Chapter-2
Review of Literature
2.1 History & discovery of tuberculosis

Tuberculosis has been present in humans since antiquity. The earliest unambiguous detection of "Mycobacterium tuberculosis" is in the remains of bison dated 18,000 years before the present. Whether tuberculosis originated in cattle and then transferred to humans, or diverged from a common ancestor infecting a different species, is currently unclear. However, it is clear that "M. tuberculosis" is not directly descended from "M. bovis", which seems to have evolved relatively recently. Skeletal remains from a Neolithic Settlement in the Eastern Mediterranean show prehistoric humans (7000 BC) had TB, (Hershkovitz et al., 2008) and tubercular decay has been found in the spines of mummies from 3000–2400 BC (Zink et al., 2003).

Over time, the various cultures of the world gave the illness different names: **yaksma** (India), **phthisis** (Greek), **consumptione** (Latin) and **chaky oncay** (Incan), each of which make reference to the "drying" or "consuming" affect of the illness, cachexia. Other classical names included "phthisis pulmonalis"; scrofula (in adults), affecting the lymphatic system and resulting in swollen neck glands; "tabes mesenterica", TB of the abdomen and "lupus vulgaris", TB of the skin; wasting disease; white plague, (because sufferers appear markedly pale); king's evil, because it was believed that a king's touch would heal scrofula; and Pott's disease, or gibbus of the spine and joints. "Miliary tuberculosis"—now commonly known as disseminated TB—occurs when the infection invades the circulatory system, resulting in lesions which have the appearance of millet seeds on X-ray. Before the
Industrial Revolution, tuberculosis was sometimes regarded as vampirism. Exact pathological and anatomical descriptions of the disease began to appear in the seventeenth century. In his Opera Medica of 1679, Sylvius was the first to identify actual tubercles as a consistent and characteristic change in the lungs and other areas of consumptive patients. The bacillus causing tuberculosis; "Mycobacterium tuberculosis", was identified and described on 24th March 1882 by Robert Koch. Tuberculosis is a bacterial disease caused mostly by infection with M. tuberculosis, an organism belonging to MTB complexes (MTBC), which include M. tuberculosis, M. bovis, M. africanum, M. microti, M. canetti.

2.2 Epidemiology of tuberculosis

TB is the second most common cause of death due to an infectious disease.

1) In 2012, there were an estimated 8.6 million incident cases of TB, equivalent to 122 cases per 100,000 population.

2) India and China combined have almost 40% of the world’s TB cases. India is included in the 22 HBCs that account for 81% of all estimated incident cases worldwide. Therefore, of the total incident TB cases about 2.3 million were estimated to have occurred in India.

3) Of the 8.6 million incident TB cases around the globe, 0.5 million were children and 2.9 million occurred among women.

4) 1.1 million cases were among people living with HIV (13%). However, the percentage of HIV infected TB patients is about 6% in India which is quiet low (about half) as compared to global average 13%.
5) There were a total of 1.4 million TB deaths in 2012. These deaths included 0.5 million among women, making TB one of the top killers of women worldwide.

6) The incidence of the disease is higher amongst the working population. Of the notifications of new cases of smear-positive pulmonary TB, 85% were aged 15–64 years and 2% were children (aged <15 years).

7) There were an estimated 5, 30,000 TB cases among children (under 15 years of age) and 74,000 TB deaths (among HIV-negative children) in 2012 (6% and 8% of the global totals, respectively).

8) According to National Tuberculosis Control Programmes (NTPs), 2.6 million new cases of sputum smear-positive pulmonary TB (PTB), 2.0 million new cases of sputum smear-negative PTB and 0.8 million new cases of extrapulmonary tuberculosis (EPTB) were observed in 2010 (WHO, 2011).

9) EPTB has become more common since the advent of human immunodeficiency virus (HIV) infection (Cabantugama et al., 2011; WHO, 2011).

10) EPTB constitutes about 15–20% of TB cases and can constitute up to 50% of TB cases in HIV-infected individuals (Noussair et al., 2009; Peto et al., 2009; Cortez et al., 2011).

11) As India has high burden of TB cases, thus proportionately higher number of EPTB cases are also observed in this country (WHO, 2011).
2.3 Classification of *Mycobacterium tuberculosis*

(A) Phylogenetic classification of *Mycobacterium tuberculosis*:-

<table>
<thead>
<tr>
<th>Kingdom</th>
<th>Bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phylum</td>
<td>Actinobacteria</td>
</tr>
<tr>
<td>Class</td>
<td>Actinobacteria</td>
</tr>
<tr>
<td>Sub class</td>
<td>Actinobacteridae</td>
</tr>
<tr>
<td>Order</td>
<td>Actinomycetales</td>
</tr>
<tr>
<td>Sub order</td>
<td>Corynebacterineae</td>
</tr>
<tr>
<td>Family</td>
<td>Mycobacteriaceae</td>
</tr>
<tr>
<td>Genus</td>
<td>Mycobacterium</td>
</tr>
<tr>
<td>Species</td>
<td><em>M. tuberculosis</em></td>
</tr>
</tbody>
</table>

(B) Classification on the basis of growth rate:-

According to Good fellow *et al.*, 1982, mycobacteria have been classified into two broad categories on the basis of growth rate:

1. **Slow Growers** - Those mycobacteria which do not produce colonies within 7 days of growth e.g.; *M. lepra* (in vivo), *M. tuberculosis*, *M. bovis*, *M. ulcerans* etc.

2. **Rapid Growers** - Those mycobacteria which produce colonies within 7 days. e.g.; *M. fortuitum*, *M. phelei*, *M. chelonae*, *M. smegmatis* etc.

The optimum temperature for growth of both types of mycobacteria ranges between 28°C-45°C.
(C) **Classification on the basis of pigment production:**

According to Runyon 1959, mycobacteria have been classified into 4 groups on the basis of pigment production:

1. **Photochromogens (Runyon Group I)** - These strains form colonies that produce no pigment in dark, but when the young culture is exposed to light for one hour in the presence of air, and re-incubated for 24-28 hours, a yellow orange pigment appears. e.g. *M. kansasii, M. simiae* and *M. marinum*. All species of this group is potentially pathogenic.

2. **Scotochromogens (Runyon Group II)** - These strains form pigmented colonies (yellow-orange-red) even in the dark. If culture is grown in light, the pigment turns orange in colour. e.g.; *M. scrofulaceum* and *M. szulgai*. Species of this group are both potentially pathogenic and common saprophytes.

3. **Non-photochromogens (Runyon group III)** - The non photochromogens are slow growing Non Tubercular Mycobacterium (NTM) whose colonies produce no pigment whether they are grown in the dark or light. Colonies may resemble those of tubercle bacilli. *M. xenopi* and *M. avium* are thermophils, capable of growth at 45°C and causes chronic disease distinguishable from tuberculosis. Though usually classified as non-chromogen, some may form scotochromogenic yellow colonies. e.g.; *M. ulcerans, M. xenopi, M. malmoense, M. terrae, M. haemophilum* and *M. genavense*.

4. **Rapid Growers (Runyon Group IV)** - This is a heterogeneous group of mycobacteria whose colonies appear on solid media within 7 days of incubation at 37°C. e.g.; *M. smegmatis, M. phlei, M. szulgai M. chelonae, M. abscessus, M. fortuitum* and *M. peregrinum*. 
(D) Classification on the basis of pathogenesis:-

They are classified into two types-

1. **Pathogens** – Mycobacterium sps. which cause diseases in humans and animals. According to clinical importance, mycobacteria can be classified into the following three principal groups (Rastogi et al., 2001):
   a) **Strict pathogens**, including the human pathogens *M. tuberculosis* & *M. leprae* and the animal pathogen *M. bovis*.
   b) **Opportunistic pathogens**, including *M. simiae, M. avium* and *M. xenopi*.
   c) **Rare pathogens**, including saprophytes such as *M. smegmatis* and *M. phlei*.

2. **Non-pathogens**- Mycobacterium which never cause diseases in humans and animals e.g.-*M. terrae, M. vacce, M. phlei, M. nonchromogenium* etc.

2.4 General characteristics of mycobacteria

Tuberculosis complex organisms are obligate aerobes growing most successfully in tissues with high oxygen content, such as the lungs. *M. tuberculosis* is a facultative intracellular pathogens usually infecting mononuclear phagocytes (e.g. macrophages), slow-growing with a generation time of 12 to 18 hours. They are hydrophobic with high lipid content in the cell wall. Because the cells are hydrophobic and tend to clump together, they are impermeable to the usual stains, e.g. Gram's stain. They are known as "**acid-fast bacilli**" because of their lipid-rich cell walls, which are relatively impermeable to various basic dyes unless the dyes are combined with phenol. Once stained, the cells resist decolorization with acidified organic solvents and are therefore called "**acid-fast**" (Madigan, 2012).
2.4.1 Cell Wall Structure:-

The mycobacterial cell wall has three unique components (Madigan, 2012):

(A) **Mycolic acids** are unique alpha branched lipids. They are among hydrophobic molecules that form a lipid shell around the organism and affect permeability properties. They prevent attack on mycobacteria by cationic proteins, lysozyme and oxygen radicals in phagocytic granules and render the cell impermeable to stains (acid-fast).

(B) **Cord Factor** is responsible for the serpentine cording. Cord factor is toxic to mammalian cells. Cord factor is made up of trehalose 6, 6 dimycolate (TDM), a glycolipid, which are composed of a non-reducing sugar trehalose linked to mycolic acids.

(C) **Wax-D** in the cell envelope is the major component of Freund's Complete Adjuvant (FCA).

![Cell wall structure of Mycobacterium](www.nature.com)
2.4.2 Virulence Mechanisms and Virulence Factors

*Mycobacterium tuberculosis* does not possess the classic bacterial virulence factors such as toxins, capsules and fimbriae. However, a number of structural and physiological properties of the bacterium have been recognized for their contribution to bacterial virulence and the pathology of tuberculosis (Smith, 2003). The following are some of the virulence mechanisms and factors present in mycobacteria:

1) MTB has special mechanisms for cell entry. The tubercle bacillus can bind directly to mannose receptors on macrophages *via* the cell wall-associated mannosylated glycolipid, LAM, or indirectly *via* certain complement receptors or Fc receptors. MTB can grow intracellularly. This is an effective means of evading the immune system. In particular, antibodies and complement are ineffective. Once MTB is phagocytosed, it can inhibit phagosome-lysosome fusion. The exact mechanism used by MTB to accomplish this is not known but it is thought to be the result of a protein secreted by bacterium that modifies the phagosome membrane. The bacterium may remain in the phagosome or escape from the phagosome, in either case finding a protected environment for growth in the macrophage (Smith, 2003).

2) MTB interferes with the toxic effects of reactive oxygen intermediates produced in the process of phagocytosis by two mechanisms:

(a) Compounds including glycolipids, sulfatides and LAM down regulate the oxidative cytotoxic mechanism.

(b) Macrophage uptake *via* complement receptors may bypass the activation of a respiratory burst.
3) **Antigen 85 complex**: This complex is composed of a group of proteins secreted by MTB that are known to bind fibronectin. These proteins may aid in walling off the bacteria from the immune system and may facilitate tubercle formation.

4) **Slow generation time**: Because of MTB’s slow generation time, the immune system may not readily recognize the bacteria or may not be triggered sufficiently to eliminate them. Many other chronic diseases are caused by bacteria with slow generation times.

5) **The high lipid concentration** in cell wall as mentioned previously, accounts for the impermeability and resistance to antimicrobial agents, resistance to killing by acidic and alkaline compounds in both the intracellular and extracellular environment, and resistance to osmotic lysis *via* complement deposition and attack by lysozyme.

6) **Cord factor**: The cord factor is primarily associated with virulent strains of MTB. It is known to be toxic to mammalian cells and to be an inhibitor of PMN migration. However, its exact role in MTB virulence is unclear.

### 2.4.3 Cultural Characteristics:-

1. Tubercle bacillus is a slow grower with the generation time *in vitro* being 14-15 hours (as compared to 15-20 minutes for *Escherichia coli*) and visible colonies usually appear within 2-8 weeks.

2. Optimum temperature is $37^0$ C and growth does not occur below $25^0$ C or above $40^0$C.

3. Optimum pH is 6.4 -7.0.
4. *M. tuberculosis* it is an obligate aerobe but growth is stimulated by 5-10% CO$_2$.

5. Addition of glycerol (0.5%) improves the growth of human strains.

6. *M. tuberculosis* does not grow in media containing 500mg/L of $p$-nitrobenzoic acid unlike other slow growing non-chromogens.

7. Cultures of tubercle bacilli are sensitive to UV, formaldehyde and gluteraldehyde.

![Fig. 2: MTB Growth on LJ medium](image)

### 2.4.4 Resistance

Mycobacteria are not heat resistant, being killed at 60°C in 15-20 minutes. Cultures may be killed by exposure to direct sunlight for 2 hours, but bacilli in sputum may remain alive for 20-30 hours. Bacilli may remain viable in droplet nuclei for 8-10 days. Culture remains viable for 6-8 months at room temperature and may be stored for 2 years in the deep freeze at -20°C (Madigan, 2012).

### 2.5 Mode of transmission of TB

Patients, suffering from active pulmonary TB are the principal source of TB transmission. Droplet nuclei particles 1 to 5 µm in diameter that contain *M. tuberculosis* complex spread via the air borne route. Droplet nuclei are produced when persons
with pulmonary or laryngeal tuberculosis cough, sneeze, speak, or sing. They also may be produced by aerosol treatments, sputum induction, and aerosolization during bronchoscopy. Droplet nuclei, containing two to three \( M. \text{tuberculosis} \) organisms, are so small that air currents normally present in any indoor space can keep them airborne for long periods of time. Droplet nuclei are small enough to reach the alveoli within the lungs, where the organisms replicate. Of these infected individuals, infection is successfully contained in 90% cases due to an efficient immune response according to (Jensen et al., 2005).

2.6 Pathogenesis of tuberculosis infection

After inhalation, the droplet nucleus is carried down the bronchial tree and implants in a respiratory bronchiole or alveolus. Whether or not an inhaled tubercle bacillus establishes an infection in the lung depends on both the bacterial virulence and the inherent microbicidal ability of the alveolar macrophage that ingests it (Ahamad., 2010). If the bacillus is able to survive initial defenses, it can multiply within the alveolar macrophage. The tubercle bacillus grows slowly, dividing approximately every 25 to 32 h within the macrophage. \( \text{Mycobacterium tuberculosis} \)
has no known endotoxins or exotoxins; therefore, there is no immediate host response to infection. The organisms grow for 2 to 12 wk, until they reach $10^3$ to $10^4$ in number, which is sufficient to elicit a cellular immune response (Glickman & Jacobs, 2001) that can be detected by a reaction to the tuberculin skin test.

### 2.6.1 Clinical manifestations of tuberculosis

The clinical manifestations of tuberculosis are quite variable and depend on a number of factors. Host and microbe-related characteristics as well as their interactions influence the clinical features of the disease. 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 (Yoon et al., 2004). Moreover, extrapulmonary involvement tends to increase in frequency with worsening immune compromise.

(A) **Systemic effects of tuberculosis:**

Tuberculosis involving any site may produce symptoms and findings that are not specifically related to the organ or tissue involved but, rather, is systemic in nature. Of the systemic effects, **fever** is the most easily quantified. The most common hematologic manifestations of tuberculosis are increases in the peripheral blood leukocyte count and anemia, each of which occurs in approximately 10% of patients with apparently localized tuberculosis (ATS, 2000).

(B) **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 the
diagnostic methods. **Hemoptysis** may rarely be a presenting symptom but usually is the result of previous disease and does not necessarily indicate active tuberculosis. Inflammation of the lung parenchyma adjacent to a pleural surface may cause **pleuritic pain**. **Dyspnea** is unusual unless there is extensive disease. Tuberculosis may, however, cause severe respiratory failure (Jeong & Lee, 2008).

(C) **Extrapulmonary Tuberculosis:**

Extrapulmonary 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 (Sharma & Mohan, 2004a). In addition, extrapulmonary 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.

**Extra pulmonary tuberculosis in HIV-infected patients:** Presumably, the basis for the high frequency of extrapulmonary tuberculosis among patients with HIV infection is the failure of the immune response to contain *M. tuberculosis*, thereby enabling hematogenous dissemination and subsequent involvement of single or multiple nonpulmonary sites. Because of the frequency of extrapulmonary tuberculosis among HIV infected patients, diagnostic specimens from any suspected site of disease should be examined for mycobacteria (Mirza *et al.*, 2011). Moreover, cultures of blood and bone marrow may reveal *M. tuberculosis* in patients who do not have an obvious localized site of disease but who are being evaluated because of fever.
Disseminated tuberculosis:- Disseminated tuberculosis occurs because of the inadequacy of host defenses in containing tuberculous infection. This failure of containment may occur in either latent or recently acquired tuberculous infection. Because of HIV or other causes of immune-suppression, the organism proliferates and disseminates throughout the body. Multi-organ involvement is probably much more common than is recognized because, generally, once *M. tuberculosis* is identified in any specimen, other sites are not evaluated. The term “miliary” is derived from the visual similarity of some disseminated lesions to millet seeds. Grossly, these lesions are 1- to 2-mm yellowish nodules that, histologically, are granulomas (Sharma *et al.*, 2012).

1. **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 (Gupta, 2004).

2. **Pleural tuberculosis**: There are two mechanisms by which the pleural space becomes involved in tuberculosis. The difference in pathogenesis results in different clinical presentations approaches to diagnosis, treatment, and sequelae. 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 (Torgersen *et al.*, 2006) 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 are bilateral. In approximately 30% of patients there is no radiographic evidence of involvement of the lung parenchyma; however, parenchymal disease is nearly always present, as evidenced by findings of lung dissections. The second variety of tuberculous involvement of the pleura is **empyema**. This is much less common than tuberculous pleurisy with effusion and results from a large number of organisms spilling into the pleural space, usually from rupture of a cavity or an adjacent parenchymal focus via a bronchopleural fistula (Boloursaz *et al*., 2010).

3. **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. In women genital involvement is more common without renal tuberculosis than in men and may cause pelvic pain, menstrual irregularities, and infertility as presenting complaints. In men a painless or only slightly painful scrotal mass is probably the most common presenting symptom of genital involvement, but symptoms of prostatitis, orchitis, or epididymitis may also occur. Acid-fast bacillus (AFB) smears of the urine should be done, but the yield is low. The suspicion of genitourinary tuberculosis should be heightened by the presence of abnormalities on the chest film. In most series, approximately 40 to 75% of patients with genitourinary tuberculosis have chest radiographic abnormalities; although in many these may be the result of previous, not current, tuberculosis (Abbara & Davidson, 2011).
4. **Skeletal tuberculosis:** The usual presenting symptom of skeletal tuberculosis is pain. Swelling of the involved joint may be noted, as may limitation of motion and, occasionally, sinus tracts. Systemic symptoms of infection are not common. Since the epiphysis region of bones is highly vascularized in infants and young children, bone involvement with tuberculosis is much more common in children than adults. Approximately 1% of young children with tuberculosis disease will develop a bony focus (Teo & Peh, 2004).

5. **Central nervous system 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. The consequences of subarachnoid space contamination can be diffuse meningitis or localized arteritis. In tuberculous meningitis the process is located primarily at the base of the brain. The other major central nervous system form of tuberculosis, the tuberculoma, presents a more subtle clinical picture than tuberculous meningitis (Brienze et al., 2001).

6. **Abdominal tuberculosis:** Tuberculosis can involve any intra abdominal organ as well as the peritoneum, and the clinical manifestations depend on the areas of involvement. In the gut itself tuberculosis may occur in any location from the mouth to the anus, although lesions proximal to the terminal ileum are unusual. The most common sites of involvement are the terminal ileum and cecum, with other portions of the colon and the rectum
involved less frequently. Tuberculous peritonitis frequently causes pain as its presenting manifestation, often accompanied by abdominal swelling (Sharma & Bhatia, 2004).

7. **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 (Mayosi et al., 2005).

2.7 **Diagnosis of tuberculosis**

There are two basic approaches for the diagnosis of tuberculosis. The direct approach includes detection of mycobacteria or its products and the indirect approach includes measurements of humoral and cellular responses of the host against tuberculosis. The diagnostic modalities should have certain desirable features *viz.* sensitivity, specificity, predictive value, speed, reproducibility, cost effectiveness, safety, simplicity, robustness and easy application for wider use. Ideally, the tests should be quantitative, at least in some measure, so that the infectiveness of the individual cases can be measured. This is especially important for decisions to isolate hospitalized patients and to provide preventive therapy to contacts. Diagnostic modalities must also be tailored to needs of the population and epidemiology of TB in that region. Epidemiologically, the countries can be grouped as non-endemic or endemic. The diagnostic algorithms are planned as per specific needs and resources available in individual countries according to (Ramachandran & Paramasivan, 2003).
Accurate and early diagnosis of TB is important for its effective treatment. A complete medical evaluation for TB disease includes the following five components: (ATS, 2000).

2.7.1 Medical history & Physical examination:

Tuberculosis is generally suspected when a pneumonia-like illness has persisted longer than three weeks, or when a respiratory illness in an otherwise healthy individual does not respond to regular antibiotics. The medical history includes obtaining the symptoms of pulmonary TB: productive, prolonged cough of three or more weeks, chest pain, hemoptysis etc. Other parts of the medical history include prior TB exposure, infection or disease; past TB treatment; demographic risk factors for TB; and medical conditions that increase risk for latent TB infection progressing to TB disease, such as HIV infection (ATS, 2000; CDC, 2011).

2.7.2 Radiography

(A) Chest X-ray Radiography

Fig. 4: Tuberculosis creates cavities visible in x-rays like this one in the patient's right upper lobe.
In active pulmonary TB, infiltrates or consolidations and/or cavities are often seen in the upper lungs with or without mediastinal or hilar lymphadenopathy or pleural effusions (tuberculous pleurisy). However, lesions may appear anywhere in the lungs. In disseminated TB a pattern of many tiny nodules throughout the lung fields is common so-called miliary TB. In HIV and other immuno-suppressed persons, any abnormality may indicate TB or the chest X-ray may even appear entirely normal.

Abnormalities on chest radiographs may be suggestive of, but are not necessarily diagnostic of, TB. However, chest radiographs may be used to rule out the possibility of pulmonary TB in a person who has a positive reaction to the tuberculin skin test and no symptoms of the disease (Reuter et al., 2005). Cavitation or consolidation of the apexes of the upper lobes of the lung or the tree-in-bud sign may be visible on an affected patient's chest X-ray. The tree-in-bud sign may appear on the chest CTs of some patients affected by tuberculosis, but it is not specific to tuberculosis.

(B) Abreugraphy

A variant of the chest X-Ray, abreugraphy (from the name of its inventor, Dr. Manuel Dias de Abreu) was a small radiographic image, also called miniature mass radiography (MMR) or miniature chest radiograph. Though its resolution is limited (it doesn't allow the diagnosis of lung cancer, for example) it is sufficiently accurate for diagnosis of tuberculosis (Rodriguez Salvador, 2013).
2.7.3 Immunological diagnosis of TB

Currently, there are two methods available for the detection of *M. tuberculosis* infection (Teixeira *et al.*, 2007).

2.7.3.1 Mantoux test (Tuberculin Skin Test)

Mantoux test is also known as tuberculin skin test, is the preferred type of skin test. In the Mantoux test an intradermal injection of 0.1ml of purified protein derivative (PPD) tuberculin containing 5 tuberculin units is given into the volar surface of the forearm. After 48 to 72 hours the area of induration is measured for hypersensitivity reaction. In general a 15 mm or more induration is considered as positive.

However, in countries with a high coverage of BCG, which also produces tuberculin hypersensitivity, tuberculin test has lost its sensitivity as an indicator of the true prevalence of infection.

2.7.3.2 Interferon-gamma release assays (IGRAs):

Interferon-Gamma Release Assays (IGRAs) are whole-blood tests that can aid in diagnosing *Mycobacterium tuberculosis* infection. They do not help differentiate latent tuberculosis infection (LTBI) from tuberculosis disease. Two IGRAs that are commercially available are:

(A) Quantiferon-Tb Gold Test

The Quantiferon-Tb Gold test (QFT-G) is a whole-blood test for use as an aid in diagnosing *Mycobacterium tuberculosis* infection, including latent tuberculosis infection (LTBI) and tuberculosis (TB) disease. Requires a single patient visit to draw a blood sample and results can be available within 24 hours and is not affected by prior BCG (Bacille Calmette-Guérin) vaccination. But, in this test, blood samples
must be processed within 12 hours after collection while white blood cells are still viable. There are limited data on the use of QFT-G in children younger than 17 years of age, among persons recently exposed to *M. tuberculosis*, and in immunocompromised persons. Errors in collecting or transporting blood specimens or in running and interpreting the assay can decrease the accuracy of QFT-G (CDC, 2007).

(B) **T-Spot.Tb Test**

TB counts the number of anti-mycobacterial effector T cells, white blood cells that produce interferon-gamma, in a sample of blood. This gives an overall measurement of the host immune response against mycobacteria, which can reveal the presence of infection with tuberculosis. Because this does not rely on production of a reliable antibody response or recoverable pathogen, the technique can be used to detect tuberculosis. This technique has the advantage that it is comparatively fast (results within 24 hours), and less influenced by previous BCG vaccination compared with the traditional testing method for latent tuberculosis, the tuberculin skin test. This is due to the fact that both tests use different antigens for stimulation. While the tuberculin skin test uses purified protein derivative, a heterogeneous mixture of more than two hundred different mycobacterial peptides, the T-SPOT. *TB* uses relatively *Mycobacterium tuberculosis*-specific antigens (peptides called ESAT-6 and CFP-10). ESAT-6 and CFP-10 are expressed by *Mycobacterium tuberculosis*, but absent are from all currently used BCG vaccines and most nontuberculous mycobacteria. Based on those test principles, it is thought that the T-SPOT.*TB* is more specific than the tuberculin skin test (Nicol *et al.*, 2009).
2.7.3.3 Serological Diagnosis

To date, serological based tests for TB are not very well developed. Those available have low sensitivity in smear-negative patients, HIV-positive cases and disease endemic countries with high infection rates. They however have a high negative predictive value and are useful as screening tests. But the tests also have difficulty in distinguishing between \textit{M. tuberculosis} and NTM (Ramchandran \textit{et al.}, 2003).

1) **Development of antigen detection assay using sputum samples:** Tests have been developed to detect lipoarabinomannan (LAM) in both pulmonary and extrapulmonary specimens. Preliminary reports from a field trial of a semi-quantitative dipstick method have shown sensitivity and specificity of 93 and 95 percent respectively (Del Prete \textit{et al.}, 1998). Other commonly used antigens detected in body fluids include mycobacterial sonicates, extracted glycolipids, PPD, Ag5 (38kDa), Ag A60, 45/47kDa Ag, Ag Kp90, 30 kDa Ag, P32 Ag and cord factor. Most of the tests, except for Ag5, use polyclonal antibodies and have sensitivity of 40-50 percent and specificity of 80-95 percent. Sandwich ELISA, inhibition ELISA, latex agglutination and reverse passive haemagglutination tests are various methods used for their detection (Arais-Bouda \textit{et al.}, 2000).

2) **Detection of antibodies:** Immune response in mycobacterial disease is associated with HLA class II allotypes. Patients recognize different antigens and thus it is unlikely that all TB patients recognize the same antigen. This is a major obstacle in the development of an antibody-based detection test for mycobacteria. Newer tests are the TB STAT-PAK, enzyme immuno-assay
for detection of anti-mycobacterial superoxide dismutase antibody, and the Insta test TB (Ramchandran et al., 2003)

2.7.4 Microbiological diagnosis of TB

The microbiological diagnosis of tuberculosis (TB) is an important tool for disease control. It consists of both conventional methods (acid-fast microscopy, culture, biochemical identification, anti-tuberculosis drug-susceptibility testing; DST) and modern molecular techniques. The targets of microbiological testing include the detection and isolation of mycobacteria, species identification, detection of drug resistance, monitoring patient responses to therapy and epidemiological typing of Mycobacterium strains.

2.7.4.1 Direct Microscopy

Detection of acid-fast bacilli in stained and acid-washed smears examined microscopically may provide the first bacteriologic evidence of the presence of mycobacteria in a clinical specimen. There are two procedures commonly used for acid-fast staining:

(A) Carbol fuchsin methods which include the Ziehl-Neelsen and Kinyoun methods (Direct microscopy):-

The most important tool in the diagnosis of tuberculosis is direct microscopic examination of appropriately stained sputum specimens for Acid-Fast Bacilli by Ziehl-Neelson staining. The Ziehl-Neelsen staining is also known as the acid-fast staining. It was first described by two German doctors; Franz Ziehl (1859 to 1926), a bacteriologist and Friedrich Neelsen (1854 to 1894), a pathologist. It is a special bacteriological stain used to identify acid-fast organisms, mainly Mycobacteria.
Acid-fastness is a physical property of some bacteria referring to their resistance to decolorisation by acids during staining procedures. It can also be used to stain few other bacteria like Nocardia. The reagents used are Ziehl-Neelsen Carbol fuchsin, acid alcohol and methylene blue. The technique is simple and inexpensive, and detects those cases of tuberculosis, which are infectious. Sputum microscopy is also useful to assess the response to treatment, and to establish cure or failure at the end of treatment. When this method is used, Acid-fast bacilli appear pink in a contrasting (blue) background.

![Mycobacteria seen in ZN smear](image)

Koch (1882), Made a formal announcement of discovery of tubercle bacilli on March 24. He published the method he had used to stain the bacilli on April 10, 1882. He used a mixture of methylene blue and potassium hydroxide for staining the smears after fixing and then counters staining with Vesuvin (Bismarck brown).

The present Ziehl Neelson (ZN) stain has evolved from attempts to improve Koch’s method. The significant contributions to this were from the following scientists - Ehrlich (1882), who improved it by adding a step of decolorisation with mineral acid; He also used a red stain, Fuchsin and changed the mordant to aniline oil.

Ziehl (1882), who changed the mordant to carbolic acid. Rindfleisch (1882), who heated the slide instead of putting it into hot water. Neelson (1883), who combined Ziehl’s mordant with Ehrlich’s red stain.
Out of various methods of staining acid-fast organisms, the Z.N staining technique is the best and is in vogue in most laboratories in the world. It is believed that the success of the method depends partly on the heat employed, which renders the bacilli permeable to aqueous dyes. Various attempts have been made to develop a cold staining method.

Kinyoun (1915) used a higher concentration of basic fuchsin and phenol for cold staining. According to Padmanabha Rao et al., (1966) Cold staining method, no attempt was made to increase the permeability of the surface barrier that is the cell wall of Mycobacteria, however in the conventional Z.N method, this barrier is probably overcome by applying heat while staining.

The smears stained by ZN staining method have to be scanned with an oil emersion objective. This limits the total area of slide that can be viewed in a given unit of time. Acid-fast stains lack sensitivity and a large number of bacilli \(10^4-10^6/\text{ml}\) are required for a positive stain. Therefore, a positive stain from a respiratory specimen is typically thought to correlate with a higher infectivity potential and patients are routinely placed in airborne isolation rooms until their acid-fast smears convert to negative. Immuno-compromised individuals often present with lower bacterial loads making detection by smear difficult (Chegou & Hoek, 2011). Up to 30% of persons (commonly children) are unable to produce sputum for a smear requiring the use of more invasive methods (gastric washing, bronchoalveolar lavage, etc). Acid-fast bacteria seen on smear may represent either \textit{M. tuberculosis} or NTM. Another drawback is that the method does not differentiate between live and dead bacillus (Katoch, 2004).
Smears can be used to follow the response to treatment in smear-positive individuals. A concentration step provides increased sensitivity over direct smear microscopy (Steingart & Henry, 2006).

(B) Fluorochrome procedure using auramine-O or auramine-rhodamine dyes (Fluorescent microscopy):

Fluorescence staining utilizes basically the same approach as Z-N staining, but carbol fuchsin is replaced by a fluorescent dye (auramine-O, rhodamine, auramine-rhodamine, acridine orange etc), the acid for decolorisation is milder and the counter stain, though not essential, is useful to quench background fluorescence. Both sensitivity and specificity of fluorescence microscopy are comparable to the characteristics of the Z-N technique. The most important advantage of the fluorescence technique is that slides can be examined at a lower magnification, thus allowing the examination of a much larger area per unit of time. In fluorescence microscopy, the same area that needs examination for 10 minutes with a light microscope can be examined in 2 minutes. This staining was recently evaluated for assessing bacilli viability in sputum smears. It has been proposed for the early and accurate detection of TB treatment failure in poor-income settings with a high TB burden (Salim et al., 2006).

Modifications of the auramine fluorochrome stain include addition of rhodamine, giving golden appearance to the cells or the use of acridine orange as counter stain resulting in red to orange background. ‘False positives’ may be due to fluorescence of non-specific tissue or cellular debris that can be mistaken for bacilli with 25X objective. High cost of fluorescent microscopy (four to five times more
expensive than light microscopy), the need for a reliable electricity supply and the presence of naturally fluorescent particles in sputum that can be confused with acid-fast bacilli are some of the drawbacks of the method (Getahun et al., 2007).

Fig 6 - Mycobacterium TB visualization using Auramine ‘O’ stain (At 40x).

2.7.4.2 Concentrated smear microscopy

In the setting of human immunodeficiency virus (HIV) infection, the diagnostic sensitivity of direct smear microscopy is further reduced because of a lower bacillary burden in the lungs, a finding confirmed in multiple studies from sub-Saharan Africa (Mugusi et al., 2006). Despite these limitations, smear microscopy is rapid, inexpensive, and highly specific and will likely remain the primary diagnostic test for TB for the foreseeable future. Improved smear microscopy is therefore a primary goal for global TB control. Several methods of smear microscopy that involve sputum liquefaction and concentration have been reported to increase diagnostic sensitivity (Morcello et al., 2008). The \textit{N-acetyl-L-cysteine-NaOH} (NALC) method has been the most widely investigated and has been reported to increase sensitivity modestly compared to direct smear microscopy (Steingart et al., 2006).
2.7.4.3 Culture based diagnostic methods

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

1. Culture is much more sensitive than microscopy, being able to detect as few as 10 bacteria/ml of material (Katoch et al., 2004).
2. Growth of the organisms is necessary for precise species identification.
3. Drug susceptibility testing requires culture of the organisms.
4. Genotyping of cultured organisms may be useful to identify epidemiological links between patients or to detect laboratory cross-contamination. In general, the sensitivity of culture is 80.85% with a specificity of approximately 98% (Baylan, 2005).

Conventional media for cultivation of mycobacteria

Three different types of traditional culture media are available:

(A) **Egg based media**: Lowenstein Jensen, Petragini or American Thoracic Society Medium has been used for primary isolation of *M. tuberculosis* from clinical samples and has been observed to be more sensitive than the agar based media. The Lowenstein-Jensen media is widely employed for routine culture as recommended by the International Union against Tuberculosis and Lung Disease (IUATLD). This consists of coagulated hen’s egg, mineral salt solution, asparagine and malachite green, the last acting as a selective agent inhibiting other bacteria.
Fig.7: Colonies of *Mycobacteria tuberculosis* on LJ media

B&C **Agar based and liquid media**: Middle brook media (7H10, 7H11) is a common agar based media, Dubo’s, Kirscheners media and Middlebrook’s (7H9) are liquid media. They used for isolation of Mycobacteria. The agar based media is transparent and permits early microscopic detection of micro colonies (including the rapid slide culture technique).

**Commercially available culture media**

(A) **Manual systems**

1. **MB redox**: This system is based on the reduction of a tetrazolium salt indicator in liquid medium (Heifets *et al.*, 2000). MB Redox® allows an easy macroscopic visualisation of the bacterial growth. The tetrazolium salt indicator forms red to violet particles when reduced by the growth of the mycobacteria.

2. **Versa Trek system (previously called ESP culture system II)** This technique for diagnosis and DST for first-line drugs is a fully automated, nonradioactive system, providing continuous monitoring of growth of mycobacteria based on the detection of gases released by the bacteria (Ruiz *et al.*, 2000).
3. **MB/Bach Alert (Organon Technika):** This method was developed by Organon Technika and is based on colorimetric detection of bacterial growth in cultures. The method is also useful for drug susceptibility testing (Brunello & Fontana, 2000).

4. **TK Medium:** Developed by Salubris, Inc., MA, USA is a novel colorimetric system that indicates growth of mycobacteria by changing its color. The method also discriminates between mycobacteria and contamination, and enables drug susceptibility testing. The test has low cost and is simple. Sensitivity of TK medium is comparable to the LJ-medium (Kocagoz et al., 2012).

5. **Septi-Chek (Roche):** This is a Bi-phase system developed by Roche. It consists of enriched selective broth and a slide having non-selective Middlebrook agar on one side and two sections on other side: one with NAP + egg-containing agar, and second with chocolate agar for detection of contaminating microbes (Katoch, 2003).

6. **Reporter phages (Biotec/Medispan):** The method is based on use of mycobacterium-specific phage(s) and a reporter gene (luciferase) for detection of growth and drug-susceptibility to anti-TB drugs. Viability detection is by either emission of light from microbe due to activation of luciferase gene or production of plaque on an indicator strain of mycobacteria. Results are available in 2 days (Krishnamurthy et al., 2002).

7. **E-test:** This method is based on the use of gradient of drug on a paper-strip; useful for drug susceptibility testing of *M. tuberculosis* (Kakkar et al., 2000).
(B) Semi automated radiometric system

1) BACTEC460 system (Becton Dickinson): Generation and detection of radioactive CO$_2$ from substrate palmitic acid. Used world-over, detection of growth in 5-7 days. Inclusion of (NAP: beta nitro alpha acetylamine beta hydroxy propiophenone) distinguishes *M. tuberculosis* [inhibition] from other mycobacteria (Rodriguez *et al.*, 2007).

2) MGIT 960 (Mycobacteria Growth Indicator Tube): Developed by Becton Dickinson, growth detection by non-radioactive fluorochrome detection; useful in drug screening, early detection of mycobacterium growth in 7-12 days (Bemer *et al.*, 2002).

3) Mycobacterial blood culture methods in common use include visual inspection of processed blood inoculated on a solid medium (e.g., the Isolator 10 system), Intermittent radiometric detection in liquid medium inoculated with blood (e.g., the BACTEC 13A system) and continuous detection in liquid medium inoculated with blood (e.g., the BacT/Alert MB system or the Bactec Myco/F Lytic system). Bactec Myco/F Lytic system). Becton Dickinson (Sparks, Md.) has developed such a medium, Myco/F Lytic medium (MFL). This medium was developed for use on the BACTEC 9000 series of instruments specifically for the recovery of fungi and mycobacteria; however, the growth of bacteria is also detected (Crump *et al.*, 2011).
(c) **Fully automated nonradiometric system:-**

1) **The BACTEC MGIT 960 System** (Becton Dickinson (BD). The BACTEC 960 instrument is an automated system that exploits the fluorescence of an oxygen sensor to detect growth of mycobacteria in culture. Analysis of the fluorescence is used to determine if the tube is instrument positive; i.e., the test sample contains viable organisms (Siddiqi & Rüsch-Gerdes., 2006).

### 2.7.4.4 Molecular (genotypic) diagnostic methods

Several molecular techniques have been developed for direct detection of mycobacteria from clinical samples. These nucleic acid amplification (NAA) techniques, such as PCR, have been extensively evaluated for the rapid diagnosis of TB. Molecular techniques have been developed to overcome the limitations associated with the traditional laboratory diagnostics (Neonakis *et al.*, 2008). PCR has evolved from end-point reaction to real-time PCR detection where detection is done while the reaction is ongoing. NAAT uses different enzymes and strategies, such as amplification of different targets within DNA or RNA. Most NAAT-based tests involve three steps: DNA/RNA isolation, amplification and detection.

Since the first publication of the polymerase chain reaction (PCR) amplification technique in 1985 (Saiki *et al.*, 1985) considerable progress has been made in pathogen detection and identification. Since then, nucleic acid amplification techniques have been refined and different approaches have been deployed in order to make it simple, cost effective and applicable for clinical use. PCR in microbial detection has been used in diagnostic laboratories in most of the cases as an “in-house” method. Assays are referred to as in-house” if they are based on a protocol
developed in a non-commercial laboratory. In addition many of the PCR based technologies have already been developed into commercially available diagnostic kits and assays for use in clinical microbiology.

(i) **In house PCR assays**

Several *in house* methods are available for rapid diagnosis of TB from clinical specimens. The insertion element **IS6110 and the 16S rDNA** are the most common targets used (Thierry *et al*., 1990a; Tortoli, 2003). Other regions used for amplification include the rpoB gene encoding the β-subunit of the RNA polymerase (Adekambi *et al*., 2003), the gene coding for the 32 kD protein (Del Portillo *et al*., 1996), the recA gene (Blackwood *et al*., 2000), the hsp65 gene (Mun *et al*., 2007), the dnaJ gene (Inyaku *et al*., 1993), the sodA gene (Domenech *et al*., 1997) and the 16S-23S rRNA internal transcriber spacer (Roth *et al*., 1998).

The need for increased sensitivity led to the use of **nested PCR methodology**, where the amplification of a large region of DNA is followed by a second amplification step, targeting a shorter interval and more specific region within the amplicon. This technique resulted in high sensitivities, although false-positive results due to cross contamination of the amplified products, were often detected. In order to improve specificity, Garcia-Quintanilla *et al*., (2000) developed a new method, named **Balanced Heminested PCR**, which avoided asymmetric amplification. This was achieved by replacing the outer primer that participated in both steps of amplification in the standard heminested procedure by another primer, containing the sequence of the inner primer, attached at its 5' end. The results showed 75% sensitivity and 100% specificity, when compared with smear-negative culture-positive sputum samples.
Over the last few years, Real-time PCR systems (Logan et al., 2009) have been increasingly used in routine mycobacteriology laboratories. A Real-time polymerase chain reaction is a laboratory technique of molecular biology based on the polymerase chain reaction (PCR), which is used to amplify and simultaneously detect or quantify a targeted DNA molecule. Real time PCR has been investigated for rapid and specific detection of *M. tuberculosis* in the clinical specimens (Soini & Musser, 2001). Quantitative real-time or qRT-PCR provides information beyond mere detection of DNA. It indicates how much of a specific DNA or gene is present in the sample. QRT-PCR allows for both detection and quantification of the PCR product in real-time, while it is being synthesized (van Guilder et al., 2008). The two common methods used to detect and quantify the product include (1) fluorescent dyes that non-specifically intercalate with double-stranded DNA and (2) sequence-specific DNA probes consisting of fluorescently labeled reports. These permit detection only after hybridization of the probe with its complementary DNA target (Logan et al., 2009).

The first attempt at emitting fluorescence was done using fluorescence dyes intercalating into DNA. As mentioned above, the first real-time PCR detection used *ethidium bromide*, which becomes fluorescent upon intercalating into DNA. Asymmetric cyanine intercalator dyes with two aromatic rings are used nowadays; they have no fluorescence in solution, but when they bind to a DNA minor groove, they become brightly fluorescent when exposed to the appropriate wavelength. Intercalating dyes widely used today are: *SYBR Green I, BEBO, LC Green, and SYTO9*. This is the simplest and most cost-effective detection system. The downside is that some dyes can inhibit PCR at certain concentrations (Monis et al., 2005).
The second approach is to use labelled primers and probes. Probes can be covalently bound to one or two dyes. Probes based on a single dye are actually fluorophores that change their fluorescence properties upon binding to the nucleic acid. In case of two dyes, fluorophores are covalently bound to the probe or primer which is structurally designed to bring one fluorophore in contact with another fluorophore or a quencher (Monis et al., 2005).

(ii) Commercially available PCR assays

1) Cobas Amplicor M. tuberculosis assay (Amplicor; Roche Diagnostic Systems, Branchburg, NJ)

Cobas Amplicor M. tuberculosis assay is based on PCR amplification of a 584-bp segment of the 16S rRNA gene followed by hybridization of the biotin-labeled amplified products to a M. tuberculosis complex specific oligonucleotide probe, coated to microtiter plates. The assay includes an internal PCR control. The turnaround time is approximately 6.5 h. The assay was reported to show high sensitivity when evaluated with smear-positive respiratory specimens (87.5–100%), but the sensitivity was much lower for extrapulmonary cases (27.3 to 85%) and smear-negative respiratory samples (17.2–70.8%) (Piersimoni & Scarparo, 2003).

2) Amplfied M. tuberculosis direct test (AMTD; bio Merieux, Genprobe, Inc., San Diego, Calif.)

The AMTD test is based on amplification of the released ribosomal RNA sequences (amplicons) from the target cell. Their detection is achieved by nucleic acid hybridization. An acridinium ester-labeled DNA probe combines with the amplicon to form a stable hybrid and the labeled hybrids are measured in a
luminometer. The test takes approximately 3.5 h. Specimens that are bloody are not suitable for testing with AMTD. The lack of internal amplification control is, also, a serious drawback. In evaluation studies, the overall sensitivity for respiratory specimens was found in the range between 86.8% sensitivity and 100% specificity for non-respiratory specimens & 90.9% and 95.2% and the specificity between 97.6% and 100% (Soini & Musser, 2001).

3) **DProbe Tec ET (energy transfer) *M. tuberculosis* Direct Detection Assay (DTB), (BDProbe Tec; Becton Dickinson Bioscience, Sparks, Md.)**

The technique is based on homogeneous Strand Displacement Amplification (SDA) and fluorescent energy transfer detection on an instrumental system. The SDA is a novel DNA amplification method (Barrett *et al.*, 2002; Piersimoni *et al.*, 2002). Target sequences of a 95-bp region in IS6110, a highly specific insertion element to *M. tuberculosis* complex, and 16S rRNA gene, common to most mycobacteria, is co-amplified isothermally. The process is based on the nicking of a modified recognition sequence in double-stranded DNA, by the restriction endonuclease BsoB1, and the extension and repair of that site by the DNA polymerase Bst, which synthesizes a new strand of DNA while displacing the existing strand. The displaced strand can then serve as a template for further amplification.

The entire process occurs at 52.2 °C. Fluorescent energy transfer detection is performed on the same instrumented system. An internal amplification control is run with each sample and is designed to verify that no inhibition of the SDA reaction is detected in the specimen. The assay is recommended by the manufacturer for use with respiratory specimens and is completed within 4 h. According to Barrett *et al.*
(2002) the BDProbe Tec ET system offers a reliable molecular biological approach for the detection of *M. tuberculosis* complex (MTBC) in respiratory samples in a semiautomated format with sensitivity and specificity of 92.7% and 96.0%, respectively. Cerebrospinal fluid samples gave sensitivity and specificity of 100 and 95%, respectively, when compared to culture, while pleural fluid samples had poor sensitivity (30%) (McHugh *et al.*, 2004).

4) **Genotype mycobacteria direct assay for detection of* M. tuberculosis* complex and four atypical mycobacteria (Hain Lifescience, Nehren, Germany)**

This novel assay is based on the nucleic acid sequence-based amplification (NASBA) applied to DNA strip technology. According to the manufacturer, the assay has three steps. The first step consists of isolation of 23S rRNA, the second step includes amplification of RNA by NASBA method, and the third step involves the reverse hybridization of the amplified products on membrane strips using an automated system. The assay has the ability for simultaneous detection of *M. avium*, *M. intracellulare*, *M. kansasii*, *M. malmoense* and MTBC. Isolation of highly specific RNA is achieved by the use of the “magnetic bead capturing” method. According to de Luna *et al.*, (2006) the assay is useful, reliable and rapid, with sensitivity and specificity of 92% and 100%, respectively.

5) **LCx MTBC assay (Abbott Laboratories, Diagnostic Division, Chicago, USA)**

Ligase chain reaction (LCx) uses thermostable DNA ligase and four primers: two pairs of forward primers and their complements. Forward primers are made in a way that once they attach to ssDNA a gap of 1-3 bases between them is formed. The
next step takes place at the temperature optimal for DNA polymerase which fills the gap between primers. Thermostable DNA ligase acts to covalently connect extended primers. Product is detected using a capture system (Coleman & Tsongalis 2006; Ausina et al., 1997; Monis et al., 2005). The assay uses the ligase chain reaction for amplification of a target sequence within the chromosomal gene that codes for protein antigen b, which is specific for members of the MTBC (Andersen & Hansen, 1989; Sjöbring et al., 1990). The whole process takes approximately 6 h. According to Moore & Curry (1998) comparing LCx with cultures, the overall sensitivity and specificity of the assay were 74% and 98%, respectively. For smear-positive samples the sensitivity reached 100%, but for smear-negative it was only 57% (Moore; Curry, 1998). In a multicenter evaluation of Amplicor and LCx, Tortoli et al., (1999) reported that the sensitivity of both methods was significantly better when only respiratory specimens were considered (78% and 88%, respectively). When non-respiratory samples were used, the sensitivity was reduced to 59% for Amplicor and 65% for LCx.

High rates of false negative (due to the presence of naturally occurring inhibitors in specimen) & false positive results (due to contamination) is a major drawback of these methods. In addition, they are more expensive than conventional culture systems. Again, the currently available NAA tests can enhance diagnostic speed, but they do not replace AFB smear or culture (Wong, 2008; Tsara et al., 2009) because these tests can only detect MTB, cultures are still needed for identification of NTM, drug susceptibility testing and to distinguish between live and dead organisms (Neonakis et al., 2008).
(iii) **Other methods based on isothermal amplification techniques**

1. **Transcription-mediated amplification**

   Transcription-mediated amplification (TMA) targets RNA molecules, abundantly present in each cell, and transforms them into cDNA, thereby starting with an about 10,000 transcriptional active DNA template instead of 1-2 copies of genomic DNA template. Amplification is an isothermal, two step process: rRNA is copied into cDNA using reverse transcriptase and then RNA polymerase is used to make amplicons of the target RNA using cDNA as a template. To begin the reaction, a primer that is complementary to target rRNA is added to initiate the synthesis of cDNA using reverse transcriptase. RNase H degrades the primer upon cDNA synthesis, leaving single stranded cDNA. Then, a DNA-specific primer initiates DNA polymerase binding and dsDNA is produced. The final product is dsDNA matching the target RNA region with an RNA polymerase binding region. RNA polymerase binds to the dsDNA and generates RNA via transcription. Finally, the labelled probe complementary to the DNA amplicon is used to detect the amplified sequence (Coleman et al., 2006).

2) **Loop-Mediated Isothermal Amplification**

   Loop-Mediated Isothermal Amplification (LAMP) is isothermal amplification based on strand displacement reaction, carried out at a higher incubation temperature at 65°C. Reaction mixture includes Bst DNA polymerase, with strand displacement activity, and a set of four primers, that together recognise six distinct sequences on the target DNA. LAMP reaction is based on a combination of cDNA synthesis, strand displacement DNA synthesis and formation of stem-loop DNA, by self-primed DNA synthesis. The product is a mixture of stem-loop DNAs with various
lengths and cauliflower-like structures with multiple loops. Detection of DNA amplification is measured as turbidity or precipitate forming. Pyrophosphate, which is produced as a by-product, yields a white precipitate of magnesium pyrophosphate that can be detected visually. Furthermore, the increase in the turbidity of the reaction mixture correlates with the amount of DNA synthesized, allowing real-time monitoring of the LAMP reaction by real-time measurement of the turbidity (Notomi et al., 2000; Boehme et al., 2007; Palomino, 2009).

(iv) Phage based systems for diagnosis of Mycobacteria

Fast plaque TB \textsuperscript{tm} This is an original phage based test, which uses the mycobacteriophage to detect the presence of *M. tuberculosis* directly from sputum specimens. It is a rapid, manual test, easy to perform and has an overall higher sensitivity when compared with sputum smear microscopy, in newly diagnosed smear positive TB patients. The test has a specificity of 98.7-99.0\% and a sensitivity of 70.3-75.2\%, when compared with smear microscopy, which has a specificity of 97.3-97.4\% and a sensitivity of 61.3-63.4\% (Albert et al., 2002; Muzaffar et al., 2002).

Two clinical diagnostic applications have been spawned by basic mycobacteriophage research. One is a phage replication system that detects live mycobacteria in clinical samples or in young liquid cultures using phages that infect and replicate in mycobacterial cells as indicators. This system appears surprisingly robust, and has the advantages of speed and the promise of high sensitivity. Whether such systems will eventually be useful in DECs will depend on the reproducibility of their performance, the training and equipment required for their use, and cost. The technical skill requirements of such tests, which can be used both for case-detection
(McNerney et al., 1998) and drug susceptibility testing (Eltringham et al., 1999) are likely to be at least as high as those for conventional culture, which will limit the number of laboratories in which this might be applied. Luciferase reporter phages have also been engineered which may be pressed into service for rapid drug susceptibility testing (Riska et al., 1999).

2.8 Identification of M. tuberculosis from culture isolates

At the beginning of the study phenotypic methods based on biochemical tests, growth characteristics and drug susceptibility profiles were used (Collins et al., 1997). During the course of the study these were replaced by a commercially available identification assay that uses PCR followed by reverse hybridization of the amplified products to a probe array (GenoType Mycobacterium; Hain Diagnostika; Padilla et al., 2004).

Phenotypic identification of the strains:-

2.8.1 Colony Morphology:-

All clinical isolates were grown on Lowenstein-Jen sen or Coletsos agar and examined for growth rate, gross and microscopic colony morphology, and pigmentation. They were also submitted in parallel to the TB DNA probe test. If this test was positive, only the niacin and nitrate reduction tests (tube tests) were performed. In other cases, isolates were subjected to the catalase (drop method), arylsulfatase, pyrazinamidase, urease, and lipase (Tween 80 hydrolysis) tests and also to additional tests for certain isolates. Other phenotypic test Tellurite reduction, oxygen preference, utilization of carbon sources, iron uptake, β galactosidase.
2.8.2 Biochemical Methods:-

According to Palomino et al., 2007 several biochemical tests have been described that are used for identification of mycobacterial species.

(a) **Niacin Test**: Devised by Kamino and modified by Runyon and Co-workers: This is based on the production of niacin; there is formation of complex compounds when niacin produced by the organism reacts with cyanogens’ bromide (CNBr) and a primary or secondary amine. Positive test show the formation of yellow color. *M. tuberculosis* and *M. simiae* are usually niacin positive. *M. marinum* may produce niacin and give weak positive result.

(b) **Catalase Test**: This biochemical test has a distinctive role in all mycobacteria except for certain Isoniazid resistant mutants of *M. tuberculosis* and *M. gastri* that possess catalase enzyme. Presence of this enzyme is detected by breakdown of hydrogen peroxide and the subsequent formation of bubbles.

(c) **Semi Quantitative Method**: This test divides mycobacteria in two groups, those producing less than 45mm of bubbles and those producing more than 45mm of bubbles. *M. kansasii*, *M. simiae* and most scotochromogens and rapid growers produces more than 45mm of bubbles in this test. Among these that produce less than 45mm of bubbles are *M. tuberculosis*, *M. marinum*, *M. avium – complex M. xenopi* and *M. gastri*.

(d) **Heat Stable Catalase Test**: (at pH 7.0 / 68 °C): Some mycobacteria lose catalase activity when suspended in pH 7 and heated to 68°C for 20 minutes. Included in this group are *M. tuberculosis*, *M. bovis*, *M. gastri* and *M. homophile*. 
(e) **Nitrate Reduction Test**: Slow growing organisms like *M. tuberculosis* and *M. kansasii* (strongly positive) are differentiated from members of *M. avium* complex organism (generally negative) by reduction of nitrate (nitroreductase). Positive test show the formation of bright red color.

(f) **Aryl Sulphatase Test**: Two methods commonly employed to detect the enzyme utilize liquid substrate CDC medium or solid medium. (Wayne modification)

(g) **Tween-80 Hydrolysis**: This test uses Tween-80 and the indicator neutral red in the buffered neutral red solution. Normally neutral red is red at pH 7.0 but when bound by lipids or Tween-80, the indicator dye takes amber or straw color that commonly has more alkaline pH. When bacterial esterase split Tween-80, it no longer acts to bind indicator dye and the neutral red than reverts to its pH 7.0 which is red and the test are called positive. *M. kansasii* give results within the five days where as many strains of *M. tuberculosis* become positive in 10-20 days.

(h) **Mac Conkey Agar Test**: Positive result is interpreted for by growth of mycobacteria on MacConkey media within 8-11 days. *M. fortuitum, M. cheloneae* show positive test while *M. phlei* shows negative test.

(i) **Iron Uptake Test**: Rusty brown color of colonies in the culture medium containing soluble iron salts indicates the positive test. *M. fortuitum* is positive while *M. cheloneae, slow growers and M. flavescens* are negative.
2.8.3 Molecular methods:

The molecular methods for mycobacterial identification provide rapid and accurate results. These include:

1) **PCR-based sequencing** is the “gold standard” for identification of mycobacterial species (Neonakis *et al.*, 2008). In this method initially, PCR amplification takes place followed by sequencing of the amplicons in an automatic sequencer. The identification of an unknown strain is completed by comparison of the nucleotide sequence with a library of known sequences provided in databases (Neonakis *et al.*, 2008).

2) **DNA probe technology** The AccuProbe (Gen-Probe, San Diego, CA, USA) assay has the ability to identify a series of clinically important mycobacteria (*M. tuberculosis* complex, *M. avium* complex, *M. avium*, *M. kansasii* and *M. gordonae*). DNA probes labeled with acridinium ester, complementary to the rRNA are added to the broken mycobacterial cells, to form a stable DNA-RNA complex. The hybridization is detected by light emission in a luminometer (Neonakis *et al.*, 2008).

3) **Line probe technology** includes PCR (with biotinylated primers), reverse hybridization with different specific DNA probes, immobilized in parallel lines on a strip and colorimetric detection in an automated instrument. The banding pattern is indicative of the species of the isolate. Two systems of line probe assay are commercially available:

   i. **Inno LiPA Mycobacteria v2** (Innogenetics, Ghent, Belgium) based on the amplification of the mycobacterial spacer region 16S-23S rRNA for the simultaneous identification (Tortoli *et al.*, 2003).
ii. **Geno Type Mycobacterium** (Hain Lifescience, Nehren, Germany) based on a multiplex PCR, followed by reverse hybridization and line probe technology (Gitti et al., 2006).

4) **PRA method (polymerase chain reaction and restriction enzyme analysis for identification of mycobacteria from culture)** Telenti et al., (1993) developed a rapid method, based on the amplification of the gene encoding the 65-kDa heat shock protein, followed by restriction-fragment-length polymorphism, using two restriction enzymes BstEII and HaeIII. Isolates from both solid and liquid cultures can be used. The fragments of the restriction enzyme digestion are analyzed by agarose gel electrophoresis and compared. The test can be completed within a day. It is a cost-effective and reliable assay that can be used by low-budget laboratories as well.

5) **Pyrosequencing**: Pyrosequencing™ (Biotage, Uppsala, Sweden) technology is a novel method of nucleic acid sequencing-by-synthesis that is based on the detection of released pyrophosphate (PPi) during DNA synthesis (Ronaghi, 2001; Tuohy et al., 2005). The cascade of enzymatic reactions generates visible light. The generated light is proportional to the number of incorporated nucleotides. The method is optimal for determining short sequences (typically 20–30 bases of a DNA) rapidly and in a semi-automated format (Ronaghi, 2001). The ability of this technology to accurately characterize common strains of mycobacteria was evaluated by Tuohy et al., (2005). Pyrosequencing targeted a 30-bp sequence of the hyper variable a region of the 16S rRNA gene. It provided an acceptable identification for 179 of 189 (94.7%) isolates tested. Although the short sequences generated
by Pyrosequencing are not as discriminating as the 300 to 500-bp sequences that can be generated by traditional sequencing, the authors demonstrated that abundant clinically useful information may be obtained.

6) **DNA microarrays (DNA chips):** The method is based on hybridization of fluorescently labeled PCR amplicons of an unknown strain to a DNA array, containing nucleotide probes for 16S ribosomal RNA gene. The hybridization pattern and intensity is determined by scanning the chip using laser confocal fluorescence microscopy. The process of generating the target, its hybridization and reading on the chip requires approximately two hours. It allows the identification of a large number of strains in one reaction (Gingeras et al., 1998). Gingeras et al., (1998) designed an array to determine the specific nucleotide sequence of 705 bp of the rpoB gene of *M. tuberculosis*. It accurately detected RMP resistance associated with mutations of 44 clinical isolates of *M. tuberculosis* (Gingeras, 1998). Troesch et al., (1999) investigated this probe array strategy focusing on mycobacterial diseases (Affymetrix, GeneChip technology, Santa Clara, Calif). Sequences of regions from the 16S rRNA and rpoB loci had been developed. Unique hybridization patterns allowed for the identification of Mycobacterium species and the RMP resistant alleles (Troesch et al., 1999). Seventy mycobacterial isolates from 27 different species and 15 RMP-resistant *M. tuberculosis* strains were tested. A total of 26 of 27 species were correctly identified as well as all of the rpoB mutants alleles (Troesch et al., 1999). A great disadvantage is, however, the current high cost of the required equipment.
2.9 Vaccination against TB (Wikipedia)

The first genuine success in immunizing against tuberculosis was developed from attenuated bovine-strain tuberculosis by Albert Calmette and Camille Guérin in 1906. It was called BCG (Bacillus of Calmette and Guérin). BCG contains a live attenuated (weakened) strain of \textit{M. bovis}. This strain was carefully sub-cultured many years. After about thirteen years the strain was seen to be less virulent for animals such as cows and guinea pigs. The BCG vaccines that are currently in use are produced at several sites throughout the world. The most controversial aspect of BCG is the variable efficacy found in different clinical trials (Zwerling \textit{et al.}, 2011). Some mainly proposed reasons for variable efficacy of vaccine are:

1) **Background frequency of exposure to tuberculosis**: In areas with high levels of background exposure to tuberculosis, every susceptible individual is already exposed prior to BCG, and the natural immunizing effect of background tuberculosis duplicates any benefit of BCG.

2) **Genetic variation in BCG strains**: Genetic variation in the BCG strains used may explain the variable efficacy reported in different trials (Brosch \textit{et al.}, 2007).

3) **Genetic variation in populations**: Differences in genetic make-up of different populations may explain the difference in efficacy.

4) **Interference by non tuberculous mycobacteria**: Exposure to environmental mycobacteria (especially \textit{M. avium}, \textit{M. marinum} and \textit{M. intracELLulare}) results in a nonspecific immune response against mycobacteria. This effect is called \textbf{masking} (Black \textit{et al.}, 2002). An alternative explanation is suggested...
by mouse studies; immunity against mycobacteria stops BCG from replicating and so stops it from producing an immune response. This is called the blocking hypothesis (Brandt et al., 2002).

5) **Interference by concurrent parasitic infection**: In another hypothesis, simultaneous infection with parasites changes the immune response to BCG, making it less effective (Rook et al., 2005).

6) **Exposure to ultraviolet light**: Concentration of ultraviolet light (particularly UVB light) from the sun may have some effect on efficacy of the BCG vaccine. The concentration gradient of UVB light increases geographically closer to the Earth's equator (Jeevan et al., 2009).

7) **WHO BCG policy**: The WHO recommends BCG be given to all children born in countries highly endemic for TB because it protects against ‘miliary TB’ and ‘TB meningitis’.

### 2.10 Previous studies conducted abroad to compare the various methods for microbiological diagnosis of TB:

Piersimoni et al., 2002 compared BDProbeTec ET *Mycobacterium tuberculosis* Complex Direct Detection Assay (DTB) with the enhanced *M. tuberculosis* Amplified Direct Test (AMTDII). They assessed total of 515 N-acetyl-L-cysteine–sodium hydroxide-decontaminated respiratory ($n_{331}$) and extrapulmonary ($n_{184}$) sediments (from 402 patients) parallel by both assays. The results were compared with those of acid-fast staining and culture (solid plus liquid media), setting the combination of culture and clinical diagnosis as the “gold standard”. The percent sensitivity, percent specificity, and positive and negative likelihood ratios for
AMTDII were 88%, 99.2%, 110, and 0.11 for respiratory specimens and 74.3%, 100%, 740, and 0.26 for extrapulmonary specimens, respectively. The corresponding values for DTB were 94.5%, 99.6%, 235, and 0.05 for respiratory specimens and 92.3%, 100%, 920, and 0.07 for extrapulmonary specimens, respectively. They concluded that both amplification assays proved to be rapid and specific for the detection of MTB in clinical samples and particularly feasible for a routine laboratory work flow.

*Honore Bouakline et al., 2003* compared two sample preparation methods, pretreatment with proteinase K (PK-Roche) and complete DNA purification (cetyltrimethylammonium bromide [CTAB]-Roche), on 144 extrapulmonary specimens collected from 120 patients to evaluate the impact on the Cobas Amplicor method. CTAB-Roche allowed the detection of more culture-positive specimens by PCR than PK-Roche. Comparison with the final diagnoses of tuberculosis confirmed that CTAB-Roche produced the best sensitivity (53.8%) compared to culture (43.3%), PK-Roche (16%), and smear (13%). However, the specificity of the PCR assay with CTAB-Roche-extracted material was always lower (78.8%) than those with culture (100%) and PK-Roche (96.5%). False-positive specimens were lung biopsy material, lymph node biopsy material and aspirate, or bone marrow aspirate, mainly from immuno-compromised patients. The authors hence concluded that despite the efficiency of complete DNA extraction for the rapid diagnosis by PCR of extrapulmonary tuberculosis, the false-positive results challenge our understanding of PCR results.
Tueller et al., 2005 analyzed the yield of bronchoalveolar lavage fluid (BALF) smear and PCR in patients with confirmed pulmonary TB. 90 out of 230 (39%) patients with culture-positive pulmonary TB had a positive sputum smear, and 120 patients underwent bronchoscopy. BALF smear was positive in 56 (47%), BALF PCR in 93 (78%) patients, and BALF smear and/or PCR was positive in 83%. In total, 71 patients who underwent bronchoscopy and had complete clinical records were further analyzed. BALF (smear or Mycobacterium tuberculosis complex-PCR) allowed a rapid diagnosis in 10 (59%) out of 17 patients who had a negative sputum smear, and 49 (91%) out of 54 patients without sputum production. Of these 71 patients, 12 (17%) were only culture positive. Rapid diagnosis of pulmonary TB by smear and/or PCR was made in 190 out of 210 patients (90%) in sputum or BALF. They concluded that combined use of bronchoalveolar lavage fluid smear and Mycobacterium tuberculosis complex-PCR has a good diagnostic yield in patients with sputum smear-negative tuberculosis or without sputum production.

Torrea et al., 2005 evaluated a one-tube nested PCR-based analysis of urine for diagnosing pulmonary tuberculosis (PTB) and extrapulmonary tuberculosis (EPTB). Urine from patients was analyzed using the DNA extraction and Sechi’s methods, both modified, for the detection of Mycobacterium tuberculosis. The sensitivity of the test for the microbiological-positive PTB, microbiological-negative PTB and EPTB was 40.5% (88/217), 66.7% (20/30) and 57.1% (48/84), respectively. The specificity was 98.2%. Differences were observed in the two populations infected and not infected by HIV. This method was not appropriate for detection of new TB cases in the routine laboratory, but it was found useful for cases where the clinical and bacteriological diagnosis of TB is not conclusive.
Khosravi & Omidian, 2006 evaluated the role of PCR for the detection of *Mycobacterium tuberculosis* (MTB) DNA as a diagnostic aid in cutaneous tuberculosis. Thirty formalin-fixed, paraffin-embedded samples belonging to 28 patients were analyzed. Tissue sections were treated by lysis buffer containing proteinase K and DNA was extracted by using standard extraction kit. PCR amplification was performed using assay based on a repetitive sequence IS6110. Using PCR technique, six out of the total specimens tested (21.4%), were positive for the presence of *M. tuberculosis* DNA. Statistically the difference between applied methods was significant (*P*<0.0016). Accounting histopathology as gold standard, the sensitivity of PCR in this study was determined as 75%. PCR assay has priority to conventional bacteriologic methods for detection of *M. tuberculosis* from cutaneous tuberculosis cases, and can be only used when the staining for acid fast bacilli is negative and there is a lack of growth on culture or when fresh material has not been collected for culture.

Takahashi & Nakayama, 2006 designed a novel technique consisting of an internally controlled quantitative nested real-time (QNRT) PCR assay that provided a marked improvement in detection sensitivity and quantification. They applied this novel technique to quantitatively detect *M. tuberculosis* DNA in CSF samples from patients with suspected TBM. For use as the internal control in the measurement of the *M. tuberculosis* DNA copy numbers in the QNRT-PCR assay, the original mutation (M) plasmid, which included an artificial random 22-nucleotide sequence within an inserted DNA fragment of the MPB64 gene of *M. tuberculosis*, was prepared. The QNRT-PCR assay showed high sensitivity and specificity that were approximately equivalent to those of the conventional nested PCR assay. Moreover,
the QNRT-PCR assay made it possible to precisely and quantitatively detect the initial copy number of *M. tuberculosis* DNA in CSF samples. Therefore, compared to the conventional PCR assay, the QNRT-PCR assay was considered a more useful and advanced technique for the rapid and accurate diagnosis of TBM.

**Magana-Arachchi et al., 2008** determined the feasibility of an in-house polymerase chain reaction (PCR) method to detect *Mycobacterium tuberculosis* in clinical samples. The study focused on diagnosing extra-pulmonary tuberculosis (EPTB) using an in-house PCR method in 465 clinical samples. This study also compared the efficacy of a standard phenol-chloroform (PC) extraction procedure and the guanidine thiocyanate with diatomaceous silica (GTCS) method of DNA extraction and purification. A sub sample of patients was used for the validation of results based on the final diagnosis. Among 373 patients with suspected EPTB, 75 specimens were positive by PCR, four by microscopy and six by culture. Of the 25 PCR-positive patients, 95% had a final diagnosis of TB. Globally, the GTCS method was found to be superior to the PC method for DNA extraction and removal of inhibitors from clinical specimens. The DNA amplification method was found to be significantly more sensitive and rapid compared to culture and microscopy for a reliable final diagnosis of EPTB.

**Ani et al., 2009** examined 101 HIV-positive patients and 40 clinical specimens (sputa, gastric wash out, ascitic fluid, pleural fluid and cerebrospinal fluid) from children (HIV status unknown), all suspected were examined by Ziehl Neelsen (ZN) smear microscopy, Lowenstein Jensen’s (LJ) egg-based culture, and PCR methods for the detection of *M. tuberculosis*. Mycobacteria was detected in 45/101 (44.6%)
of the specimens from the HIV-positive patients and comprised of 6% ZN+culture+ PCR+, 4% ZN-culture+ PCR-, 16% ZN-culture+ PCR+ and 19% ZN-culture-PCR+. Twenty-two of forty (55%) children were positive with 0% smear microscopy; 4/40 (10%) culture+PCR+; and 18/40 (45%) culture- PCR+. The sensitivity and specificity of the PCR for the HIV-positive patients were 85% and 74% respectively against 23% and 100% for ZN smear microscopy. The IS\textit{6110} PCR was found to be rapid and sensitive method that is specific for the \textit{M. tuberculosis} complex group. They suggested its use for the detection of \textit{M. tuberculosis} in high TB and HIV burden areas.

\textbf{Khorshidi et al., 2009} evaluated the polymerase chain reaction (PCR) technique, using primers directed against the IS\textit{6110} gene, for the detection of \textit{M. tuberculosis} in the sputum samples, and calculate the sensitivity and specificity of PCR. They studied a total of 248 sputum samples from patients suspected of mycobacterial diseases. DNA was extracted by boiling method. IS\textit{6110} PCR method by a specific pair of primers designed to amplify 123bp and 245bp sequences of the insertion sequence, IS\textit{6110}, in the \textit{M. tuberculosis} genome was used to analyze sputum samples. Totally, 32 (12.9\%) samples had positive culture. PCR yielded a sensitivity of 93.8\% and specificity of 99.1\% for the diagnosis of TB, when diagnosis was confirmed by culture. There were 2 out of 32(6.3\%) PCR-positive cases among the patients with non-TB disease.IS\textit{6110} PCR assay was found valuable in the rapid diagnosis of tuberculosis.

\textbf{Massoud et al., 2009} examined 2123 specimens who were referred from private clinical laboratories between 2006 till 2008. All specimens were stained for acid fast bacilli, cultured with standard procedures and tested with PCR using signal
based method after ensuring of nucleic acid purification. One hundred and thirteen patients were positive for TB, pulmonary tuberculosis were proved in 48 patients whilst the remaining 65 cases were classified as extrapulmonary tuberculosis. Positive rates for PCR, culture and staining were 41, 23 and 12 respectively. The respective figures for extrapulmonary tuberculosis were 46, 26 and 14. This study demonstrated that PCR had a high sensitivity in diagnosis of TB than traditional method.

**Khosravi et al., 2010** evaluated the diagnostic value of nested PCR in genitourinary tuberculosis (GUTB) compared with acid fast staining and culture method. 200 urine samples from suspected cases of GUTB were collected Urine pellets were used for smear preparation, culture and DNA extraction by ether-chloroform method. Nested PCR was performed according to standard protocol using primers based on IS6110 gene fragment. The positivity rate of urine samples in this study was 5.0% by using culture and PCR methods and 2.5% for acid fast staining. Four out of total samples showed positive results in all three methods (2%). The sensitivity of PCR in this study was estimated as high as culture equal to 100%, while the sensitivity for direct smear staining was 41.6%. Since the detection rate of culture and nested PCR was identical, they suggested PCR as a rapid alternative to culture especially for confirmed cases of GUTB.

**Akram et al., 2012** conducted a study on 43 CSF specimens from highly probable TBM patients, 33 were positive by PCR (76.7%), whereas only 5 was acid-fast microscopy (AFM) positive (11.6%) and 22 were culture positive (55.2%). No positive results were found by AFM, culture or PCR in the non-tuberculous control group. This study indicated that application of PCR is extremely useful for the
diagnosis of TBM. The PCR was found to be superior to the currently available techniques for the diagnosis of tuberculous meningitis in terms of sensitivity, specificity and rapidity and plays a critical role in the diagnosis of suspected cases.

**Zakham et al., 2012** compared the utility of the Polymerase Chain Reaction (PCR) using the Insertion Sequence IS6110 as target to conventional methods for early TB diagnosis and rapid detection of *Mycobacterium tuberculosis* (MTB) in the clinical specimens. Out of 305 patients with different clinical manifestations: suspected, new, drug relapse, drug failure and chronic cases were enrolled in this study and tested by mycobacteriological and PCR techniques for the investigation about the tubercle bacilli. The results of the in house IS6110 PCR showed a good sensitivity (92.4%) and high specificity (98.0%), the positive and negative predictive values were 96.4 % and 95.3 % respectively.

**Afrasiabian et al., 2013** examined the usefulness of serum adenosine deaminase levels as a diagnostic parameter for EPTB. The work included 116 patients with fever of unknown origin in which tuberculosis or infectious mononucleosis was not proven and 51 person who had proven EPTB. They Correlated adenosine deaminase levels between these two groups and obtained significantly higher values in patients with EPTB. The calculated sensitivity was 0.56, specificity 0.89, positive predictive value 0.80 and negative predictive value 0.72. Certain reducing of the values observed during anti TB therapy increased concentrations of serum adenosine deaminase have shown the potential of usable screening test and can be used as an indicative EPTB parameter.
2.11 Previous studies conducted in India to compare the various methods for microbiological diagnosis of TB

Michael et al., 2002 evaluated polymerase chain reaction (PCR) using primers directed against the 16S6110 gene of *M. tuberculosis* in the diagnosis of tuberculous meningitis (TBM). PCR was performed on CSF samples from 34 patients suspected to have tuberculous meningitis (TBM) and 68 likely to be having meningitis due to other causes (controls). The first group was further divided into definite TBM and probable TBM using culture as the gold standard. In 17 culture positive patients (definite TBM), PCR was positive in 13 while in 17 patients who were diagnosed clinically, PCR picked up 11 cases all of whom were culture negative. For final diagnosis of tuberculous meningitis, the overall sensitivity of microscopy, culture and PCR were 3%, 50%, and 71% and specificity 100%, 100% and 97% respectively. PCR was found valuable in the rapid diagnosis of tuberculous meningitis.

Chakravorty et al., 2005 evaluated USP on 99 extrapulmonary specimens collected from 87 patients. USP-processed specimens were submitted to smear microscopy for detection of acid-fast bacilli (AFB), culture, and two PCR tests targeting devR (*Rv3133c*) and IS6110 gene sequences. Although USP smear and culture were significantly superior to conventional microbiology, which was not optimized (*P* < 0.0001), these approaches fell short of PCR tests (*P* < 0.0001). The low yields by smear and culture are attributed to the paucibacillary load in the specimens. The highest sensitivity in PCR was achieved when devR and IS6110 test results were combined; the sensitivity and specificity values were 83 and 93.8%, 87.5 and 100%, and 66.7 and 75%, respectively, in pleural fluid, needle-biopsied
pleural tissue, and lymph node specimens. They concluded that application of USP technology, together with clinic pathological characteristics, promises to improve the accuracy and confidence of extrapulmonary tuberculosis diagnosis.

**Pahwa et al., 2005** analyzed 100 cases in which Fine needle aspiration cytology was done in all cases and the smears prepared were processed for Giemsa, Ziehl–Neelsen’s, Kinyoun and Papanicolaou stains. Parts of the aspirated materials were assessed by fluorescent staining, culture and PCR. Seventy four percent of aspirates were positive by fluorescent stain while only 22% were positive by culture. PCR could be performed in 55 cases, out of which 22 (40%) were positive. When compared to culture, the sensitivity and specificity of PCR were found to be 89.5% and 86.1%, respectively. Fluorescent stain was found to be the most sensitive (81.8%) of the conventional methods but showed poor specificity (28.2%). Interestingly, PCR detected 80% of smear-negative but culture positive cases.

**Oberoi & Aggarwal, 2007** conducted a study on Two hundred and thirty samples from suspected pulmonary and extra pulmonary cases of tuberculosis. Samples were processed for detection of *Mycobacterium tuberculosis* by ZN smear examination, LJ medium culture, BACTEC radiometric culture and Polymerase Chain Reaction tests. A significant difference was seen in the sensitivities of different tests, ie.73.9% for PCR tests, 34.78% for ZN smear examination, 52.17% for LJ culture and 58.69% for BACTEC culture. However, there was no significant difference in specificity of different tests (P>0.05). PCR test sensitivity in pulmonary and extrapulmonary clinical samples was 74.0% and 78.5% respectively and found to be significantly higher (P<0.05) when compared with those of other tests. The
mean detection time for *M. tuberculosis* was 24.03 days by LJ medium culture, 12.89 days by BACTEC culture and less than one day by PCR test.

**Sekar et al., 2008** compared IS6110 sequence based polymerase chain reaction (PCR) with conventional bacteriological techniques in the laboratory diagnosis of extra-pulmonary tuberculosis (EPTB). One hundred and ninety one non-repeated clinical samples of EPTB and 17 samples from non-tuberculous cases as controls were included. All the samples were processed for Ziehl-Neelsen staining for acid fast bacilli (AFB) and 143 samples were processed by culture for *M. tuberculosis*. All the samples were processed for PCR amplification with primers targeting 123 bp fragment of insertion element IS6110 of *M. tuberculosis* complex. Of the total 191 samples processed, 34 (18%) were positive by smear for AFB. Culture for AFB was positive in 31 (22%) samples among the 143 samples processed. Either smear or culture for AFB was found positive in 51 (27%) samples. Of the total 191 samples processed 120 (63%) were positive by PCR. In 140 samples, wherein both the conventional techniques were found negative, 74 (53%) samples were positive by PCR alone. Among 51 samples positive by conventional techniques, 46 (90%) were found positive by PCR. PCR assay targeting IS6110 was found useful in establishing the diagnosis of EPTB, where there is strong clinical suspicion, especially when the conventional techniques are negative.

**Chawla et al., 2009** evaluated the efficiency of PCR in 104 different tissue samples compared to histopathology that was considered gold standard. PCR showed 74.1% sensitivity and 96.1% specificity. False positive and false negative results were observed in three (2.88%) and seven (6.73%) samples, respectively.
Positive agreement between histopathology and PCR was observed as 0.737, indicating substantial good agreement between two tests. PCR can be used for early diagnosis of tuberculosis in tissue samples that can help to initiate timely antitubercular treatment and prevent progression to irreversible changes.

**Haldar et al., 2009** analysed one hundred and sixty-seven CSF samples from patients with ‘suspected’ TBM (n=581) and a control group including other cases of meningitis or neurological disorders (n=586). CSF ‘sediments’ and ‘filtrates’ were analysed individually for *M. tuberculosis* DNA by quantitative real-time PCR (qRT-PCR) and conventional PCR. Based on these, TBM was diagnosed with 87.6% and 53.1% sensitivity (P 0.001) in ‘filtrates’ and ‘sediments’, respectively, and with 92% specificity each. Conventional devR and IS6110 PCR were also significantly more sensitive in ‘filtrates’ versus ‘sediments’ (sensitivity of 87.6% and 85.2% vs. 31% and 39.5 %, respectively; P, 0.001).

**Iqbal et al., 2010** subjected 205 patients of suspected TB (pulmonary or extra-pulmonary) to ZN smear examination, LJ medium culture, and PCR test by amplifying 541 bp fragment of *Mycobacterium tuberculosis* complex genome. The sensitivity of different tests was found to be significantly different, which was 67.32 percent for PCR test, 27.81 percent for LJ medium culture and 12.20 percent for ZN smear examination. PCR test sensitivity in pulmonary and extra-pulmonary clinical samples was 77.15 and 61.6 percent respectively, being significantly higher, when compared with sensitivity of other tests.

**Shukla et al., 2011** compared sensitivity and specificity of the PCR from smear and culture in the diagnosis of suspected cases of pulmonary and extra
pulmonary tuberculosis. The various specimens collected from these patients included 74 sputum, 38 endometrial biopsies, 16 CSF and 12 gastric aspirates. All the specimens were tested by ZN staining, culture was on L-J medium and PCR was performed for targeting IS6110 sequence. In these 140 patients, 40 (28.5%) were ZN smear positive for AFB, 48 (34.2%) were culture positive for AFB and 104 (74.2%) were sensitive to nested PCR for *Mycobacterium tuberculosis*. They observed significant difference in sensitivity of PCR for smear positive and negative cases, and also for culture positive and negative cases.

**Rosso et al., 2011** studied 150 consecutive patients with pleural effusion diagnosed by chest radiography, had a pleural fluid specimen submitted for real-time PCR testing. Overall, 98 patients had pleural TB and 52 had pleural effusion secondary to other disease. Sensitivity, specificity, positive and negative predictive values of PCR testing for pleural TB diagnosis were 42.8% (95% CI 38.4 - 44.8), 94.2% (95% CI 85.8 - 98.0), 93.3% (95% CI 83.6 - 97.7), and 48.5% (95% CI 44.2 - 50.4), respectively. The real time PCR test improved TB detection from 30.6% to 42.9% when compared to AFB smear and culture methods performed on pleural fluid specimens, although the best sensitivity was achieved by combining the results of culture and histopathology of pleural tissue specimens.

**Thangappah et al., 2011** evaluated the efficacy of PCR technique, culture and histopathological examination in the diagnosis of GTB in female infertility. This study included 72 infertile women. Endometrial samples from were allocated for AFB smear, culture and HPE examination. Only 49 samples were available for PCR using IS6110 and TRC4 primers. In seven patients peritoneal fluid was also taken
for culture and PCR. Based on the clinical profile and laparoscopic findings, a diagnostic criteria was derived to suspect GTB. Specific diagnostic tests were evaluated against this diagnostic criterion. Based on the diagnostic criteria, GTB was suspected in 28 of the 49 cases. On evaluating against the diagnostic criteria, the sensitivity of PCR, HPE and culture were 57.1, 10.7, 7.14 per cent respectively. Their results showed that conventional methods of diagnosis namely, HPE, AFB smear and culture have low sensitivity. PCR was found to be useful in diagnosing early disease as well as confirming diagnosis in clinically suspected cases. However, false negative PCR was an important limitation in this study.

Crump et al., 2011 compared the performance of the BacT/Alert MB system, that of the manual Bactec Myco/F Lytic procedure, and that of the Isolator 10 lysis-centrifugation system in the detection of Mycobacterium tuberculosis bacteremia. Mean times to detection were 16.4 days for BacT/Alert MB versus 20.0 days for Myco/F Lytic, 16.5 days for BacT/Alert MB versus 23.8 days for Isolator 10, and 21.1 days for Bactec Myco/F Lytic versus 22.7 days for Isolator 10. There were no significant differences in yields. The mean (range) magnitude of mycobacteremia was 30.0 (0.4, 90.0) CFU/ml and was correlated with the time to positivity in the BacT/Alert MB system ($r \_ 0.4920$). M. tuberculosis bacteremia was detected more rapidly in a continuously monitored liquid blood culture system, but the mean time to positivity exceeded 3 weeks.

Jain, 2011 conducted a study on 300 patients of EPTB over a period of 5 years. These patients were suspected cases of tubercular meningitis, tubercular ascites and tubercular lymphadenitis. Samples analyzed were cerebrospinal fluid,
ascetic fluid and lymph node fine needle aspirate. A two step PCR targeting hup B gene was used. Clinical response to anti tubercular therapy (ATT) was taken as positive (gold standard). PCR for hup B gene was positive in 147 samples out of 155 ATT responders. Of these 85.71% were infected with M. tuberculosis, 9.52% with M. bovis alone and 4.76% showed co infection with both M.tb and M. bovis. The sensitivity and specificity of PCR was 90.32 and 94.48% respectively.

Maurya et al., 2012 collected seven hundred fifty-six specimens from the suspected cases of EPTB which were processed for Mycobacteria by Ziehl Neelson (ZN) staining and BACTEC culture. All the specimens were also processed for IS6110-based PCR amplification with primers targeting 123 bp fragment of insertion element IS6110 of the M. tuberculosis complex. Of these 756 specimens, 71(9.3%) were positive for acid fast bacilli (AFB) by ZN staining, 227(30.1%) were positive for mycobacteria by BACTEC culture and IS6110 PCR were positive for M. tuberculosis complex in 165 (20.7%) isolates. PCR using IS6110 primer was able to pick up more EPTB patients compared to conventional L-J culture method for detection of M. tuberculosis.

Verma et al., 2012 assessed the 170 different clinical specimens suspected of tuberculosis, (100 pulmonary and 70 Extrapulmonary) by PCR using MPB 64 primer, culture and microscopy. All specimens were processed using USP methodology for inhibitors free PCR. TB Ig G, Ig M and Ig A was determined using PATHOZYME MYCO Kit. Response to ATT on clinical follow up was considered as gold standard. Total pulmonary specimens found positive by any of the four tests was 87 (out of 100) while that for extrapulmonary samples was 63(out of 70). For
Pulmonary Specimens the diagnostic accuracy of microscopy was 88.3%, for culture 88.3%, for ELISA 67.4% and for PCR 94.1%. For extrapulmonary samples the diagnostic accuracy of microscopy was 30.1%, for culture 49.2%, for ELISA 44.4% and for PCR 87.3%. For Extrapulmonary specimens PCR can be used as an effective screening tool as conventional methods are mostly negative.

**Bunger et al., 2013** processed a total of 200 samples for direct AFB smear examination, and culture on M 960 and LJ media. Acid fast staining of the specimens was done using the Ziehl-Neelsen method. Of the 24 positive specimens, the highest rate of Mycobacterial recovery was by MGIT (91.6%) as compared to LJ media (58.3%). From smear negative samples LJ and MGIT media detected 6.06 % and 10.10 % isolates respectively. For smear-positive specimens, the mean turnaround time was 8 days by MGIT whereas on LJ medium, it was 36 days. For smear-negative specimens, the same was 18 days for MGIT and 40 days for LJ medium. The MGIT system was found to be more sensitive in detecting *mycobacterium* in smear-negative samples. The MGIT system detected mycobacterium significantly earlier than the LJ medium.

**Siddiqui et al., 2013** compared the sensitivity, specificity and turnaround time of conventional diagnostic modalities, BACTEC culture and polymerase chain reaction test for diagnosis of extra-pulmonary tuberculosis. Out of the hundred extra pulmonary samples processed, 5% were positive by ZN staining, 15% were positive by both LJ culture and BACTEC MGIT 960 TB culture and 70% were positive by PCR. Those samples positive by ZN smear, LJ culture and BACTEC culture were all found to be 100% positive by PCR. The mean detection time for *M. tuberculosis*
was 23.13 days by LJ Medium culture, 9.86 days by BACTEC MGIT 960 TB culture and less than one day by PCR. PCR as a diagnostic tool was found to be more sensitive and useful in diagnosis of extra-pulmonary form of tuberculosis when compared to conventional methods or BACTEC culture by definitely shortening the time with early initiation of anti-tubercular treatment and can prevent disease progressing towards irreversible tissue damage.

Makeshkumar et al., 2014 conducted to evaluate the use of polymerase chain reaction (PCR) in diagnosis of definitive and probable extrapulmonary tuberculosis patients, and to assess the performance of insertion sequence (IS) 6110 based PCR assay as compared to conventional culture by Lowenstein-Jensen (LJ) method for the diagnosis of EPTB. They studied a total of 178 non repeated clinical specimens from clinically suspected extrapulmonary tuberculosis patients. All these clinical samples were subjected to Ziehl-Neelsen staining (ZN) for acid fast bacilli (AFB) and culture on LJ medium. PCR was performed by targeting 123bp fragment of insertion sequence IS6110 of *Mycobacterium tuberculosis* (MTB). Of the 178 specimens, 10 (5.61%) were ZN smear positive for AFB, six (3.37%) were L-J culture positive from 10 AFB smear positive cases and 48 (26.96%) were PCR IS 6110 positive for *M. tuberculosis*. PCR using IS6110 primer was able to pick up more EPTB patients compared to conventional L-J culture method for detection of *M. tuberculosis*. False positive PCR IS6110 in three CSF samples may be due to latent TB infection which was limitation in this study.

Jain et al., 2014 correlated clinico-radiological, bacteriological, serological, molecular and histological methods for diagnosis of osteoarticular tuberculosis.
They analysed fifty clinico-radiologically diagnosed patients of osteoarticular tuberculosis with involvement of dorsal spine (n = 35), knee (n = 8), shoulder (n=1), elbow (n = 2) and lumbar spine lesion (n = 4). Tissue was obtained after decompression in 35 cases of dorsal spine and fine needle aspiration in the remaining 15 cases. Tissue obtained was subjected to AFB staining, AFB culture sensitivity, aerobic/anaerobic culture sensitivity histopathological examination and polymerase chain reaction (PCR) using 16srRNA as primer. Serology was performed by ELISA in 27 cases of dorsal spine at admission and one and three month’s postoperatively. AFB staining (direct) and AFB culture sensitivity was positive in six (12%) cases. Aerobic/anaerobic culture sensitivity was negative in all cases. Histology was positive for TB in all the cases. The PCR was positive in 49 (98%) cases. All dorsal spine tuberculosis cases showed fall of IgM titer and rise of IgG titer at three months as compared to values at admission. Histopathology and PCR was diagnostic in all cases of osteoarticular tuberculosis. The serology alone was not found diagnostic.