to the RD1 selected peptides and to QFT-IT, independent of the ability to respond to the mitogen, as previously shown by others [35]. This higher sensitivity may be due to the fact that IP-10 is mainly secreted by monocytes/macrophages [30] while IFN-γ and IL-2 are secreted mainly by $CD4^+$ T-cells. Hence, IP-10 has the probability of being less affected by HIV infection and less influenced by low $CD4^+$ T-cell counts. However IP-10-based tests, especially those based on QFT Antigen, show a lower specificity for detecting active TB. This result may be due to the fact that IP-10 is detected in contacts of patients with active TB (in both adults [28,34] and children [29],) and its levels in healthy contacts are not significantly different than in those with active TB [34]. These results may be important to underline the high sensitivity of IP-10 in detecting *M. tuberculosis* infection compared to IFN-γ. In fact, although active TB was excluded from the HIV-infected individuals of the study population classified as being without active disease, it was impossible to rule out contact with *M. tuberculosis* in a country like India.

Therefore it is unclear whether the higher proportion of positive IP-10 results is related to a higher sensitivity in detecting "infection per se" or to a lower specificity of the assay.

The specificity of the assay based on RD1 selected peptides is higher when evaluated either by IFN-γ or IP-10 detection, compared to QFT-IT, and this is not unexpected. In fact, QFT-IT uses a greater variety of epitopes to elicit *M. tuberculosis* immune responses by effector memory T-cells and is based on pools of overlapping peptides spanning the whole length of CFP-10 and ESAT-6 proteins and an additional peptide from RD11 [3,4]. Conversely, the selective approach of the design of the test based on RD1 selected peptides reduces the false positive test results among those without active TB, with a loss of diagnostic sensitivity for the detection of active TB [12–18]. Whether it is more acceptable to have false positive test results that may lead to over treatment, or false negative test results that could potentially lead to missing cases with active TB to be treated is a matter of debate and is largely dependent upon the prevalence of *M. tuberculosis* infection and the pre-test probability of TB in a community.

According to the literature generated in HIV-uninfected children [29], in this study on HIV-infected adults we observed that the stimulation index of IP-10 was lower than that of IFN-γ, despite the higher levels of cytokines produced. This was due to the higher background level of IP-10 in unstimulated samples compared to IFN-γ (data not shown).

The rate of indeterminate results in our study is 25.7% (17/66), similar to the rates observed in other studies in which patients with active TB disease [5–8, and reviewed by 11] and those not receiving antiretroviral treatment have similar $CD4^+$ T-cell counts. It is worthwhile to note that IFN-γ response to either RD1 selected peptides or QFT-IT was detected in a small proportion of patients defined as mitogen-unresponsive. This may depend on fact that a better cut-off point for the mitogen should be provided in HIV-uninfected subjects as suggested by Harada [36] and in HIV-infected persons, as suggested by Syed Ahamed Kabeer [35]. The new CDC guidelines for using IGRAs indicate that besides the mitogen score, the QFT-IT is scored positive based on the specific response to the antigen [37]. In addition, the IP-10-based assays are scored positive in the majority of the individuals classified as "mitogen-unresponsive".

It is also important to take into account that we found that the cut-off point for IP-10 in response to the QFT-antigen in this study performed in India is very similar (698 pg/ml) to what was reported by Ruhwald (673 pg/ml) in studies conducted in Europe with another detection readout [27]. Moreover, the sensitivity of the assay for active TB diagnosis in the HIV-uninfected individuals in our study was 90%, close to what was reported by Ruwald [27]. These data are very interesting and may indicate the solidness of the IP-10-based assay worldwide. Differently, the response of MCP-2 to the QFT-antigen did not show any diagnostic value. This data is different than what was previously shown in a European setting [27] and at the moment, the reason is unclear.

Kabeer at al [35] obtained similar results among the HIV-infected individuals, however in that study, the cut-off point was found in a less stringent way than in this study and only few data regarding the $CD4^+$ T-cell counts were available.

The study has some limitations. Subjects were selected according to a certain TB status and comparison between the *in vitro* assays and the $CD4^+$ T-cell counts were made, although $CD4^+$ T-cell counts were not performed on the total population. Nonetheless, although BCG coverage in India is high and we may expect that the majority of the population studied is BCG-vaccinated [38], the BCG status of a large number of the individuals (96.4%) was unknown. However, despite these limitations, the prospective design of the study, the evaluation of 6 *in vitro* assays for TB diagnosis (5 experimental and 1 commercial) and the relatively large number of HIV-infected individuals enrolled to evaluate the performance of the different immune based tests in clinical practice render the results solid.

In conclusion, we show that HIV infection significantly impairs the IFN-γ response to the mitogen and RD1 tests in those with active TB, with little impact on IP-10 responses. At the moment it is unclear whether higher detection of IP-10 is related to higher sensitivity or to lower specificity of the assay. Further studies are needed to elucidate it in both high endemic and low endemic TB countries.

Materials and Methods

Population of patients

Study individuals were prospectively recruited from April, 2007 to March, 2008 in India at: i) Tuberculosis Research Centre, Chetput, Chennai and ii) P.D.Hinduja National Hospital and Research Center, Mumbai. The study was approved by the local ethical committees: the Institutional Ethical Committee of Tuberculosis Research Centre in Chennai (TRC-IEC No: 2006005) and the Institutional Review Board at Hinduja Hospital, Mumbai (No: 316 -05-CR). Informed signed consent was required to participate to the study. Medical information and heparinised blood were obtained from individuals at enrolment after signing an informed consent. For this particular study however, we did not use the results from the Mumbai site because the data were

incomplete in terms of $CD4^+$ T-cell counts and detection of biomarkers different from IFN-γ.

The demographic details and information on previous tuberculin skin test (TST) results were collected. Individuals with a previous history of TB, silicosis, end stage renal disease, leukaemia/lymphoma, who had TST in the past 16 months or who had received anti-TB therapy or immunosuppressive therapy for more than two weeks were excluded from the study. Pregnant and lactating patients were also excluded.

Active TB was defined as microbiologically confirmed if a sputum smear was positive for acid-fast bacilli (AFB) on microscopy by Ziehl-Neelsen method and/or *M. tuberculosis* was identified in sputum culture in conventional Lowenstein Jensen (BioMérieux Inc., Marcy I'Etoile, France) and/or in liquid BacT/ALERT MP medium (BioMérieux Inc., Marcy I'Etoile, France). Conversely, patients were classified as having "clinical TB" if the diagnosis was based on clinical and radiologic criteria (after excluding other diseases) including appropriate response to anti-tuberculosis therapy.

Subjects classified as "no TB" were free of TB symptoms and were checked to have a normal chest X-Ray and to be AFB smear and culture negative. These individuals did not report having had close contacts with patients with active TB in the past and at this time of the study. The presence of HIV infection was evaluated by two ELISA (Retroquic Comb Aids-RS, Span Diagnostics, India and HIV TRI-DOT, J. Mitra & Co, India) in serum.

Tuberculin Skin Test

TST was performed by intradermally injecting 2 TU (tuberculin unit) of purified protein derivative (PPD) RT23 (Staten Serum Institut, Copenhagen, Denmark) by Mantoux method; the induration was measured by trained professionals between 48–72 hrs after PPD injection. The cut-off point for TST positivity was considered as 5 mm for the HIV-infected individuals in accordance with Indian guidelines [39].

RD1 selected peptides and stimuli used for cell cultures

The selection of Human Leukocytes Antigens (HLA)-class II restricted epitopes of ESAT-6 and CFP-10 *M. tuberculosis* proteins was performed by a quantitative implemented HLA peptide-binding motif analysis as previously described for ESAT-6 [12,13]. The whole blood test was carried out as previously described [34]. The two centres were provided with RD1 selected peptides from the same batch, detailed protocol and both received personal training from INMI's laboratory staff. Inter-site communication was present throughout the study to solve any potential problems. Clinicians were blinded to the laboratory test results and the laboratory staff was blinded to the status of the patients. Throughout the test, the assay based on RD1 selected peptides is also called "experimental test".

IP-10, MCP-2, IL-2 and IFN-γ ELISA

The levels of IP-10, MCP-2 and IL-2 were measured in the plasma of whole blood stimulated with or without the mitogen, RD1 selected peptides and QFT-Antigen, using Duoset ELISA Development kits as per the manufacturer's instructions (R&D Systems Inc, MN, USA). To detect the chemokines, plasma was diluted 1:10 as a starting dilution. Further dilutions were performed when necessary. The levels of IFN-γ were evaluated by a commercial ELISA (CMI, Cellestis Limited, Carnegie, Victoria, Australia). In general, the data from stimulated whole blood reported in the text and figures are reported after the subtraction of the relative unstimulated control, which is either the whole blood with the same concentration of Dimethyl Sulfoxide (DMSO) used to dissolve the peptides for the RD1 stimulated conditions [12,13,34] or the "nil" for the QFT antigen.

Commercially available assays

QFT-IT (Cellestis) was performed and the results were scored as indicated by the manufacturers. In particular, the results were scored as indeterminate if the IFN-γ response to the mitogen after subtracting the nil IFN-γ response was <0.5 IU/ml or if the nil IFN-γ response was >8 IU/ml.

Statistical analysis

The median and range of values were calculated. The Mann-Whitney U test was used to compare continuous variables, and Chi square was used for categorical variables. Analysis was carried out with SPSS v 14 for Windows (SPSS Italia SRL, Bologna, Italy). Receiver-operator characteristic analysis was performed using Prism 4 software (GraphPad PRISM, version 4.03, La Jolla, CA, USA).

Author Contributions

Conceived and designed the experiments: DG AR PHL. Performed the experiments: BSAK CR AS. Analyzed the data: DG SC ST EG. Contributed reagents/materials/analysis tools: DG GV CL GI ST ML. Wrote the paper: DG AR BSAK CR EG PHL.

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BMC Infectious Diseases

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IP-10 response to RD1 antigens might be a useful biomarker for monitoring tuberculosis therapy

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Abstract

Background: There is an urgent need of prognosis markers for tuberculosis (TB) to improve treatment strategies. The results of several studies show that the Interferon (IFN)-γ-specific response to the TB antigens of the QuantiFERON TB Gold (QFT-IT antigens) decreases after successful TB therapy. The objective of this study was to evaluate whether there are factors other than IFN-γ [such as IFN-γ inducible protein (IP)-10 which has also been associated with TB] in response to QFT-IT antigens that can be used as biomarkers for monitoring TB treatment.

Methods: In this exploratory study we assessed the changes in IP-10 secretion in response to QFT-IT antigens and RD1 peptides selected by computational analysis in 17 patients with active TB at the time of diagnosis and after 6 months of treatment. The IFN-γ response to QFT-IT antigens and RD1 selected peptides was evaluated as a control. A non-parametric Wilcoxon signed-rank test for paired comparisons was used to compare the continuous variables at the time of diagnosis and at therapy completion. A Chi-square test was used to compare proportions.

Results: We did not observe significant IP-10 changes in whole blood from either NIL or QFT-IT antigen tubes, after 1-day stimulation, between baseline and therapy completion ($p = 0.08$ and $p = 0.7$ respectively). Conversely, the level of IP-10 release to RD1 selected peptides was significantly different ($p = 0.006$). Similar results were obtained when we detected the IFN-γ in response to the QFT-IT antigens ($p = 0.06$) and RD1 selected peptides ($p = 0.0003$). The proportion of the IP-10 responders to the QFT-IT antigens did not significantly change between baseline and therapy completion ($p = 0.6$), whereas it significantly changed in response to RD1 selected peptides ($p = 0.002$). The proportion of IFN-γ responders between baseline and therapy completion was not significant for QFT-IT antigens ($p = 0.2$), whereas it was significant for the RD1 selected peptides ($p = 0.002$), confirming previous observations.

Conclusions: Our preliminary study provides an interesting hypothesis: IP-10 response to RD1 selected peptides (similar to IFN-γ) might be a useful biomarker for monitoring therapy efficacy in patients with active TB. However, further studies in larger cohorts are needed to confirm the consistency of these study results.

Background

The T cell-based assays using region of difference (RD)1 antigens, such as early secreted antigenic target, 6 kDa (ESAT-6), and culture filtrate protein, and 10 kDa (CFP-10), have an evolving niche in detecting *Mycobacterium tuberculosis* infection. The RD1 sequence is missing from *M. bovis* Bacille Calmette-Guerin (BCG), and this omission makes these antigens more specific for *M. tuberculosis* infection diagnosis than the purified protein derivative (PPD) [1-3]. The two RD1 antigens, ESAT-6 and CFP-10, have been shown to induce strong Interferon (IFN)-γ response during short term incubation *in vitro* [3,4]. Based on this principle, there are two commercial kits available for diagnosing tuberculosis (TB) infection, the QuantiFERON-TB GOLD In-Tube® (QFT-IT) (Cellestis Ltd., Carnegie, Australia) and T-SPOT.*TB*® (Oxford Immunotec, Abingdon, UK). The performance of these assays is extensively reviewed [5-8] indicating that they are at least as sensitive as the tuberculin skin test (TST) in detecting latent TB infection (LTBI) and active TB cases.

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Animal and human studies have shown a relationship between the magnitude of IFN-γ responses and mycobacterial bacillary load [9,10]. It has, therefore, been postulated that a decrease in the magnitude of IFN-γ responses to *M. tuberculosis* specific antigens might be used as a biomarker of treatment response [11]. However, studies using serial QuantiFERON-TB Gold tests or IFN-γ ELISPOT assay in adults (performed during treatment of either LTBI [12-17] or active TB disease [9,18-26]) in various settings have shown conflicting results, with IFN-γ responses decreasing [14,15,17-20,22,26], increasing [12,16] or remaining almost unchanged [13,21,23-25] in response to treatment.

We developed an *in vitro* IFN-γ immune diagnostic assay for active TB disease, the novelty of which consists of the use of multiepitopic RD1 peptides selected by computational analysis [27-29]. IFN-γ response to these RD1 selected peptides can be detected in individuals with ongoing *M. tuberculosis* replication (such as during active TB disease and/or recent TB infection) and has been shown to significantly decrease in Human Immunodeficiency Virus (HIV) uninfected [17,18] or infected individuals [30] during TB prophylaxis and therapy.

It has recently been shown (by others and us) that IFN-γ-inducible protein (IP)-10 is a potential diagnostic marker [31-37]. An enzyme linked immunosorbant assay (ELISA), which measures levels of IP-10 in whole blood after overnight stimulation with TB Antigens of the QFT-IT format (hereinafter referred to as "QFT-IT antigens"), has demonstrated to have similar sensitivity for detecting active TB compared to QFT-IT and to the IFN-γ assay based on RD1 selected peptides in HIV-uninfected subjects [32]. Higher sensitivity in diagnosing TB cases for both IP-10-based assays has been described in HIV-infected subjects compared to the corresponding IFN-γ-based tests [34,36,38]. However, to our knowledge, the kinetics of IP-10 secretion in response to QFT-IT antigens before and after treatment has never been investigated.

Azzurri et al [39] have described a decline in the levels of IP-10 in plasma after successful anti-TB treatment. Furthermore, the previous observations have demonstrated that patients with active TB had higher IP-10 levels in the NIL tube (unstimulated whole blood culture tube used in the QFT-IT assay) compared to healthy controls [40].

Thus, in this exploratory study involving 17 enrolled subjects (a subgroup of the 41 HIV-uninfected individuals previously described [33]), we evaluated whether or not IP-10 can be a good biomarker for monitoring TB therapy. Therefore, we assessed the changes of IP-10 levels from the NIL and QFT-IT antigen tubes and RD1 selected peptides stimulated whole blood in patients with active TB disease at the time of TB diagnosis and after successful specific treatment. IFN-γ response to QFT-IT antigens and RD1 selected peptides were evaluated as controls.

Methods

Study subjects

This study has been approved by the Institutional Ethical Committee of the Tuberculosis Research Centre, Chetput, Chennai TRC-IEC (No: 2006005) and written consent was obtained from each study subject. Study subjects were prospectively recruited from the Revised National Tuberculosis Control Program (RNTCP) centers from April, 2007 to March, 2008. Subjects who were diagnosed as pulmonary TB patients at the RNTCP center were assessed for the study. Individuals with a previous history of TB, who had undergone TST in the past 16 months, who had HIV infection, silicosis, end stage renal disease, leukemia/lymphoma or who were undergoing immunosuppressive therapy were excluded from the study.

After registering, the eligible subjects underwent radiological examinations, and three sputum samples were collected from each. The collected sputum samples were processed [41], stained for acid fast bacilli (AFB) microscopy by Ziehl-Neelsen method and cultured in Lowenstein Jensen (BioMérieux Inc., Marcy I'Etoile, France) and in liquid MP BacT medium (BioMérieux Inc). The presence of *M. tuberculosis* in the positive culture samples was further confirmed by Gen-probe based PCR (BioMérieux Inc., Marcy I'Etoile, France) method. Therefore, active TB was defined as microbiologically confirmed if the criteria stated above was fulfilled. Conversely, patients were classified as having "clinical TB" if the diagnosis was based on clinical and radiologic criteria (after excluding other diseases) including appropriate response to anti-TB therapy.

Blood was drawn from all the recruited study subjects for a total blood count, HIV testing and IFN-γ- and IP-10-based assays. All subjects were treated with a standard regimen of rifampicin, isoniazid, ethambutol and pyrazinamide for 2 months and then, if the clinical conditions and chest X-rays improved and AFB sputum conversion occurred, rifampicin and isoniazid were continued for an additional 4 months [42]. At the end of six months, sputum samples and blood samples were collected once again and assessed for their response to treatment and to the in vitro test, respectively.

Stimulation of whole blood with QFT-IT antigens

A commercial QFT-IT assay (Cellestis) was used to evaluate the QFT-IT antigen-specific IFN-γ and IP-10 secretion. Briefly, one ml of blood was taken into each of the three tubes: pre-coated either with QFT-IT

antigens, phytohemaglutinin for the positive control or no antigen for the negative control (NIL). The blood samples were drawn between 10 and 11 am and taken to the laboratory within 2 hours of phlebotomy. The tubes were incubated for 16-24 hours at 37°C and plasma were collected after centrifugation and stored at 4°C until tested.

RD1 selected peptides and whole blood cultures

The selection of Human Leukocyte Antigens (HLA)-class II restricted epitopes of ESAT-6 and CFP-10 *M. tuberculosis* proteins was performed by a quantitative, implemented, HLA peptide-binding motif analysis as previously described for ESAT-6 [27-29]. Peptides were synthesized as free amino acid termini using Fmoc chemistry (ABI, Bergamo, Italy). All synthetic peptides were purified by reverse-phase chromatography to have at least 90% purity. Sequence and purity were confirmed by mass spectrometry and analytical reverse-phase chromatography [27]. Lyophilized peptides were diluted in Dimethyl Sulfoxide (DMSO) at stock concentrations of 10 mg/mL for each peptide and stored at -80°C. RD1 selected peptides were used as follows: a pool of the two ESAT-6 peptides (at 10 μg/mL each) and a pool of the three CFP-10 peptides (at 2 μg/mL each). DMSO was used as a negative control at 10 μg/mL. The whole blood test was carried out as described [33,36]. Briefly, aliquots of 0.5 ml per well of heparinised blood in monoplicate were seeded in a 48-well plate and stimulated with or without RD1 selected peptides, as described above. Samples were then incubated for 16-24 hours at 37°C in the presence of 5% CO_2 when 100 μl of plasma was harvested.

Indian collaborators were provided with RD1 selected peptides from the same batch, detailed protocol and personal training by INMI's laboratory staff. Inter-site communication was present throughout the study to solve any potential problems. Clinicians were blinded to the laboratory test results and laboratory staff was blinded to the status of the patients.

IP-10 assay

The IP-10 levels were measured in the plasma samples using human IP-10 ELISA Set (R&D Sysytems, USA) as per the manufacturer's instructions [33,36]. To detect the chemokines, plasma was diluted 1:10 as a starting dilution. Further dilutions were performed when necessary. The IP-10 data from QFT-IT antigens or RD1 peptides stimulated culture provided in the text and figures are reported after subtracting the respective unstimulated controls, which is either the whole blood culture incubated with the same concentration of DMSO used to dissolve the peptides for the RD1 selected peptides stimulated conditions [33,36] or the NIL tube for the QFT-IT antigens.

Measurement of IFN-γ

The QFT-IT ELISA (Cellestis) was performed to measure the IFN-γ levels in the plasma samples following the manufactures instructions (Cellestis Ltd., Victoria, Australia). The test results were interpreted using software supplied by the manufacturer (Cellestis Ltd., Victoria, Australia). Values above 10 IU/ml were considered as equal to 10 IU/ml, as indicated by the manufacturers. The IFN-γ data from QFT-IT antigens or RD1 peptides stimulated culture provided in the text and figures are reported after subtracting the respective unstimulated controls, which is either the whole blood culture incubated with the same concentration of DMSO used to dissolve the peptides for the RD1 peptides stimulated conditions [33,36] or the NIL tube for the QFT-IT antigens.

Longitudinal analysis of the IP-10 and IFN-γ data

A longitudinal analysis of the IP-10 and IFN-γ data was made considering the highest IP-10 response to either ESAT-6 or CFP-10 selected peptides per single patient at both baseline and end of treatment.

Eligibility criteria for the study

Enrolled patients were defined as "eligible" if the experimental data at both, baseline and after 6 months treatment were available. Data analysis was performed only on the subjects that met the eligibility criteria.

Statistical analysis

The main outcome of the study on IP-10 and IFN-γ production in response to QFT-IT antigens and RD1 selected peptides was expressed as continuous (IU/ml) or dichotomous (positive/negative) measures. For continuous measures, the median and interquartile range (IQR) was calculated. A non-parametric Wilcoxon signed-rank test was used for paired comparisons. Differences were considered significant at p values ≤ 0.05.

For dichotomous measures, chi square was used. For pair-wise comparisons, differences were considered significant at p values ≤ 0.05. SPSS v 14 for Windows (SPSS Italia Srl, Bologna, Italy) and Prism 4 software (GraphPad Software 4.0, San Diego, CA, USA) were used in the analysis.

Results

Characteristics of the subjects included in the study

As previously described [33], a total of 41 HIV-uninfected individuals were assessed for this report. However, only 17 subjects met the eligibility criteria for the

Table 1 Demographic and clinical characteristics of the subjects enrolled in the study

Parameter	Total N. 17
Age, mean in years (IQR)	32 (25-52)
Sex	
Male, Number (%)	9 (52.9)
Smear Positivity, Grade Number (%)	
1+	6 (35.3)
2+	4 (23.5)
3+	2 (11.8)
0	5 (29.4)
Culture Results, Number (%)	
Positive	16 (94.1)
Negative	1 (5.9)
TB Severity, Number (%)	
Mild/Moderate	12 (70.6)
Severe	5 (29.4)

Abbreviations: IQR: interquartile range; TB: tuberculosis; IP: inducible protein; IFN: interferon; RD: region of difference.

study. The median duration of TB treatment was 6 months (IQR: 6.0-6.1).

The median age of the eligible 17 subjects was 32 years and 9 of them were males (Table 1). Regarding the microbiological data, 12 (71%) were positive for AFB sputum microscopy and 16 (94%) were positive to sputum culture. In the remaining 1 subject, diagnosis was made based on clinical criteria.

All 17 subjects completed the anti-tuberculosis therapy by end of six months. They were negative for AFB sputum microscopy after 2 months of treatment and at therapy completion. Radiological examination also confirmed their healthy status.

Longitudinal analysis of IP-10 secretion to NIL and DMSO samples in patients with active TB who were followed until therapy completion

Azzurri et al [39] have described a decline in the IP-10 levels in plasma after successful anti-TB treatment. Furthermore, the previous observations have demonstrated that patients with active TB had higher IP-10 levels in NIL tubes when compared to healthy controls [40]. Assuming that successful treatment reverts the IP-10 levels in the unstimulated cultures, we evaluated the IP-10 level in the plasma from NIL tubes and DMSO whole blood cultures, after 1-day stimulation, in the patients at the time of TB diagnosis (T0) and at therapy completion (T6). As shown in Figure 1, no significant changes were observed when comparing the IP-10 levels in the NIL tubes at T0 (median: 1630; IQR: 212-2330) to T6 (median: 722; IQR: 326-1581) (p = 0. 0833) (Figure 1A) or comparing the IP-10 levels in the DMSO cultures at T0 (median: 1250; IQR: 221-1898) to T6 (median: 830; IQR: 269-1206) (p = 0.1148) (Figure 1B). These data indicate the absence of significant IP-10 changes in the unstimulated culture in this longitudinal analysis. Therefore, we evaluated the changes in QFT-IT and RD1 selected peptides stimulated whole blood.

Longitudinal analysis of IP-10 secretion in response to the QFT-IT antigens in patients with active TB who were followed until therapy completion: comparison with IFN-γ results

The level of IP-10 secretion in response to the QFT-IT antigens did not significantly change from the time of TB diagnosis (T0) (median: 7137 pg/ml; IQR: 2527-9756) to the end of treatment (T6) (median: 6969 pg/ml; IQR: 2299-10148) (p = 0.7) (Figure 2A) Using the cut-off point of 698 pg/ml, previously found by ROC analysis in the same Indian setting [35] (Table 2), 16 out of 17 subjects (94.1%) scored positive for IP-10 at the time of TB diagnosis (Table 3, Figure 2A). At therapy completion, the one subject who scored negative to IP-10 turned positive and three subjects who scored positive at enrolment became negative (Table 3). Hence, the number of IP-10 positive subjects at the end of treatment was (14/17, 82.3%) (Table 3).

IFN-γ secretion was tested as a control. The level of IFN-γ secretion in response to QFT-IT did not significantly change between the baseline (median: 4.38 IU/ml; IQR: 2.58-10.35) and therapy completion (median: 2.66 IU/ml; IQR: 0.82-4.89) (p = 0.7) (Figure 2B). Using the commercial cut-off value of 0.35 IU/ml (Table 2), 17 out of 17 subjects (100%) were positive to QFT-IT. At therapy completion, 3 subjects turned negative. The proportion of positive responders between baseline and therapy completion (14/17, 82.3%) was not statistically significant (p = 0.2) (Table 3).

Longitudinal analysis of IP-10 secretion in response to the RD1 selected peptides in patients with active TB who were followed until therapy completion: comparison with the IFN-γ results

When considering the highest IP-10 response to either ESAT-6 or CFP-10 selected peptides per single patient, the IP-10 secretion was significantly higher at the time of diagnosis (median: 5116 pg/ml; IQR: 2207-7063) than at therapy completion(T6) (median: 73 pg/ml; IQR: 0-5222) (p = 0.0060) (Figure 3A) Using the cut-off point of 350 pg/ml, previously found by ROC analysis [38] (Table 2), 16 out of 17 subjects (94.1%) scored positive for IP-10 at the time of TB diagnosis. At therapy completion, 9 subjects turned negative, whereas the individual who scored negative at enrolment was still negative after therapy completion. Therefore the proportion of positive responders significantly differed between

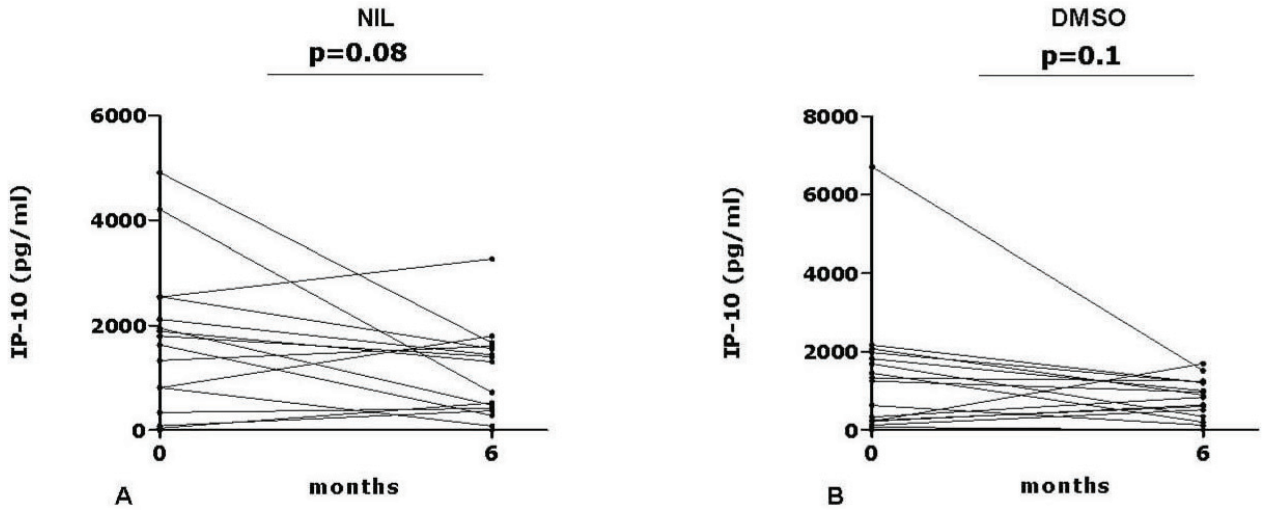

Figure 1 Longitudinal analysis of IP-10 levels in NIL and DMSO conditions after 1-day culture in samples from patients with active TB who were followed until specific therapy completion. IP-10 levels in the NIL (**A**) and DMSO (**B**) conditions after 1-day culture in samples from patients with active TB evaluated before therapy (T0) and at therapy completion (T6). No significant changes were recorded, as indicated by the p values. **Abbreviations**: IP: interferon inducible protein. DMSO: Dimethyl Sulfoxide.

baseline and therapy completion (7/17, 41.1%) (p = 0.002) (Table 3).

IFN-γ secretion was tested as a control. When considering the highest IFN-γ response to either ESAT-6 or CFP-10 selected peptides for single patient, the IFN-γ secretion was significantly higher at the time of diagnosis (median: 2.56 IU/ml; IQR: 1.20-4.18) than at therapy completion (median: 0.42 IU/ml; IQR: 0.02-1.72) (Figure 3B) (p = 0.0003). With the cut-off value of 0.57 IU/ml, previously found by ROC analysis [32] (Table 2), 16 out of 17 subjects (94.1%) scored positive in response to RD1 selected peptides at the time of TB diagnosis. At therapy completion, 9 subjects turned negative, whereas the individual who scored negative at enrolment was still negative after therapy completion. Therefore, the proportion of positive responders significantly differed between baseline and therapy completion (7/17, 41.1%) (p = 0.002) (Table 3).

When we used the same cut-off value used by the QFT-IT (0.35 IU/ml), no change was found in the score of RD1 selected peptides responders at the time of TB diagnosis. At the end of treatment, 7 subjects (instead of 9) turned negative, and the individual who scored negative at enrolment was still negative after therapy

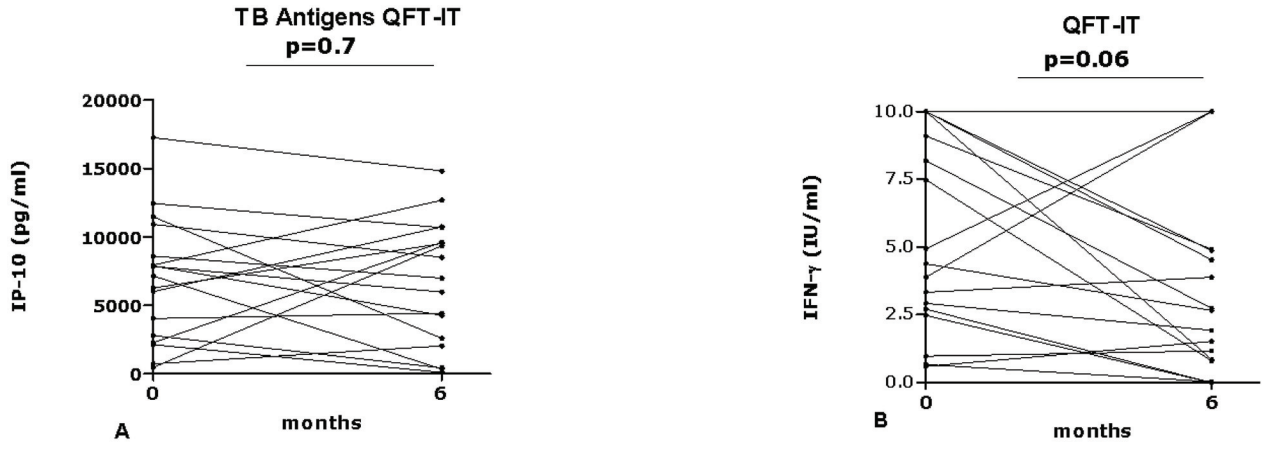

Figure 2 Longitudinal analysis of IP-10 secretion in response to the QFT-IT antigens in patients with active TB who were followed until specific therapy completion: comparison with IFN-γ results. IP-10 response to the QFT-IT antigens (**A**) and IFN-γ response (QFT-IT) (**B**) in patients with active TB evaluated before therapy (T0) and at therapy completion (T6). No significant changes were recorded, as indicated by the p values. **Abbreviations**: IP: interferon inducible protein; IFN: interferon; IU: international units.

Table 2 IP-10 and IFN-γ cut-off points employed for the assays

	IP-10		IFN-γ	
Antigen	**IP-10 (pg/ml)**	**Cut-off point provided by:**	**IFN-γ (IU/ml)**	**Cut-off point provided by:**
QFT-IT antigens*	698	ROC analysis [36]	0.35	[company, 44]
RD1 selected peptides	350	ROC analysis [36]	0.57	ROC analysis [33]

Abbreviations: *antigens used in QuantiFERON TB Gold In tube test format; TB: tuberculosis; IP: inducible protein; IFN: interferon; RD: region of difference; ROC: Receiver Operator Characteristics.

completion. The proportion of positive responders was still significantly different between baseline and therapy completion (9/17, 52.9%) (p = 0.02).

The changes in the secretion of IFN-γ or IP-10 in response to QFT-IT antigens or RD1 selected peptides did not depend on the grade of smear positivity, sputum culture positivity or severity of TB disease based on the chest X-ray results at the time of recruitment (data not shown).

Discussion

In this exploratory study, we demonstrated (for the first time to our knowledge) that the IP-10 secreted response to selected RD1 peptides decreases during specific treatment in patients with active TB. A significant quantitative decrease in the level of IP-10 in response to the RD1 selected peptides was found between the baseline and end of TB treatment accompanied by a significant decrease in the positive rate of the test. Similar results were obtained by the detection of IFN-γ, confirming our previous reports [18,30]. Differently, the IP-10 response to the QFT-IT antigens did not significantly change by either quantitative or qualitative analysis. Interestingly, when considering the IFN-γ data, the quantitative responses to QFT-IT decreased, although not significantly, from baseline to the end of TB treatment whereas no change was found in the proportion of responders.

Previous studies have reported a decline in the levels of IP-10 in plasma after successful anti-TB treatment [39] and other earlier observations have demonstrated that patients with active TB have higher IP-10 levels in the plasma of unstimulated culture when compared to controls [40,43]. However in this study we were unable to confirm these data, probably due to the small number of patients analyzed.

The earlier studies conducted to assess the secretion of IFN-γ in response to QFT-IT ended up with conflicting results among those with active TB. While some of the studies reported significantly reduced IFN-γ secretion at the time of therapy completion compared to the baseline [9,18-20,22,26,30], other reports showed unchanged or minimal IFN-γ secretion upon effective therapy [21,23-25]. The probable reasons for this controversy might be due to several factors including re-infection, persistent infection, persistent exposure to mycobacteria, and possible maintenance of the circulating pool of effector memory T cells, rather than technical factors [7].

IP-10 secretion was elevated in active TB patients after stimulation with *M. tuberculosis* antigens [31-36,40,43,44]. However, similar to IFN-γ, the IP-10 secretion in response to the QFT-IT antigens did not change upon effective therapy. This is not unexpected, as IP-10 secretion is mainly induced by antigen-specific IFN-γ secreting T cells. Furthermore, previously [33,33], we found a good correlation between the level of IFN-γ and IP-10 in subjects with TB infection. Interestingly, in contrast to QFT-IT antigens, we found decreased IFN-γ and IP-10 levels after overnight

Table 3 Serial response to the IP-10-based and IFN-γ-based assays in patients with active TB

	QFT-IT antigens Time points			RD1 selected peptides IFN-γ cut-off 0.57 IU/ml Time points		
	T0	**T6**	***p* value**	**T0**	**T6**	***p* value**
IP-10 Positive over total (%)	16/17 (94.1)	14/17 (82.3)	0.6	16/17 (94.1)	7/17 (41.1)	0.002
IFN-γ Positive over total (%)	17/17 (100)	14/17 (82.3)	0.2	16/17 (94.1)	7/17 (41.1)	0.002
IP-10 N of reversion over total (%)	-	3/17 (17.6)		-	10/17 (58.8)	
IP-10 N of conversion over total (%)	-	1/17 (5.8)		-	0/17 (0)	
IFN-γ N of reversion over total (%)	-	3/17 (17.6)		-	9/17 (52.9)	
IFN- γ N of conversion over total (%)	-	0/17 (0)		-	0/17 (0)	

Footnotes: TB: tuberculosis; QFT-IT: QuantiFERON TB Gold In tube; IP: inducible protein; IFN: interferon; RD: region of difference; N: number; T0: baseline; T6: end of treatment.

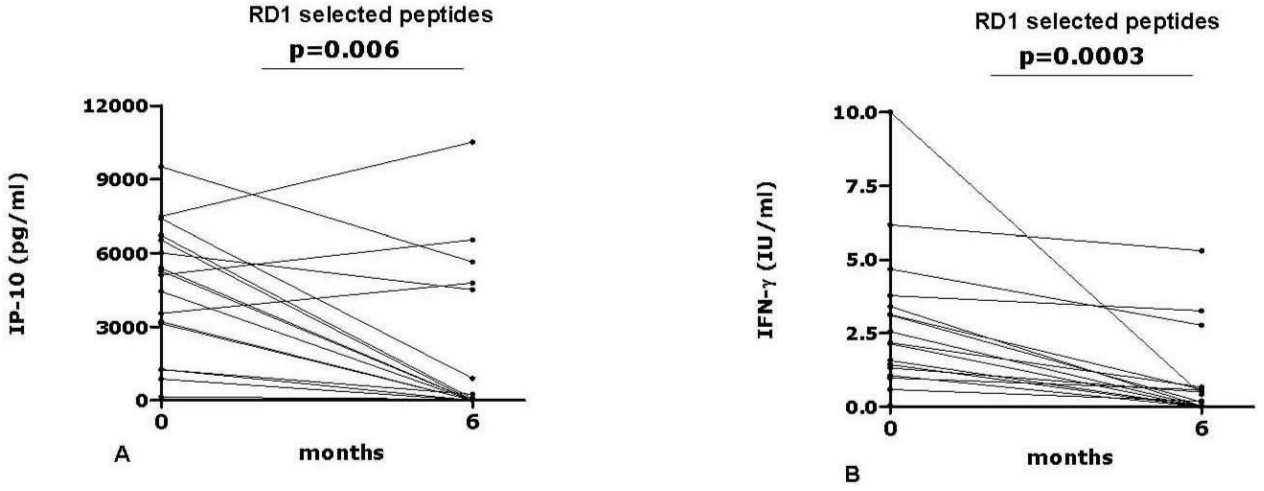

Figure 3 Longitudinal analysis of IP-10 secretion in response to the RD1 selected peptides in patients with active TB who were followed until specific therapy completion: comparison with the IFN-γ results. IP-10 (**A**) and IFN-γ response (**B**) to the RD1 selected peptides in patients with active TB evaluated before therapy (T0) and at therapy completion (T6). Significant changes were recorded, as indicated by the p values. **Abbreviations**: IP: interferon inducible protein; IFN: interferon; IU: international units.

stimulation with selected RD1 peptides after successful therapy.

The difference in the levels of IP-10 and IFN-γ secretion between QFT-IT antigens and selected RD1 peptides might be related to the amount and the composition of epitopes covered by the peptides used in the two different tests. For example, the peptides employed in the QTF-IT cover the whole CFP-10 and ESAT-6 intact proteins (in addition to having a peptide from TB7.7 from the RD11 region) [41] whereas the peptides used in our assay are few and selected in order to be highly immunogenic [27,28]. The response is mediated by the CD4+ T cells with an effector memory phenotype, as previously shown [45]. Based on our data, this oligoclonal response (more than polyclonal against all RD1 epitopes) appears to be a sensitive tool for monitoring *M. tuberculosis* replication [17], as well as active TB disease [18,30].

In the present study we show that both the proportion of IFN-γ and IP-10 positive responders to RD1 selected peptides was significantly lower after successful therapy compared to baseline. It is important to note that the CFP-10 selected peptides induced a stronger and more frequently observed immune response compared to ESAT-6 peptides (data not shown) which emphasizes the need of pooling CFP-10 and ESAT-6 peptides together.

This exploratory study has some limitations. It was conducted on a small number of subjects (17 out of the 41 initially enrolled). The proportion of responders to RD1 selected peptides was higher compared (94%) to previous studies conducted in HIV-uninfected subjects (around 70%) [29,33], and the BCG status of the patients was unknown. BCG coverage in India is high though and we may expect that the majority of the population studied is BCG-vaccinated [46]. Another limitation is related to the cut-off points used to evaluate the response to treatment. These were found by ROC analysis after comparing the results obtained in healthy subjects with patients with active TB before treatment [30,33,36,47]. Consequently they may not be correct when evaluating the response to treatment and greater efforts to find more accurate cut-off points for treatment efficacy should be made. Indeed based on the cut-off used, the assay based on RD1 selected peptides is inferior to the sputum smear as a means to detect failure. However, despite these limitations, the prospective design of the study, the evaluation of 4 *in vitro* assays for TB diagnosis (3 experimental and 1 commercial) and the consistency of the data found between the 2 markers used to evaluate the RD1 responses render the results solid and interesting.

Conclusions

In conclusion, we are showing (for the first time to our knowledge) that IP-10 response to the QFT-IT antigens might be a useful biomarker for monitoring therapy efficacy in patients with active TB. Similar results were obtained in our previous reports using IFN-γ [18,30]. Therefore, there is no real difference between the two biomarkers (IFN-γ and IP-10) other than the magnitude of the response (greater than 20 fold). Additional studies performed on a larger number of individuals in both high and low burden TB settings are needed to evaluate the consistency of these results.

List of Abbreviations

AFB: Acid fast Bacilli; BCG: Bacille Calmette-Guerin; CFP-10: Culture filtrate protein-10; ELISA: Enzyme linked immunosorbant assay; ESAT-6: Early secreted antigenic target-6; HIV: Human immunodeficiency virus; HLA: Human leukocyte antigen; IQR: Interquartile range; IFN-γ: Interferon gamma; IP-10: Interferon gamma inducible protein-10; LTBI: Latent tuberculosis infection; PPD: Purified protein derivative; QFT-IT: QuantiFERON-TB Gold In tube; RD: Region of Difference; RNTCP: Revised national TB control programme; T0: Time of diagnosis; T6: End of treatment; TB: Tuberculosis.

Acknowledgements

The authors wish to thank all the study subjects, clinical nurses, lab technicians, counsellors and physicians who took part in this study. The study was supported by grants from *Fondation Mérieux* and the Italian Ministry of Health "Ricerca Corrente", "Ricerca Finalizzata" 06.76.1, 07.103, RF-IMI-2009-1302952, 40H58. The sponsors had no role in the decision to publish the study, in analysing the data or in drafting the manuscript. We are deeply grateful to Ms. Andrea Baker for the editing.

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Authors' contributions

Conception and design of the experiments: DG and AR. Study subject recruitment: BR. The experiments were performed by: BSAK. Data acquisition: ST, ML. Data analysis: DG, EG, PHL and AR. Contribution of reagents/materials/analysis tools: DG, AR, ST, ML, GI. Writers of the paper: BSAK and DG. Critical revision of the manuscript: ST, ML, BR, GI. Final approval of the version to be published: BSAK, AR, BR, ST, ML, GI, EG, PHL, DG.

Conflict of interests

DG and EG have European patent N. 1723426 on T-cell assay based on selected RD1 peptides.

Received: 8 October 2010 Accepted: 19 May 2011
Published: 19 May 2011

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Pre-publication history
The pre-publication history for this paper can be accessed here:
http://www.biomedcentral.com/1471-2334/11/135/prepub

doi:10.1186/1471-2334-11-135
Cite this article as: Kabeer *et al.*: IP-10 response to RD1 antigens might be a useful biomarker for monitoring tuberculosis therapy. *BMC Infectious Diseases* 2011 **11**:135.