Tuberculosis is a disease of great antiquity and has almost certainly caused more suffering and death than any other bacterial infection.\textsuperscript{20} Despite the availability of effective chemotherapy, it is still a major health problem in most countries of the world. In the past tuberculosis has been referred to as ‘the great white scourge’ and, by John Bunyon, as ‘the captain of all of these men of death’. The clinical features of both pulmonary and spinal tuberculosis were well described by Hippocrates in about 400 B.C.,\textsuperscript{21} accounts of the disease appeared in the Vedas and other ancient Hindu texts, in which it was sometimes termed Rajayakshman – the king of diseases (Petersen, 1919)\textsuperscript{22} and it afflicted Neolithic man and pre Columbian Amerindians (Clark et al, 1987)\textsuperscript{23}.

The transmissible nature of tuberculosis was clearly established by Jean-Antoine Villemin, a French military doctor. In 1868 Villemin published the results of a series of studies in which he convincingly demonstrated that tuberculosis could be produced in rabbits by inoculating them with tuberculous material from man or cattle. The disease could be passaged from animal to animal and differences in virulence were
observed between human and bovine material. In addition, Villemin established that scrofula (tuberculous cervical lymphadenitis) and pulmonary tuberculosis were different manifestations of the same disease.  

Villemin’s prediction that causative agent of tuberculosis would be isolated was realized in 1882 when Robert Koch succeeded in culturing it on inspissated serum. By a large series of inoculation’s with pure cultures of the bacillus, several generations removed from the primary one, Koch transmitted the disease to many animals of different species. His classical study established without doubt that the bacillus he isolated was the cause of tuberculosis. To this day its demonstration affords the sole infallible criterion for diagnosing tuberculosis in all its diverse form.  

In addition to culturing the causative organism, Koch succeeded in staining it with alkaline methylene blue for 24 hours, subsequently this was modified by Ehlerich and further by Ziehl-Neelsen. After Koch’s discovery, acid fast bacilli were isolated from cases of tuberculosis like disease in various animals and were named after the host from which they were isolated. Five main types of ‘tubercle bacilli’ were recognized –
human, bovine, vole, avian and cold blooded. The first 4 correspond to *Mycobacterium tuberculosis*, *M.bovis*, M. microti and *M.avium* respectively. The ‘Cold blooded’ tubercle bacilli comprise two species of rapidly growing mycobacteria, namely *M.fortuitum* (synonym *M.ranae*, the frog tubercle bacillus) and *M.chelone* (the turtle tubercle bacillus), and the slow growing fish tubercle bacillus (*M.marinum*).  

The generic name Mycobacterium (fungus bacterium) was proposed by Lehmann and Neumann (1896) in reference to the mould like pellicle formed by *M.tuberculosis* on liquid media. The genus contains over 50 well-defined species. Though essentially a genus of free-living saprophytes, the mycobacteria include the causative agents of tuberculosis, leprosy and chronic hypertrophic enteritis (Johne’s disease) of cattle. Some of the saprophytic species occasionally cause disease in animals and man; these have a number of unsatisfactory epithets including ‘atypical’, opportunistic, tuberculoid and ‘MOTT’ (mycobacteria other than typical tubercle) bacilli.  

The more aesthetic term ‘nyrocine mycobacteria’ proposed by Grange and Collins (1983) has not,
unfortunately, entered general usage; perhaps the best name at present is ‘environmental’ mycobacteria.

**The Genus Mycobacterium**:

**Definition**:

The only genus in the family Mycobacteriaceae. Straight or slightly curved rods, but coccobacillary, filamentous, and branched forms also occur. Cells are gram positive (though not easily stainable by this method), acid fast, nonmotile and nonsporing. Some strains produce yellow pigment in the dark or after exposure to light. Aerobic or microaerophilic. Acid is produced from sugars oxidatively. Nutritional requirements and temperature range of growth vary considerably. Two major subdivisions are recognized: rapid growers and slow growers. Cell wall contain large amount of lipid. The genus is distinguished by characteristic antigenic patterns and mycolic-acid structures. G+C content of DNA 66-72 mol% (except *M.leprae* which has 55 mol%). Type species: *M.tuberculosis*.25
**Mycobacterium Species**:

The genus *Mycobacterium* is very well classified as a result of extensive studies undertaken by International Working Group of Mycobacterial Taxonomy (Wayne et al, 1971\textsuperscript{27}, 1981\textsuperscript{28}; Kubica et al, 1972\textsuperscript{29}; Meissner et al, 1974\textsuperscript{30} and Saito et al, 1977\textsuperscript{31}). The approved lists of bacterial names (Skerman et al, 1980)\textsuperscript{32} includes 41 mycobacterial species (Table-A), several others have been described and reintroduced subsequently (Table-B).
**Table-A**: Alphabetical list of approved mycobacterial names

<table>
<thead>
<tr>
<th>Name</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. africanum</em></td>
<td><em>M. malmoense</em></td>
</tr>
<tr>
<td><em>M. asiaticum</em></td>
<td><em>M. marinum</em></td>
</tr>
<tr>
<td><em>M. aurum</em></td>
<td><em>M. microti</em></td>
</tr>
<tr>
<td><em>M. avium</em> (avian tubercle bacillus)</td>
<td><em>M. neoaurum</em></td>
</tr>
<tr>
<td><em>M. bovis</em></td>
<td><em>M. nonchromogenicum</em></td>
</tr>
<tr>
<td><em>M. chelonei</em> (or <em>M. cheloneae</em>)</td>
<td><em>M. parafortuitum</em></td>
</tr>
<tr>
<td><em>M. chitae</em></td>
<td><em>M. paratuberculosis</em> (Johne’s bacillus)</td>
</tr>
<tr>
<td><em>M. duvalii</em></td>
<td><em>M. phlei</em></td>
</tr>
<tr>
<td><em>M. farcinogenes</em></td>
<td><em>M. scrofulaceum</em></td>
</tr>
<tr>
<td><em>M. flavescenes</em></td>
<td><em>M. senegalense</em></td>
</tr>
<tr>
<td><em>M. fortuitum</em></td>
<td><em>M. simiae</em></td>
</tr>
<tr>
<td><em>M. gadium</em></td>
<td><em>M. smegmatis</em></td>
</tr>
<tr>
<td><em>M. gastri</em></td>
<td><em>M. szulgai</em></td>
</tr>
<tr>
<td><em>M. gilvum</em></td>
<td><em>M. terrae</em></td>
</tr>
<tr>
<td><em>M. gordonae</em></td>
<td><em>M. thermoresistibile</em></td>
</tr>
<tr>
<td><em>M. haemophilum</em></td>
<td><em>M. triviale</em></td>
</tr>
<tr>
<td><em>M. intracellulare</em></td>
<td><em>M. tuberculosis</em> (the tubercle bacillus)</td>
</tr>
<tr>
<td><em>M. kansasii</em></td>
<td><em>M. ulcerans</em></td>
</tr>
<tr>
<td><em>M. komossense</em></td>
<td><em>M. vaccae</em></td>
</tr>
<tr>
<td><em>M. leprae</em> (the leprosy bacillus)</td>
<td><em>M. xenopi</em></td>
</tr>
<tr>
<td><em>M. lepraemurium</em> (the rat-leprosy bacillus)</td>
<td></td>
</tr>
</tbody>
</table>
Table-B: Species of mycobacteria published subsequently to the Approved Lists of Bacterial Names of 1980.

<table>
<thead>
<tr>
<th>Species</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. agri</em></td>
<td><em>M. aichense</em></td>
</tr>
<tr>
<td><em>M. chubuense</em></td>
<td><em>M. obuense</em></td>
</tr>
<tr>
<td><em>M. rhodesiae</em></td>
<td><em>M. tokaiense</em></td>
</tr>
<tr>
<td><em>M. austroafricanum</em></td>
<td><em>M. diernhoferi</em></td>
</tr>
<tr>
<td><em>M. fallax</em></td>
<td><em>M. porcinum</em></td>
</tr>
<tr>
<td><em>M. pulveris</em></td>
<td><em>M. shimoidei</em></td>
</tr>
<tr>
<td><em>M. shinshuense</em></td>
<td><em>M. sphagni</em></td>
</tr>
</tbody>
</table>

With few exceptions each species is a well-defined and distinct taxonomic entity, and speciation by numerical or Adansonian taxonomic correlated closely with that obtained by Antigen analysis (Standford and Grange, 1974)\textsuperscript{33} and DNA relatedness (Baess, 1979)\textsuperscript{34}. Difficulties arise in the case of *M. tuberculosis* group (*M. tuberculosis*, *M. bovis*, *M. africanum* and *M. microti*) which are so closely related that they should really be considered as variants of one species and the *M. avium* group (*M. avium*, *M. intracellulare*, *M. paratuberculosis*, *M. lapramurium*) which could likewise be regarded similarly.\textsuperscript{35}
Humans are the only reservoir for *M. tuberculosis* despite its ability to infect other primates. *M. tuberculosis* is a strict aerobe, non-spore forming, non-motile rod that grows slowly (doubling time 12 to 18 hours). On complex solid media (LJ or MB 7H 10 agar) incubated at 37°C buff coloured colonies are visible in 3 to 6 weeks. Virulent strains form strands or cords. A high lipid content – 25 percent, in contrast to 0.5% for gram positive bacteria and 3% for gram negative bacteria ones – characterizes all mycobacteria. Mycolic acid is the principle component of the complex lipids of mycobacteria such as mycosides, wax D, cord factor and sulfolipids. The contribution of each constituent to virulence remains obscure. For instance, wax D a complex of peptides, polysaccharides and mycolic acid, enhances cell mediated immune response against mycobacterial proteins. Cord factor is lethal to mice and inhibits polymorphonuclear leucocyte migration. Sulpholipids inhibit activation of macrophages and thus, microbicidal molecules. Therotically, a permeability barrier consequent to the complexing of carbfuchsins with mycolic acid residues in the cell wall of mycobacteria is the basis for the phenomenon of acid fastness, a property shared by some strains of Nocardia.
*M. tuberculosis* can be differentiated from other mycobacteria by its production of niacin, reduction of nitrates, growth in presence of paranitrobenzoic acid. Most strains of *M. bovis* do not produce niacin (96%) and reduce nitrates (91%).

**The laboratory approach to the recovery and identification of mycobacteria**:

The classic laboratory approach to the diagnosis of mycobacterial infections involves microscopic identification and culture on solid media which is time consuming. New techniques and revised algorithms for the recovery, identification and susceptibility testing of mycobacteria are being implemented in many clinical laboratories in view of the changes in the clinical manifestations and epidemiology of tuberculosis. In the short term, conventional methods will be used; however, rapid techniques for the recovery and identification of mycobacterium species from clinical specimens are being introduced. The following is a review of both the conventional and newer techniques for laboratory diagnosis of mycobacterial infections.
Specimen collection and Processing:

Specimen Collection:

Mycobacteria may be recovered from a variety of clinical specimens, including respiratory specimens, urine, faeces, blood, cerebrospinal fluid, tissue biopsies and aspirations of any tissue or organ. Thus successful isolation of mycobacteria from clinical specimens begins with properly collected and handled specimens. All specimens should be transported to the laboratory and ideally should be processed as soon as possible after collection. If immediate transport is not possible, the specimen should be refrigerated, but no longer than overnight. Delays in processing lead to false-negative cultures and increased bacterial contamination.37

In suspected mycobacterial disease as in all other infectious diseases, the diagnostic procedure begins at the patient’s bedside. Collection of proper clinical specimens requires careful attention to detail by health care professionals. Specimens should be collected in sterile, leak proof, disposable and appropriately labeled containers.36
The spectrum of illness caused by mycobacterium species is so broad that almost any site may yield an acceptable specimen. Each specimen type even when properly collected, transported and processed, may have an intrinsic maximal yield. This can be the result of tubercle burden at the collection site or by environmental factors such as pH that may affect recovery. Emphasis should be placed on collecting the number and types of specimens that, when transported and processed correctly maximize diagnostic yield.

Types of clinical specimens acceptable for mycobacteriological diagnosis.\textsuperscript{36,37}

\textbf{Respiratory :}

Spontaneous expectorated sputum

Normal saline – nebulized [induced sputum]

Tracheal aspirate

Bronchoalveolar lavage (BAL).

Gastric aspirate

Laryngeal swab

Nasopharyngeal swab.

Bronchoalveolar brushings.
**Body Fluids:**

- Pleural fluid
- Pericardial fluid
- Joint aspirate
- Peritoneal fluid
- Cerebrospinal fluid
- Feces
- Urine
- Pus

**Body Tissues:**

- Blood, bone marrow biopsy / Aspirate, Solid organ, Lymph node biopsy, Bone, Skin.

**Collection of specimens:**

1. **Pulmonary Specimen:**

   a) Sputum expectorated spontaneously:

   To raise sputum, patient must be instructed to take a deep breath, hold it momentarily, and then cough deeply and vigorously. Patients must also be instructed to cover their mouths carefully while coughing and to discard tissues in appropriate receptacle. Saliva and nasal secretions are not to be collected nor is the patient to use oral antiseptics during the period of collection. Sputum samples collected by expectoration are best obtained shortly after the patient awakens in the morning, when mycobacteria are in the highest concentration.
b) Other Pulmonary Specimens:

When sputum is not obtainable, bronchoscopy may be performed at which time samples such as bronchial washings, bronchoalveolar lavage are obtained. Brushings appears to be more diagnostic than washings. This may be the result of an inhibitory effect on the mycobacteria of the volumes of lidocaine used in adult during bronchoscopy or of dilution of the specimen with saline. Often patients are able to produce sputum for several days after bronchoscopy, these samples should be collected and examined.\textsuperscript{37}

Gastric aspirates are used to recover mycobacteria that may have been swallowed during the night. This type of specimen only be used for patients who fail to produce sputum by aerosol induction, children under 3 years of age and non ambulatory individuals.\textsuperscript{36,37} Children with primary pulmonary tuberculosis typically have closed caseous lesions with relatively small number of organisms.\textsuperscript{37} The most desirable gastric lavage is collected at the patient’s bedside before the patient arises and before exertion empties the stomach. A series of three specimens are collected within 3 days.\textsuperscript{36,37} Abadco and colleagues (1992)\textsuperscript{38} have suggested that gastric lavage is better
than bronchoalveolar lavage for the detection of mycobacteria in children. They reported that a bacteriological diagnosis of childhood pulmonary tuberculosis could be made in 10% versus 50% respectively, when BAL was compared with three morning samples of gastric lavage.

The aerosol (saline) induced sputum procedure can be best done on ambulatory patients who are able to follow instructions. Aerosol induced sputum have been best collected from children as young as age 5 years. This procedure should be performed only in an enclosed area with appropriate airflow and by operators taking all appropriate safety measures to avoid exposure. The patient is instructed to inhale slowly and deeply through the mouth and to cough at will, vigorously and deeply, coughing and expectorating into a collection tube. The procedure is discontinued if the patient fails to raise sputum after 10 minutes or feels any discomfort. Ten milliliters of sputum should be collected and submitted. Specimens should be delivered promptly to the laboratory and refrigerated if processing is delayed. If the patient continues to raise sputum, a second specimen should be collected and submitted.36
2. Urine Specimen:

For examination of urine a first morning mid stream specimen is preferred. The entire volume of voided urine, or a minimum of 15 ml, is collected in a sterile container. Some have suggested collecting of such samples for 3 successive days. Twenty-four hour urine specimens are undesirable because of excessive dilution, higher contamination and difficulty in concentration.\textsuperscript{36,37}

3. Fecal Specimen:

Examination of stool specimens for the presence of acid-fast organism can be useful in identifying patients (such as individuals with AIDS) who may be at risk of developing disseminated mycobacterial disease resulting from \textit{M. avium} complex. Stool sample should be collected in clean container (not necessarily sterile) without preservative or diluents and not contaminated with urine.\textsuperscript{36}

4. Blood:

Immunocompromised patients, particularly those who are infected with HIV, can have disseminated mycobacterial infection; the majority of these infections are caused by
*M. avium* complex. Recovery of the organism from blood is associated with clinical disease. Blood for culture of mycobacteria should be collected in a manner as for routine blood culture.\textsuperscript{36,37}

5. **Tissue and Body Fluids**:

Tissue or body fluids should be collected aseptically and placed in a sterile container. If the tissue is not processed immediately, a small amount of sterile saline should be added to prevent dehydration. For body fluids like CSF, pleural, peritoneal, pericardial, synovial the amount of fluid collected is critical and the maximum amount possible should be collected for better results. It is recommended that if possible up to 10-15 ml of fluid may be collected. It may be necessary to collect fluid containing fibrinogen into container with an anticoagulant.\textsuperscript{36,37}

6. **Wounds, Skin Lesions and Aspirates**:

If attempting to culture a skin lesion or wound, an aspirate is the best type of specimen to collect. If volume is insufficient for aspiration, pus and exudates may be obtained
on a swab and then placed in a transport medium such as Amie’s or Stuart’s (dry swabs are unacceptable).36

**Specimen Processing:**

To ensure optimal recovery of mycobacteria from clinical specimens, many specimens must be processed before inoculation onto culture media. Each step must be carried out with precision. Specimens from sterile sites can be inoculated directly to media after concentration to reduce volume. However, specimens that may contain commensalistic bacteria should be decontaminated, concentrated and digested.37

Most clinical specimens such as sputum, contain mucin or organic debris that surround the bacteria within the sample. An abundance of non-mycobacterial organism as well as possible mycobacteria make up the microflora of these specimens. When placed into culture medium, the abundant non-mycobacterial organism can quickly overgrow the more slowly growing mycobacteria. The purpose of decontamination and digestion process are:
1) To liquefy the sample through digestion of the proteiniaceous material.

2) To allow the chemical decontamination of the sample by killing the non-mycobacterial organism.

3) Concentrate the sample.

The high lipid content in the cell walls of mycobacteria makes them somewhat less susceptible to the killing action of various chemicals. Additionally, liquefying the mucin enables the mycobacteria to come in contact and use the nutrients of the medium to which they are subsequently inoculated.\textsuperscript{36,37,39}

The optimal decontamination procedure requires an agent that is mild and yields growth of mycobacteria while controlling contaminants. The use of selective or antibiotic treated media may diminish the need for harsh decontamination procedures. The bactericidal action of decontaminating agent is influenced by the concentration of the chemical agent, exposure time and temperature; therefore alteration in any of these factors may increase or decrease the bactericidal effect. In general, a range that is considered acceptable in this delicate balance is between 2% to 5% of bacterially contaminated mycobacterial cultures.\textsuperscript{37}
The specific gravity of the tubercle bacilli ranges from 1.07 to 0.79. Because of the low specific gravity of the acid-fast bacilli a low centrifugal force has a buoyant rather than sedimenting effect. Kent and Kubica (1985) suggested that 95% sedimentation efficiency should be the goal for recovery. Therefore concentration centrifugation speeds must be at least 3000 g to maximize recovery. Disadvantage of high speeds is generation of considerable heat, which may affect recovery of mycobacteria. This may be overcome by using refrigeration centrifuge.

The standard digestion decontamination procedure are listed below:

Specimens that require digestion and decontamination are sputum, gastric washings, bronchoalveolar lavage, transtrachial aspirates, urine, autopsy tissue, abdominal fluid and any fluid known to contain contaminants.

**Sodium Hydroxide**:

The traditional decontamination method by Petroff (1915) in which sputum or other material are treated with 4% sodium hydroxide for 15-30 minutes before being neutralized.
Concentration of NaOH used may be varied from 2% to 4% and time of exposure also varied by different laboratories. NaOH acts both as digestant and decontaminating agent.

**N-acetyl – L-Cysteine – Sodium hydroxide (NALC-NaOH):**

A combination of a liquifying agent such as NALC or dithiothreitol and sodium hydroxide is commonly used. Contamination is controlled with a lower concentration of sodium hydroxide, the recovery of mycobacteria is indirectly improved. The addition of phosphate buffer makes strong shifts in pH less likely. Buffer also serves to wash the specimen, dilute toxic substances and decreases the specific gravity of the specimen so that centrifugation is more effective in sedimentation of organisms.

**Benzalkonium Chloride:**

Another digestant – decontamination procedure uses benzalkonium chloride (Zephiran) combined with trisodium phosphate (Z-TSP). Trisodium phosphate liquefies sputum rapidly but requires long exposure to decontaminate the specimen. Benzalkonium chloride shortens the exposure time and effectively destroys many contaminants, with little
bactericidal effect on the tubercle bacilli.\textsuperscript{37} Specimens containing large numbers of \textit{M.tuberculosis} can withstand the action of these agents for as long as overnight, and careful timing of exposure is not required.\textsuperscript{39}

The addition of phosphate buffer to digested specimens results in greater isolation of mycobacteria. Zephiran is bacteriostatic for tubercle bacilli, necessitating either neutralization before plating or use of egg-based media to exploit its inherent neutralization capacity. Neutralization is achieved by addition of lecithin.\textsuperscript{37,39}

**Dithiotheritol plus 2\% NaOH :**

Dithiothreitol is also an effective mucolytic agent when used with 2\% NaOH. Trade name of Dithiotheritol is sputolysin. Sputolysin is more expensive than NALC. Exposure is limited to 15 minutes.\textsuperscript{39}

**Cetylpyridium Chloride (1\%) :**

Effective as a decontaminant solution for sputum specimens mailed from outpatient clinics. Tubercle bacilli have survived 8-day transit without significant loss. It contains 2\% sodium chloride.\textsuperscript{39}
**Oxalic Acid (5%)**

Oxalic acid is used to decontaminate specimens contaminated with *Pseudomonas aeruginosa*, such as sputum specimens from patients with cystic fibrosis.\textsuperscript{37,39}

**Microscopic Examination**

Robert Koch (1882) stained the tubercle bacillus with hot alkaline methyl blue as the primary stain and Vesuvin as the decolorizer and counterstain. Shortly afterwards Ehrlich (1882) discovered the now well known ‘acid fast’ property, staining the bacilli with hot fuchsin in the presence of aniline oil as a mordant and destaining with dilute mineral acid. Ziehl changed the mordant to phenol and Neelsen combined the dye and mordant to form carbol fuchsin. Thus the staining technique, although pioneered by Ehrlich, is now known as Ziehl-Neelsen (ZN) method.\textsuperscript{20,25}

**Staining of Acid Fast Bacilli**

Mycobacteria possess cell walls that contain mycolic acids, which are long-chain, multiple cross-linked fatty acids. These long chain fatty acids probably serve to complex basic dyes, contributing to the characteristic of acid-fastness that
distinguishes mycobacteria from other bacteria. Mycobacteria are not the only group with this unique features. Species of Nocordia, Rodococcus, Legionella miedadei, are partially acid fast. Cyst of the genera Cryptosporidium and Isospora are distinctly acid fast. The mycolic acids and lipids in the mycobacterial cell wall probably account for the unusual resistance of these organisms to the effects of drying and harsh decontaminating agents, as well as acid-fastness. When Gram stained mycobacteria usually appear as slender, poorly stained, beaded gram positive bacilli; sometimes they appear as “gram neutral” or “gram-ghosts” by failing to take-up either crystal violet or safranin.36

Staining Methods:

Two types of staining procedures are used in the laboratory for rapid detection and confirmation of acid-fast bacilli.39

1) Carbolfuchsins stains: a mixture of fuchsin with phenol (carbolic acid).

   a) Ziehl-Neelsen stain (hot stain)

   b) Kinyoun (cold stain)
2) Flurochrome stain: Auramine O, with or without a second flurochrome, rhodamine.

Although workers in various laboratories may be partial to either the carbolfuchsin or the fluorescent staining method, the specificity of detecting mycobacteria by the two methods seems about the same, with the possible exception of *M. fortuitum* which may be missed by the later one.\(^\text{39}\)

Flurochrome stain is more sensitive than the conventional carbolfuchsin stains, because the fluorescent bacilli standout brightly against the dark background. Smears can be scanned with 25 X objective thereby increasing the field of view and reducing the time needed to scan a given area of the slide. In addition a positive fluorescent smear may be restained by the conventional Ziehl-Neelsen or Kinyoun procedure.\(^\text{39}\)

**Carbolfuchsin Stains:**

The classical carbolfuchsin (ZN) stain requires heating the slide for better penetration of stain into the mycobacterial cell wall; hence it is also known as hot stain procedure. The Kinyoun acid-fast (cold) stain is similar to ZN stain but without heat, hence the term “cold stain”. The only difference is that in
cold stain the concentration of basic fuchs in and phenol in 
carbolfuchsin is increased, therefore heating the smear is 
avoided. If present typical acid fast bacilli appear as purple to 
red, slightly curved, short or long rods (2 to 8 µm); they may 
also appear beaded or banded or pleomorphic, usually coccoid. 
The background may be blue or green depending on the 
counter stain used i.e. methylene blue or malachite green. 
Although the ZN stain and the Kinyoun stain are theoretically 
the same the former is more sensitive in detecting lightly 
staining organisms particularly some strains of the rapidly 
growing *M.fortuitum – chelonei* complex due to thin waxy 
capsule.\(^{39}\)

**Fluorescence Staining :**

Fluorescence microscopy was introduced by Hagemann 
(1937)\(^ {41}\) who originally used berberine sulphate as the dye but 
later (1938) recommended auramine. Traunt et al (1962)\(^ {42}\) used 
2 arylmethane dyes, auramine O and rhodamine B together. 
This combined staining method detected AFB in 358 of 3000 
samples of sputum as against 274 by ZN staining (Somlo et al, 
1969)\(^ {43}\).
**Microscopic Examination:**

Although microscopy alone does not usually distinguish between members of the *M. tuberculosis* group and other acid fast bacilli, it is none the less a rapid and simple means of detecting pulmonary disease that are a source of infection.\(^{20}\)

The visualization of acid fast bacilli in sputum or other clinical material should be considered only a presumptive evidence of tuberculosis because stain does not specifically identify *M. tuberculosis*. For example M.gordonae, a non pathogenic scotochromogen commonly found in tap water, has been a problem when tap water or deionized water has been used in the preparation of smear or even when patients have rinsed their mouths with tap water before the use of aerosolized saline solution for inducing sputum.\(^{36}\) Cross contamination of slides may also occur. However the incidence of false positive smears is very low when good quality control is maintained. Acid-fast stained smears of clinical sputum require atleast $10^4$ acid-fast bacilli per ml for detection by conventional microscopy.\(^{36,39}\)

The overall sensitivity of an acid-fast smear ranges from 20% to 80%.\(^{36,37}\) Acid-fast smears are also useful in following a
patient’s response to treatment. After antimycobacterial drugs are started, cultures become negative before the smears do, suggesting that the organisms are not capable of replicating but are capable of binding the stain. With continued treatment, more organisms are killed and fewer shed, so that assessing the number of organisms in the sputum during treatment can provide an early objective measure of response. Should the number of organisms fail to decrease after therapy is started, the possibility of drug resistance must be considered and additional cultures and susceptibility studies should be conducted.

Deshmukh et al (1996) compared modified Schaeffer and Fulton stain, Ziehl-Neelsen stain and cold stain for 187 sputum samples. They found 67 (35.82%) positive by ZN stain and cold stain method while 66 (35.29%) were positive by modified Schaeffer and Fulton method. Considering ZN staining as a standard method the positivity rate was 100% with cold staining and 98.50% by modified Schaeffer and Fulton method.

In a study by Venkataraman et al (1998) who processed 205 clinical specimens, 129 (62.9%) smears were found positive and 76 (37.1%) smears were found negative by Ziehl-Neelsen
staining. Bhargava et al (2001)\textsuperscript{1} studied a total of 100 clinical specimens which consisted of 73 pulmonary and 27 extrapulmonary. Z-N staining was positive in 54 (73.97\%) pulmonary and 5 (18.51\%) extrapulmonary specimens. A total of 125 sputum specimens stained by Ziehl-Neelsen method by Selvakumar et al (1995)\textsuperscript{46} found 27 that is (21.6\%) were positive by microscopy.

Jain et al (2002)\textsuperscript{47} compared ZN staining and fluorescent auramine-rhodamines (AR) staining for 715 clinical samples, (222 extrapulmonary and 493 sputum). Overall 32.3\% AFB positivity was observed by microscopy using both the techniques, 42.2\% in sputa and 9.9\% in extrapulmonary specimens. ZN staining showed 23.4\% AFB smear positivity (32.7\%) in sputa and 1.4\% in extrapulmonary specimens. Auramine Rhodamine staining showed 31.87\% AFB smear positivity (41.6\% in sputa and 9.9\% in extrapulmonary specimens). Overall 208 cases were discovered, out of which ZN contributed only 164 (78.8\%) cases, which included 3 cases (1.4\%) missed by auramine-rhodamine method. The AR found 205 (98.5\%) and missed 3 cases; the difference in case-yields
was found to be highly significant (p<0.001). They found AR staining to be more sensitive then ZN method.

In another study by Lakshmi et al (2006)\textsuperscript{48} who studied 3,597 clinical samples (Pulmonary 1,568 & extrapulmonary 2,029) by ZN staining, got a positive result in 559 (35.65\%) pulmonary and 111 (5.47\%) extrapulmonary samples.

Of the 159 samples tested by Negi et al (2007)\textsuperscript{49} smear microscopy by Ziehl Nelsen staining 35 (49.2\%) were positive in the 71 pulmonary samples and 21 (23.86\%) were positive in 88 extrapulmonary samples. The overall positivity was 56 (35.2\%) for the 159 samples.

**Culture of Specimens for Recovery of Mycobacteria :**

Robert Koch grew the tubercle bacillus on heat coagulated bovine or sheep serum – a culture medium invented by the Irish Physist John Tyndall. Nocard later introduced glycerol – Beef broth that Koch used for his studies on tuberculin.\textsuperscript{25}

The recovery of mycobacteria from agar culture media was poor when first attempts were made late in the 19\textsuperscript{th} century. Through experimentation it was found that a culture medium containing whole egg, potato flour, glycerol and salts solidified
by heating to 85° to 90°C for 30 to 45 minutes was effective in isolating *M. tuberculosis*. The process of solidifying protein containing medium by heat is known as inspissation. An inspissated culture medium is more subject to liquefaction from the effects of proteolytic enzymes produced by contaminating bacteria than a medium solidified by the addition of agar. However it was soon discovered that the use of aniline dyes, such as malachite green or crystal violet in the inspissated medium helped control contaminating bacteria. The concentration of the dye must be carefully controlled; if too high, the growth of mycobacteria may also be inhibited along with the contaminating bacteria. Malachite green is the dye most commonly incorporated into nonselective culture media, in concentrations ranging between 0.0025 g/100 ml and 0.052 g/100 ml.39

The many different media available for the recovery of mycobacteria from a clinical specimen are variations of three general types; egg-based medium, serum albumin agar medium and liquid medium. Within each general type, there are nonselective formulations and formulations that have been made selective by addition of antimicrobial agents.37
Various media available are mentioned below:

<table>
<thead>
<tr>
<th>Medium</th>
<th>Composition</th>
<th>Inhibitory agents</th>
</tr>
</thead>
<tbody>
<tr>
<td>American Thoracic Society (ATS)</td>
<td>Fresh whole eggs, potato flour, glycerol</td>
<td>Malachite green (0.02%)</td>
</tr>
<tr>
<td>Lowenstein-Jensen (LJ)</td>
<td>Fresh whole egg, defined salts, glycerol potato flour</td>
<td>Malachite green (0.025%)</td>
</tr>
<tr>
<td>Petragnani</td>
<td>Fresh whole eggs, egg yolk, whole milk, potato, potato flour, glycerol</td>
<td>Malachite green (0.052%)</td>
</tr>
<tr>
<td>Middlebrook 7H 10</td>
<td>Defined salts, vitamins, cofactors, oleic acid, albumin, catalase, glycerol, glucose.</td>
<td>Malachite green (0.00025%)</td>
</tr>
<tr>
<td>Middlebrook 7H 11</td>
<td>Defined salts, vitamins, cofactors, oleic acid, albumin, catalase, glycerol, 0.1% casein hydrolysate</td>
<td>Malachite green (0.0001%)</td>
</tr>
<tr>
<td>Middlebrook 7H 9, 7H 12 (BACTEC 12 B)</td>
<td>Broth base, casein hydrolysate, bovine serum albumin catalase, C-14 labelled palmitic acid, deionised water</td>
<td>Polymyxin B, Amphoterecin B, Nalidixic acid, Trimethoprim, Azlocillin</td>
</tr>
<tr>
<td>Middlebrook 7H 9, 7H 13 (BACTEC 13 A)</td>
<td>Broth base, casein hydrolysate, bovine serum albumin catalase, C-14 palmitic acid, deionized water</td>
<td>Polymyxin B, Amphoterecin B, Nalidixic acid, Trimethoprim, Azlocillin</td>
</tr>
<tr>
<td>Gruft (modification of LJ)</td>
<td>Fresh whole eggs, defined salts, glycerol, potato flour, RNA.</td>
<td>Malachite green, Penicillin, Nalidixic acid</td>
</tr>
<tr>
<td>Mycobacttosel (BBL) LJ</td>
<td>Fresh whole eggs, defined salts, glycerol, potato flour.</td>
<td>Malachite green, Cyclohexamide, Lincomycin, Nalidixic acid</td>
</tr>
<tr>
<td>Middlebrook 7H 10 (selective)</td>
<td>Defined salts, vitamins, cofactors, oleic acid, albumin, catalase, glycerol, glucose</td>
<td>Malachite green, Cyclohexamide, Lincomycin Nalidixic acid</td>
</tr>
<tr>
<td>Mitchison’s selective 7H 11</td>
<td>Defined salts, vitamins, cofactors, oleic acid, albumin, catalase, glycerol, glucose, casein hydrolysate</td>
<td>Carbencillin, Amphotericin B, Polymyxin B. Trimethoprim lactate.</td>
</tr>
</tbody>
</table>
**Egg Based Media:**

Of the several egg-based culture media for the recovery of mycobacteria Lowenstein-Jensen (LJ) medium is most commonly used in most clinical diagnostic laboratories. The basic ingredient in an inspissated egg medium such as LJ, Petragnani, and American Thoracic Society (ATS) media, are fresh whole eggs, potato flour, and glycerol, with slight variations in defined salts, milk and potato. Each contains malachite green to suppress the growth of gram positive bacteria.\(^{37,39}\)

Lowenstein-Jensen medium is less inhibitory to the growth of mycobacteria than is Petragnani medium, which is used primarily to recovery mycobacteria from specimens heavily contaminated with bacteria. Conversely, the American Thoracic Society (ATS) medium, which contains only 0.02 g/100 ml of malachite green is less inhibitory to the growth of mycobacteria and is recommended for use in usually sterile specimens, such as CSF, pleural fluid and tissue biopsies.\(^{39}\)

Selective egg media that contain antibiotics, such as Gruft modification of LJ and Mycobactosel (Becton Dickinson Microbiology System, Cockeysville Md), are some times used in
combination with nonselective media to increase isolation of mycobacteria from contaminated specimens.\textsuperscript{37}

**Serum Albumin Agar Media** (Media of Cohen and Middlebrook)

During the 1950s, Cohen and Middlebrook developed a series of defined culture media. These media were prepared from defined salts and organic chemicals; some contained agar, but all had albumin.\textsuperscript{39}

Serum albumin agar media such as Middlebrook 7H10 and 7H11 agars, are prepared from a basal medium of defined salts, vitamins, cofactors, glycerol, malachite green, and agar combined with an enrichment consisting of oleic acid, bovine albumin, glucose, and beef catalase (Middle Brook OADC enrichment). Middlebrook 7H11 also contains 0.1% casein hydrolysate, which improves recovery of isoniazid resistant strains of *M.tuberculosis*.\textsuperscript{37}

Middlebrook agar media are transparent and allow early detection of growth after 10-12 days instead of 18-24 days incubation required on egg media. This is partly due to the inclusion of biotin and beef catalase to stimulate revival of damaged bacilli in specimens. Albumin is also incorporated to
bind toxic amounts of oleate and other compounds that might be released from spontaneous hydrolysis of Tween 80. The albumin does not appear to be metabolized by the bacilli.  

Although essentially all culture media yield more growth and larger colonies of mycobacteria when incubated in 5% to 10% CO₂, the Middlebrook media absolutely require capneic incubation for proper performance. Chromogenic studies and biochemical studies are more accurate when performed on subculture from LJ medium. In contrast to opaque egg based media, clear agar-based media can be examined using a dissecting microscope for early detection of growth (micro colonies) and observing certain well defined morphological features within 10 days.  

The addition of antimicrobial agents to either 7H10 and 7H11 makes the medium more selective by suppressing the growth of contaminating bacteria and fungi. Selective 7H11 is a modification of oleic acid agar medium first described by Mitchison. The medium was originally designed for use with sputum specimens without the use of decontaminating agent. Mitchisons medium contains carbencillin, polymyxin, trimethoprim lactate, and amphotericin B. McClatchy suggested
reducing the concentration of carbencillin from 100 μg/ml to 50 μg/ml and using 7H11 medium instead of oleic acid agar. He called this modification 7H11 selective or S7H11. Similarly middle brook 7H10 selective is also available and contains malachite green (Absent in S7H11), cycloheximide (to prevent fungal contamination), lincomycin and nalidixic acid.³⁹

**General Growth Characters of Mycobacteria:**

Mycobacteria are strictly aerobic and grow more slowly than most bacteria pathogenic for humans. The generation time of the mycobacteria is more than 12 hours, *M.tuberculosis* has the longest replication time at 20 to 22 hours. The most rapidly growing species generally grows on simple media in 2 to 3 days at temperatures of 20° to 40°C.³⁷

Inoculated culture media are usually incubated for at least 8 weeks, with weekly inspection for growth. Most strains of *M.tuberculosis* appear within 4 weeks, but they may not be visible for 8 weeks or more if they originated from patients treated with antituberculous agents. Extended incubation is required for the isolation of very slowly growing species such as *M.xenopi* and *M.malmoense*. If incubated for 6-12 weeks there is an increased isolation by 4.1% for *M.tuberculosis* and 10.5% for
MOTT except for *M. malmoense* 70% (Ispahani & Baker, 1968). Cultures should be incubated at 35°C and those of specimens from skin lesions should be incubated at 33°C as some skin pathogens (*M. marinum, M. haemophilum* and *M. ulcerans*) do not grow at the higher temperatures. Culture techniques may detect as few as 10-100 organisms/ml of sputum.

In a study by Chia et al (1990) who studied 284 clinical specimens (236 sputum and 48 pleural fluids) found culture on LJ to be positive in 69 specimens (61 sputum and 8 pleural effusion) i.e. 17.3%. Banavalikar et al (1998), cultured on LJ medium a total of 85 clinical specimens and got a culture positive result in 25 out of 135 (18.51%) sputa, 2 out of 10 (20%) bronchial aspirates, 2 out of 10 lymphnode aspirates (20%), 2 out of 10 pleural fluids (20%) none (0%) out of 20 blood specimens. Altogether 31 specimens were culture positive on LJ medium. Of the 35 pulmonary cases studied by Beerbal et al (1999), 27 (77.14%) were found to positive by culture on LJ medium and were confirmed as *M. tuberculosis*. Similar results were obtained by Prasad et al (2001) who reported a culture positivity of 70.7% (46 specimens) in 65 sputum specimens studied by him.
Borun et al (2001) found 127 specimens positive for growth on LJ medium out of 265 clinical specimens (48%).

Shenai et al (2004) studied 94 respiratory and 45 non-respiratory specimens, positive cultures were found in 64 (68%) and 28 (62%) specimens respectively. In a study by Chakravorty et al (2005) no culture was found to be positive in 53 pleural fluid specimens, 8 pleural tissue specimens and 15 lymph node aspirates.

**Use of Broth/Liquid Culture Media**:

Mycobacteria species grow more rapidly in liquid media. Middle brook 7H9 broth is a nonselective liquid medium used for subculturing stock strains, picking single colonies, and preparing inoculum for in vitro testing.

In general the use of a liquid media system reduces the turn around time for isolation of acid-fast bacilli to approximately 10 days on an average 2 to 14 days. These detection times are an obvious improvement in the recovery time from conventional media.

Three semi-automated and automated mycobacteria detection systems are currently available. The time honored
and tested radiometric BACTEC system, and the recently introduced non-radiometric MB/BacT system and the ESP Mycosystem. Two viable alternates are the manual Septi-Chek system and the MGIT system for use in laboratories without automation.\textsuperscript{36,39}

**BACTEC AFB System**:

The most sensitive and rapid primary isolation liquid media are Middlebrook 7H12 and 7H13 (BACTEC 12B and 13B, Becton Dickinson Diagnostic Instruments System, Towson, Md). The BACTEC is automated radiometric culture system for detecting the growth of Mycobacteria species.

The BACTEC medium contains BACTEC 12B broth containing an antibiotic ‘Cocktail’ of Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim, Azocillin (PANTA) and the substrate $^{14}$C-labeled palmitic acid. The vial is inoculated with 0.5 ml of the processed specimen and placed in a 35°C incubator. If mycobacteria are present in the inoculum, $^{14}$Co$_2$ is released into the headspace, the amount of which is a direct reflection of the amount of growth in the vial. When a designated period of incubation has passed, usually about 3 days, the vials are placed on the track of the BACTEC 460
instrument in preparation for reading. The amount of radioactivity is measured in the aspirated head gas, which is translated into a numerical value called the growth index (GI). A GI higher than 10 is considered positive; however, acid fast staining must be performed on a small aliquot of broth to confirm the presence of AFB.\textsuperscript{39}

Disadvantages of the system include the cost of the instrumentation, inability to observe colony morphology and detect mixed cultures, overgrowth by contaminants, need for disposal of radioactive materials, extensive use of needles.\textsuperscript{32} False positive results from cross contamination do occur and may not be a good recovery medium for M. fortuitum and \textit{M. avium} complex.\textsuperscript{39}

The value of the BACTEC in detection of mycobacteria from sputum, blood and other clinical specimens has been demonstrated in several field trials and clinical co-relation studies.\textsuperscript{56,57} In a multicenter collaborative study, the recovery of \textit{M. tuberculosis} from clinical specimens known to be smear positive can be accomplished by BACTEC system in 14 days compared with 21 days by standard culture method.\textsuperscript{58} Similarly smear negative culture positive specimens were positive for
growth of *M. tuberculosis* in an average 14 days by the BACTEC method compared with 25 days by the standard culture method. These detection times are an obvious improvement in the recovery time from conventional media.59

Initial studies of the sensitivity of this system in monitoring mycobacterial growth showed that an inoculum of 200 viable *M. tuberculosis* bacilli could be detected in 12 to 13 days, whereas if one waited for 14 to 17 days as few as 20 viable bacilli could be detected.60

The capability of performing rapid mycobacterial drug susceptibility studies is an additional advantage of the BACTEC system. In multicentric collaborative study the total time for isolation and drug susceptibility testing of *M. tuberculosis* was completed in an average of 18 days by the radiometric method in comparison with an average of 38 days with standard procedures.59

The BACTEC instrument also allows the differentiation of *M. tuberculosis* from other mycobacteria in inoculating one additional bottle containing p-nitro-acetylamino hydroxy-propiophenone (NAP). *M. tuberculosis* and *M. bovis* do not grow in
NAP containing culture media and therefore will not produce a positive GI after several days of incubation.\textsuperscript{39}

The Middlebrook 7H13 medium (BACTEC 13B) was introduced for the culture of larger volumes of blood or bone marrow. Its components are similar to those of the BACTEC 12B vial except that an anticoagulant, sodium polyethenol sulfonate (SPS) and polycarbonate 80 have been added. Five ml of blood may be added directly to this 30 ml vial.\textsuperscript{37}

In a study carried out by Venkataraman et al (1998)\textsuperscript{45} on 130 sputum and 109 extrapulmonary specimens the difference in the isolation rate of LJ and BACTEC was not statistically significant for smear positive samples. But for smear negative samples in the pulmonary group 3 were positive by the conventional method as to 7 by the BACTEC method. Considering the negatives, 51 specimens were negative by the conventional method while only 35 by the BACTEC method. The above difference was statistically significant. For extrapulmonary specimens also, the number of positives by both the culture methods was similar. As for the growth rate BACTEC was superior with 87 percent of the positives being obtained by 7 days and 96\% by 14 days, compared to 2 to 6
weeks by LJ. In extrapulmonary 81% positives were detected by 14 days as against none by conventional method by 2 weeks the difference being highly significant. Majority of specimens positive by conventional methods were obtained between 3 and 5 weeks.

In a study carried out by Bhargava et al (2001), number of positive samples detected by LJ and BACTEC was almost similar but the growth rate (mean isolation time) of mycobacteria by BACTEC method was reported to be 20 days and five weeks for LJ medium.

Shenai et al (2004) compared BACTEC (BACTEC460 instrument) with LJ for 94 respiratory and 45 non-respiratory specimens. In case of respiratory specimens comparable results were obtained in 32 cases with LJ positive in 64 (68%) and BACTEC in 74 (79%) specimens with a sensitivity, specificity, PPV, NPV and concordance of 98.4%, 63.3%, 85.1%, 95.0%, 87.2% respectively. In case of non-respiratory specimens LJ was positive in 28 (62%) and BACTEC in 29 (64%) specimens with a sensitivity, specificity, PPV, NPV and concordance of 92.9%, 82.4%, 89.7%, 87.5% and 88.8% respectively. TB BACTEC was found superior in detecting 11
additional TB cases, which were negative by LJ. All these patients were found to be on anti-TB treatment indicating that BACTEC (12B) liquid medium supported growth of tubercle bacteria better than conventional media. Whereas LJ detected 2 new cases which were missed in BACTEC, the author explained by saying that some strains of \textit{M. tuberculosis} require egg-based media for growth and egg based media allow traces of toxic material to be neutralized enhancing the growth of mycobacteria. A average detection time for TB-BACTEC was found to be 13.16 days compared to 31.18 days by LJ method in case of respiratory specimens and 15.30 days compared to 35.25 days by LJ for nonrespiratory specimens.

Lakshmi et al (2006)\textsuperscript{48} studied 3,597 specimens (1568 pulmonary and 2029 extrapulmonary) over a period of 5 years. Significantly greater number (p<0.001) of mycobacteria could be isolated from the clinical specimens by BACTEC 681/3597 (18.93\%) than by the LJ medium 140/3597 (3.98\%). The recovery time of \textit{M. tuberculosis} by the BACTEC was shorter for smear positive specimens (pulmonary 3.2 days) than for smear negative specimens (pulmonary 15.9 days), while for extrapulmonary smear positive or negative, it was same (21.8
The average recovery time on the LJ medium was four weeks for any type of specimen.

A similar experience was of Negi et al (2007) who tested 71 pulmonary samples, 37 (57.11%) were positive for *M. tuberculosis* on LJ and in case of BACTEC system 42 (56.75%) were positive for *M. tuberculosis*. Of the 88 samples for extrapulmonary tuberculosis LJ detected 38 (42.18%) and BACTEC system 43 (48.63%) tuberculosis cases. The mean detection time for *M. tuberculosis* was 24.03 days by LJ medium and 12.89 days by BACTEC.

Rodrigues et al (2007) evaluated the efficacy of BACTEC 460 TB system by studying 12,726 clinical specimens for detection of *M. tuberculosis* over a period of six years. Of this 7,153 were respiratory with positive rate of 3987 (56%) specimens with BACTEC and 2970 (42%) specimens by LJ. Of the total 5,573 non-respiratory specimens positive rate of 1019 (18%) specimens was seen by BACTEC and 737 (13%) specimens by LJ method. This difference was found to be statistically significant. An average detection time for TB-BACTEC was found to be 13.3 days compared to 31.2 days by LJ method in case of respiratory specimens. For nonrespiratory
specimens an average detection time was found to be 15.3 days by TB BACTEC compared to 35.3 days by the LJ method.

Venkataswamy et al (2007) evaluated BACTEC 460 TB system and LJ for isolation of *M.tuberculosis* from 2325 CSF specimens over a period of 3 years. They found 256 yielded growth on both BACTEC and LJ combined. BACTEC 12B medium could successfully support the growth in 237 (93%) CSF specimens and LJ supported growth in 101 (39%) of the specimens. All the strains isolated were *M.tuberculosis*. One hundred and fifty one specimens yielded growth only in the BACTEC as against 19 (7%) only on LJ medium. The average recovery time for BACTEC was 15 days, while it was 35 days by the LJ medium. The contamination rate for BACTEC was 4% and that for LJ medium was 10%.

Thus from the above studies it proves that BACTEC system is similar in its sensitivity to detect cases of tuberculosis although some studies here reported a marginal better result with BACTEC. The main advantage of BACTEC system lies in mean recovery time which is shown by the above studies to be definitely superior to conventional LJ culture.
MB/BacT Mycobacterial Detection System:

MB/BacT Mycobacterial Detection System is an automated system to detect mycobacteria. The MB/BacT process bottles containing 10 ml of enhanced Middlebrook 7H9 broth in an atmosphere of Co$_2$, nitrogen and O$_2$ under vacuum. This bottle therefore provides suitable nutritional and environmental conditions to recover the more commonly encountered Mycobacterium species from clinical specimens other than blood. It contains antibiotics and proprietary growth factors added to each bottle just before use to enhance the growth of mycobacterium species and curtail the growth of contaminating bacteria that may survive the decontamination and concentration procedure. The bottom of each broth bottle is fitted with a gas permeable sensor that changes from a dark green to a bright yellow when Co$_2$ is produced in the broth by metabolizing mycobacteria. Bottles are placed bottom down within individual wells in the incubator chamber and reflected light is used to continuously monitor the production of microbial generated Co$_2$.$^{39}$

Although multicenter field trials sponsored by the manufacture indicate that the MB/BacT system recovered a
higher percentage of mycobacteria with less time to detection when compared with conventional and compared favourably in parallel cultures with the BACTEC 460 TB system, the overall performance when used in clinical setting remains to be determined. Advantage is that it eliminates the need for handling and disposing of radioisotopes and is fully automated and so less labour intensive.\textsuperscript{39}

Mahadev et al (2001)\textsuperscript{63} processed 205 specimens by MB/BacT-240 system and LJ culture. A total of 101 isolated were detected by both the methods. The colorimetric method picked up 117 (57.1\%) positives and the conventional method 113 (55\%). The contamination rate were 1.0\% (2) and 6.8\% (14) respectively for the colorimetric and conventional method. The mean detection time of mycobacteria by the colorimetric and conventional method was 14 and 24 days respectively. The advantage of MB/BacT over LJ was found to be in higher yield by 15.3\% from extrapulmonary specimens. Out of the 26 extrapulmonary isolated, 10 specimens were positive by MB/BacT where as only 6 positive by LJ.
The ESP Myco system:

Each culture bottle contains modified Middlebrook 7H9 medium, casitone, glycerol and cellulose sponges. The sponges increases the culture surface area for the mycobacteria, simulating the alveoli of the lungs. Immediately prior to inoculation of specimen, each bottle is supplemented with antibiotic mixture polymyxin B, vancomycin, nalidixic acid and amphotericin B (PVNA).

Each culture bottle when placed in a special drawer in the incubation module, attached to a sensor, consisting of a plastic housing, a recessed needle and a hydrophobic membrane. Thus each bottle is constantly monitored for any change in gas pressure due to the metabolic activity of the micro-organisms. Significant pressure change may be signaled early from the consumption of oxygen; or, later with the production of gas by the metabolizing mycobacteria.37,39

Field trials sponsored by manufactures indicated the ESP Myco System agrees favourably with BACTEC 460 system. Off note, specimens from patients receiving antituberculous therapy were at times not positive in either both systems, indicating the need to inoculate solid culture media in parallel
with the broth bottles. The results from this study further indicate that the ESP Myco System detected positive cultures first about 3 times more frequently than the BACTEC 460 system and greater than 7 days sooner in about ½ of the cultures, presumably based on the early signal derived during the oxygen consumption phase of the growth cycle. Although the results of this field trial are impressive, the overall performance in clinical settings remains to be determined. Another advantage is no use of radioactive material.\textsuperscript{39}

**Septi-Chek AFB System:**

The septi Chek AFB system is a capped bottle containing 20.0 ml of modified Middlebrook 7H9 broth under 20% CO\textsubscript{2}, an enrichment consisting of growth enhancing factors and antimicrobial agents. A second component is a plastic tube, fitted on one end with a removable screw cap, within which is enclosed a two faced paddle, on both surfaces of which are embedded solid culture media. One surface of the paddle is covered with nonselective Middlebrook 7H11 agar, the reverse side is divided into two sections, and containing modified LJ medium and other containing chocolate agar to detect the growth of contaminating bacteria.
The biphasic media system provides for growth for rapid identification and drug susceptibility testing without the need for routine subculturing. The agar surface and the culture media are examined every third day for the presence of growth, after which if negative, the assembly is again inverted to reinoculate the agar media and incubated in an upright position at 35°C.\textsuperscript{36,37,39}

In a four center study in which over 3000 clinical specimens of various sources were studied, Isenberg and coworkers (1991)\textsuperscript{64} found that the Septi-Chek system was more sensitive than LJ and 7H11 and BACTEC Broth in the percentage of mycobacterial isolates recovered. The authors concluded that this better recovery rate could be attributed to the biphasic nature of the system and the advantage gained from repeated early exposure of the agar media to actively proliferating organisms in the broth phase. Compared with LJ and 7H11 the average number of days for recovery of mycobacteria was less for the Septi-Chek AFB system averaging 3 days less for the detection of \textit{M.tuberculosis} and 12 days for \textit{M.avium–intracellulare}. Although the time of recovery for the BACTEC system was less by an average of 3 days for
M. tuberculosis and 12 days for M. avium-intracellulare compared with Septi-Chek system, the former does not provide for isolated colonies. Therefore, the time to final identification was about equal between the two systems. Similar results were found by D’Amato and associates (1991) in which LJ agar was added to the paddle.

Sewell et al (1993), also confirmed that both the rate and time of recovery of M. tuberculosis, M. avium-intracellulare, and M. gordonae from smear positive respiratory secretions were improved with the Septi-Chek AFB system compared with LJ medium. In this study BACTEC 12 A bottles detected growth of the Mycobacterium specifies earlier than Septi-Check, however the later provided isolated colonies and did not require special equipment for detection of growth.

**Mycobacteria growth indicator tube (MGIT) system:**

This system consists of 16 x 100 mm round bottomed glass tube containing 4 ml of modified 7H11 broth base, to which has been added 0.5 ml of OADC enrichment (oleic acid, bovine albumin, dextrose and catalase) and 0.1 ml PANTA antibiotic mixture. A fluorescent compound is embedded in silicone on the bottom of the tube. The fluorescent compound
is sensitive to dissolved oxygen in the broth; that is, the presence of oxygen in the uninoculated medium serves to quench the emission of fluorescent light. As the actively growing bacteria consume the dissolved oxygen, the fluorescence is unmasked and can be detected by observing the tube under long-wave ultraviolet light (woods lamp). Tubes are read with a woods lamp, placing the tube with the test mixture between a positive (sodium sulphite solution) and a negative (uninoculated MGIT tube) control. Bright orange colour in the bottom of the tubes with an orange reflection also seen on the meniscus indicates positive result. Positive tubes are stained for AFB with ZN staining.\textsuperscript{39} A fully automated version of this, BACTEC MGIT 960 (M960) is also available with Middlebrook 7H9 media. MGIT PANTA and MGIT growth supplement (OADC) are added to it. The M960 detects the growth automatically, flashing red light to indicate instrument positive tubes and green for negative one.\textsuperscript{67}

Rodrigues et al (1997)\textsuperscript{8} studied 101 clinical specimens and found culture results of MGIT and LJ similar except for three CSF samples which did not grow on MGIT but were positive on LJ. MGIT helped significantly in the earlier
detection of *M.tuberculosis* (within 8-9 days) as compared to LJ (minimum 3 weeks).

Rishi et al (2007) demonstrated that BACTEC MGIT 960 (M960) system provided better isolation rate of mycobacteria (98.06%) from a variety of clinical samples than the LJ media (63.95%). This higher positivity for BACTECT MGIT (M960) was both in pulmonary and extrapulmonary samples, not only in smear positive but also in smear negative samples. Besides higher isolation rate, even the time to detect mycobacteria was shorter on MGIT, 9.66 days (2-39) as compared to 28.81 days (7-48) with LJ media.

**Isolator lysis centrifugation system :**

Isolator (Wompole Laboratories, Cronbury, N.J.) is a collection system that contains saponin to liberate intracellular organisms. After treatment with saponin, the sample is inoculated into mycobacteria media plates and tubes. The system allows for higher yields and shorter recovery times for mycobacteria. It offers the advantage of yielding isolated colonies and the ability to quantitate mycobacteria, which may be useful in monitoring the effectiveness of therapy in disseminated *M.avium* complex infection. David et al (2004)
recommends the use of lysis centrifugation technique followed by direct smear of the sediment along with subsequent subculturing on to modified LJ medium and selective Kirchner’s medium followed by subculturing onto the modified Lowenstein Jensen medium for diagnosing mycobacteremia.

Witebsky et al (1988)\textsuperscript{69} compared BACTEC 13 A medium and Du pont isolator for detection of mycobacteremia. Concentrate from the isolator system were inoculated on Middlebrook 7H11 plates and also in 12B bottle. Their results for the two systems was comparable. BACTEC 13A medium has an advantage over the isolator system in requiring less laboratory manipulation of the specimen but has the disadvantage of not providing isolated colonies or quantitation of organisms. Similar results were found by Kietin and Cammarata (1998)\textsuperscript{70} for the recovery of \textit{M.avium} and \textit{M.intracellulare} from patients with AIDS. Average time for detection was 16 and 14 days for the isolator and BACTEC 13A systems respectively. There was no significant difference between the two blood culture systems in sensitivity or time to detection.
The BACTEC MYCO/F LYTIC:

The BACTEC MYCO/F LYTIC culture bottle contains a lytic agent to release the mycobacteria that have been phagocytosed by white blood cells. It is incubated and monitored automatically in a manner similar to the other BACTEC blood culture bottles. In a direct comparison of many of the commercially available products, Crump et al (2003) evaluated the performance of the BACTEC 13A (BD Diagnostics), BACTEC MYCO/F LYTIC (BD Diagnostics), BacT/ALERT MB (Bio Merieux) and ISOLATOR 10 Lysis centrifugation (Wompole Laboratories) systems for the detection of mycobacterimia in adults. There were no significant differences found in the yields between the systems. However, there were differences in the mean time to detection among the systems. The time to detection was shorter for BacT/Alert MB, followed by BACTEC MYCO/F LYTIC and BACTEC 13A and then ISOLATOR 10 lysis system. They found the continuously monitored systems (BACTEC MYCO/F LYTIC and BacT/Alert MB) to be a sensitive, and on balance, faster for the detection of \textit{M. avium - intracellulare} bacteremia than the standard manual isolator 10 and radiometric BACTEC 13A system.
**T.K. Medium :**

T.K. Medium (Salubris, Inc, MA, USA) is a novel colorimetric system that indicates growth of mycobacteria by changing its colour. Metabolic activity of growing mycobacteria changes the colour of the medium and this enables an early positive identification before bacterial colonies appear. The test can distinguish between mycobacteria and contamination (By difference in colour). T.K. Medium also enables susceptibility testing for drug resistance and can allow for differentiation between *M. tuberculosis* and NTM. The test is not currently FDA approved. Data from a multicenter study in Turkey demonstrated that the T.K. medium’s sensitivity for mycobacterial detection was comparable to that of the Lowenstein – Jensen medium. The average time to detection with TK medium was 2 weeks as compared to 4 weeks with LJ medium.73

**Identification of mycobacterial isolates :**

The definitive diagnosis of tuberculosis requires recovery of the causative organisms in culture. Several mycobacterium species other than *M. tuberculosis* have emerged as important pathogens, each with different potential for producing disease
and often with unique antimicrobial drug susceptibility profiles. Therefore identification of species plays an important part in the patients treatment strategies. The various methods of identification of mycobacterial isolates are mentioned below.

1. Conventional methods: The two things which are used to identify are growth characters and biochemical tests. Growth characters include, growth rate, colony texture, pigment production, and in some instances the permissive incubation temperature (25°C, 37°C and 45°C) for mycobacteria. Biochemical tests include tests like niacin test, nitrate reduction, catalase, tween 80 hydrolysis, tellurite reduction, arylsulphatase, growth inhibition on media containing para-nitrobenzoic (PNB) acid, growth inhibition on media containing thiophene-2-carboxylic acid hydrazide (TCH), iron uptake, urease pyrazinamidase, growth on MacConkeys agar, growth in 5% NaCl. These are however time consuming and sometime ambiguous results are obtained. They require patience, familiarity with the endpoints of different identification characteristics and a collection of control strains. There is no universally accepted scheme for this and the techniques vary considerably from laboratory to laboratory. Some workers use a
battery of tests to identify all isolates at species level while others use a small number of tests to group strains into groups or complexes of species of similar clinical significance. Most clinical isolates belong to the *M.tuberculosis* group and most workers limit identification to separation of *M.tuberculosis* group from other mycobacterial species. For this the test used are niacin, nitrate reduction, growth on media containing para-nitrobenzoic acid (PNB). A similar differentiation of *M.tuberculosis* group from other species is done in BACTEC radiometric culture system by doing the p-Nitro-acetylaminobetahydroxypropiophenone test (NAP). Morgan et al (1985) evaluated the NAP test for the identification of *M.tuberculosis* complex for 480 clinical isolates by the direct and indirect NAP test. For the indirect test carried out by inoculating two Middlebrook 12 A BACTEC test vials (one control). These bottles were incubated at 37°C for 1 to 3 days or until the growth index (GI) reached 50; then 1 ml of the medium was removed and added to an NAP test vial containing 5 ug of NAP per ml. GI readings were taken daily for a maximum of 5 days from the NAP test vial and compared with normal growth in the control vial. The TB complex is inhibited
in the presence of NAP and the other species of mycobacteria are not, resulting in a decrease or no significant increase in GI as compared with the control vial, in which the GI increased.

In the direct method, when the BACTEC culture vial containing a specimen from a patient registered a GI of 50 or greater, a small volume of the medium was removed and stained for acid-fast bacilli. If smear was positive the specimen was checked for bacterial contamination by subculturing to a blood agar plate and observing for growth after 18 hours incubation. A 1 ml sample of culture medium from a non-contaminated test vial with a GI between 50 and 100 was directly transferred into an NAP test vial and incubated. GI readings were taken daily for a maximum of 5 days on the NAP and control vials. Data were interpreted as for the indirect test method. The NAP test was compared with conventional identification methods. The NAP test correctly identified 399 of the 400 isolates (99.8%) with only one isolate of *M. tuberculosis* incorrectly classified in the indirect NAP test. Once adequate growth was obtained for inoculation into the NAP test vial, test results are available in 5 days.
2. **Analysis of lipid profiles**: Mycobacteria have characteristic lipid profiles. These lipid profiles can be analysed by high performance liquid chromatography (HPLC). Tinsdall et al (1979)\textsuperscript{75} performed identification of 18 mycobacterial species by gas liquid chromatography. By using this technique 58% of specimens were correctly identified to the species level and additional 41% were correctly identified to a group of two or three organisms, for eg, *M.tuberculosis*, *M.bovis*, *M.xenopi* or *M.fortuitum* and *M.chelonei*. In another study by Thibert and Lapierre (1993)\textsuperscript{76} HPLC was used to identify clinical isolates of mycobacteria. HPLC identified 96.1%, whereas the biochemical procedures and/or the commercial DNA probe, identified 98.3% of strains. HPLC allowed early detection and identification of the rare mycobacterial species, M.haemophilum, *M.malmoense*, M.shimoidei, *M.fallax* as well as uncharacteristic strains of *M.simiae*. After 18 months of routine use, HPLC proved to be reliable, easy to perform, rapid and less costly than other identification methods.

3. **Use of nucleic acid probes**: Based on information about specific gene sequence well defined oligonucleotide probes for identification of various clinical relevant mycobacteria have
been used and are readily available. When used along with newer methods for detection of the early growth (such as BACTEC, septi-check, MGIT) these are of great help in rapidly confirming the diagnosis as identification of isolate can be established within 1 to 2 days. The probes may be DNA or RNA probes. RNA targeting probes are 10-100 fold more sensitive than DNA targeting probes and may be used to confirm the diagnosis directly in the clinical specimens in a good proportion of cases, the lowest detection limit is around 100 organisms. At present they are useful mainly for rapid identification of mycobacterial isolates. Genproge Inc, SanDiego California pioneered and brought to market single stranded 125 I-labeled DNA probes complementary to the rRNA of the following target organisms; \textit{M.tuberculosis} complex, \textit{M.avium-intracellulare}, \textit{M.Kansasii M.gordonae}. Recently nonisotopic probes (e.g. acridinester-labelled probe, chemiluminicent probe etc.) have become available, directed towards the identification of the mycobacterial species.

Kiehn and Edwards (1987) combined the BACTEC TB system with the nucleic acid probe targeted to the rRNA of \textit{M.avium} to rapidly identify this species when recovered from
blood cultures of patients with AIDS. In another study by Ellner et al (1988) the combination of BACTEC recovery and DNA probe assay identified 89% of 176 isolates of *M.tuberculosis* and 89% of 110 isolates of *M.avium*. Most impressive was the reduction, by 5 to 7 weeks of the time to give final report as compared with their conventional isolation and biochemical methods. To decrease the time of detection even more, Forbes & Hicks (1994) applied PCR amplification to detect *M.tuberculosis* recovered in BACTEC 12 B broth cultures. By using PCR, positive BACTEC 12 B vials could be assessed when the growth index (GI) reached 10, shortening the time of incubation required for the GI to reach 100 or more. The use of PCR in this study resulted in a mean time to detection of *M.tuberculosis* of 9 days compared with 14 days by using nucleic acid probes from growth of BACTEC 12 B subculture on solid media.

4. **Gene amplification methods for identification**: Different strategies to identify the isolates from cultures and also directly from the clinical specimens have been described. These include amplification of gene regions followed by hybridization with species specific probes, sequencing and RFLP (Restriction
fragment length polymorphism) analysis.\textsuperscript{68,69} These PCR-RFLP assays help in quick identification of pathogenic mycobacteria including \textit{M. tuberculosis} from the culture isolates as well as from the clinical specimens. While PCR sequencing approach can be applied by reference laboratories, the RFLP approaches are easily practicable in clinical mycobacterial laboratories.\textsuperscript{77}

Katoch et al (2007)\textsuperscript{81} used gene amplification RFLP analysis using primers encoding 16S as well as 23S rRNA gene. Distinct gene amplification restriction analysis patterns were obtained by restriction of amplicons with three distinct restriction endonucleases (Hha1, Hinf and Rsal) which could differentiate various mycobacterial species. The assay could easily distinguish \textit{M. tuberculosis} complex from other non-chromogenic mycobacteria (\textit{M. avium, M. intracellulare}).

5. \textbf{In situ hybridization} : This technology uses an oligonucleotide probe labeled with a detector molecule. If the detector molecule is fluorescein and interpretation is made by direct observation using a fluorescence microscopy, it is referred to as fluorescence in situ hybridization (FISH). If, however, detection of the hybridized probe is achieved through secondary reaction and colour, rather than light produced, the reaction is
termed chromogenic in situ hybridization (CISH). Recently FISH has been used to identify bacterial and yeast pathogens in positive blood cultures. This technique has been used to identify *M.tuberculosis* in culture that become positive and in direct respiratory specimens that contain acid fast bacilli. This technology has been used by pathologists to identify mycobacteria in formalin fixed, paraffin embedded tissues and to confirm the identity of *M.leprae*.$^71$

**Immuno-diagnostic tests:**

There is a need for a sensitive and specific immunodiagnostic test, especially when sputum smear microscopy is not helpful. New reagents, both purified antigens and monoclonal antibodies may provide the means to obtain better sensitivity and specificity. Serological test may be antigen based or antibody based.$^9$

**Antigen detection:**

Free mycobacterial antigens may be detected in the various types of body fluids at a minimum concentration of 3-20 ug/ml. the most commonly used antigens are purified protein derivative (PPD), glycolipids, sulpholipids,
lipopolyscharides, antigen 5 (38 KDa), antigen A60, 45/47 KDa antigen, antigen KP90, 30 KDa antigen, P32 antigen, lipoarabinomannan (LAM), cordfactor (trehalose-6, 6’dimycolate) and phenolglycolipid – lipid antigen (PLG TP 1).³

The methods for detecting antigens of *M. tuberculosis* include – sandwich ELISA, inhibition ELISA, latex agglutination and reverse passive haemagglutination tests. ELISA is useful for early diagnosis of all forms of TB. Capture ELISA detects LAM in urine samples.³² Tiwari et al (2007)⁹ have found the sensitivity and specificity of Dipstick method to be 93% and 95% respectively.

A vast array of potential diagnostic antigens is available, but no single antigen gives 100% sensitivity. In a recent study a “cocktail” of purified cell wall antigens incorporated on the surface of liposome particles is used. Assure TB rapid test uses “cocktail” of novel antigens MTB 11 (CF10), MTB8, MTB48, MTB81 and 38 KDa protein immobilized on a membrane, in lateral flow devices. The proteins are conjugated to colloidal gold. MTB81 has been found to be promising antigen in serodiagnosis of HIV-TB co-infection.⁸³
Karin et al (2005) identified four superior sero-antigens by cloning and expressing 35 *M. tuberculosis* proteins as recombinant proteins in *Escherichia coli* and analysed their serodiagnostic potential. By this method they could identify four superior serodiagnostic antigens TB9.7, TB 15.3, TB 16.3 and TB 51, none of them described earlier. They were tested with sera from both smear positive and smear negative patients from TB endemic and non-endemic areas. The single most potent antigen was found to be TB 16.3, with sensitivity of 48-55% with Danish samples and 88-98% with samples from African patients.

Biswas et al (2002) examined for the presence of anti ES-31 IgG antibody and free and immune complexed antigen ES-31 by stick penicillinase ELISA test in a group of 40 patients with tuberculosis. Antibody was detected in 30 of 40 patients (75%) using affinity purified anti-ES-31 antibody. Free antigen could be detected in 25 of 40 cases (62.5%) and immune complex in 20 (50%). Respective specificity values for free, immune complexed antigen and antibody detection test were 85%, 95% and 80%.
With histochemical localization of *M. tuberculosis* complex antigen with antibody to 38 KDa antigen Goel and Budhwar (2007)\(^8^6\) found 100% positivity as compared to 36.1% positivity with AFB smear.

Shende et al (2007)\(^8^7\) isolated and identified tuberculosis antigen from sputum positive and negative patients. 170KDa, 140 KDa, 85 KDa, 55 KDa, 43 KDa, 20 KDa, and 16 KDa were positive in sputum positive sera, while 85 KDa, 55 KDa, 43 KDa and 20 KDa were positive in sputum negative cases.

**Antibody detection:**

Antibodies to mycobacterial antigens, present in clinical specimens are detected by using monoclonal and polyclonal antibodies. Cross-reaction by environmental mycobacteria may produce false positive results. The currently available methods for purifying mycobacterial antigens are not reproducible and therefore, the results of antibody detection assay are variable in different settings. It is also unlikely that the immune system of all patients will recognize a single mycobacterial antigen alike.\(^8^2\)
Some of the commercially available antibody detection tests are listed below:

<table>
<thead>
<tr>
<th>Test</th>
<th>Antigen used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mycodot (Dot blot assay)</td>
<td>Lipoarabinomannan (LAM)</td>
</tr>
<tr>
<td>Detect TB (ELISA)</td>
<td>Recombinant peptide.</td>
</tr>
<tr>
<td>Pathozyme-Myco (ELISA)</td>
<td>38 KDa (recombinant) &amp; LAM</td>
</tr>
<tr>
<td>Pathozyme – TB (ELISA)</td>
<td>38 KDa (recombinant)</td>
</tr>
<tr>
<td>Antigen-A – 60 (ELISA)</td>
<td>Antigen 60</td>
</tr>
<tr>
<td>ICT diagnostics (Membrane-based)</td>
<td>38 KDa (recombinant)</td>
</tr>
</tbody>
</table>

In comparison a combination of two tests (ICT & pathozyme-myco) yielded the best results, with a sensitivity of 66% and specificity of 86%. Isocitrate dehydrogenase (ICD) encoded by icd-2 gene of BCG has been reported to be sensitive diagnostic reagent in antibody based assay.\(^8\)

Alifano et al (1997)\(^8\) evaluated IgA mediated humoral immune response against mycobacterial antigen P90 in diagnosis of pulmonary tuberculosis. This was performed by enzyme immunoassay. The overall sensitivity and specificity of the test were 70.9% and 91.9% and respectively higher titres
were shown by patients with active tuberculosis as compared to control group.

Radio-immunoassay for antibody detection in pulmonary tuberculosis was performed by Kadival et al (2000). They found it to be sensitive in 81% and specific in 96%. Differentiation between patient and control population was possible. The assay was user friendly and rapid. Antigen used was sonicated extract of autoclaved H₃₇RV.

In another study by Zuber Ahmed et al (2002) anti A60 IgG titres were measured in 120 FNAC confirmed patients of tuberculous lymphadenitis. Overall sero-positivity was 78.33%. Significantly low sensitivity (66.7%) was seen in patient with less than three months of duration of illness, while all patients with more than six months of illness were serpositive. Patients up to 1 cm lymph nodes showed low positivity (66.67%) compared with 83.35% in patients with more than 1 cm lymph nodes. Antibody titers declined or increased at variable rates after anti-tuberculous treatment. Decline in IgG titers was seen in all patients after six months of treatment and 70% of the patients became seronegative. They observed high false negativity in patients with shorter duration of illness.
Maekura et al (2003)\textsuperscript{92} did prospective clinical evaluation of tuberculous glycolipid test along with nucleic acid amplification test. It was done with smear negative active tuberculous patients. They found that individually these tests were not useful for rapid diagnosis of active TB.

Kunter et al (2003)\textsuperscript{93} determined whether IgG and IgM antibodies against mycobacteriial antigen A60 could be detected in serum and pleural fluid together with mantoux tuberculin test (TST) and tuberculous pleurisy could be diagnosed. Sensitivity and specificity of TST were 65\% and 68\% respectively. Pleural fluid anti A60 IgM had the highest sensitivity (77\%) and specificity (94\%) in patients with negative TST. When diagnosis of tuberculosis is definite IgM and IgG levels are invariably higher in the pleural fluid than in serum.

Analysis of three antigens for serodiagnosis of TB was done by Yoshinari et al (2004).\textsuperscript{94} These antigens were tuberculous glycolipid antigen, lipoarabinomannan polysaccharide antigen, antigen A60. The percentage of patients positive in all three tests were 58.8\% out of the total 131 patients with active tuberculosis and 71.4\% with chronic active pulmonary tuberculosis. When the results of the three tests were
combined, the sensitivity increased to 91.5% in patients with active pulmonary tuberculosis and to 86% in smear and culture negative patients. It was suggested to use combined test with three separate antigens to maximize the effectiveness of serodiagnosis.

Karen et al (2007)\(^\text{95}\) conducted a multi-national study (Metanalysis of 68 studies) to assess the accuracy of commercial antibody detection tests for the diagnosis of pulmonary tuberculosis. They concluded that overall the commercial tests vary in performance. Sensitivity is higher in smear positive samples, in these patients. Anda TB IgG by ELISA showed limited sensitivity (63% to 85%), specificity is higher in healthy volunteers and there are insufficient data to determine the accuracy of most commercial tests. They concluded that discovery of new antigens is necessary to intensify immunodiagnostic potential. The editor summarized that the commercial antibody tests were not useful in diagnosis of tuberculosis as compared to conventional tests microscopy and culture.
**Other methods:**

**Gamma Interferon Assay:**

The tubercular skin test (TST) is one of the few tests that has been in use for nearly 100 years in clinical medicine. Therefore it is not surprising that the test has important limitations. The TST uses a relatively crude mix of antigens from *M. tuberculosis*. As a result, false positive reactions can occur because of previous BCG vaccination or sensitization to non-tuberculous mycobacteria. False negative results on TST can occur because of severe illness including active tuberculosis or immune suppression, often due to HIV infection. Initial testing can affect results of subsequent tests because of anamnestic recall of immunity (Boster effect). Errors in administration or reading can lead to incorrect results.

In the past decade two new T-cell based test for diagnosis of latent TB infection have been developed and license for commercial distribution in many countries. One test QuantiFERON (QFT)-TB Gold (cellestis, Victoria, Australia), uses enzyme linked immunosorbant assay to measure antigen – specific production of interferon-γ by circulating T cells in whole
blood. The other test T-spot TB (Oxford Immunotec, Oxford, United Kingdom), uses the ELISPOT technique to measure peripheral blood mononuclear cells that produce IFN-\(\gamma\). Current version of both IFN-\(\gamma\) release assay (IGRAs) use more specific \textit{M.tuberculosis} antigens – ESAT-6, CFP-10 and TB 7.7. The genes encoding these antigens are found in the region of difference 1 (RD1) of the \textit{M.tuberculosis} genome, which is detected from the genome of \textit{M.bovis} BCG, and most strains of non-tuberculous mycobacteria (Except with the exception of \textit{M.kansasii}, \textit{M.marinum} and \textit{M.szulgai}). This antigen is more specific for \textit{M.tuberculosis} than PPD.\textsuperscript{98} The US Centres for Disease Control and Prevention has recommended that QFT replace the TST.\textsuperscript{99}

Interferon-\(\gamma\) release assays have several important advantages over the TST. Testing requires only one patient visit and these assays are ex vivo tests, which reduce the risk for adverse effects and eliminate potential boosting when testing is repeated.\textsuperscript{97}

Shams et al (2005)\textsuperscript{100} found ELISPOT to be as sensitive as the tuberculin skin test for the detection of LTBI in a large group of contact of patients with tuberculosis. ELISPOT was
more specific than the TST in BCG vaccinated persons. They found ELISPOT to be technically feasible and robust and likely to be more accurate means to diagnose LTBI than the TST.

Janssens et al (2007) performed T-SPOT TB IFN-\(\gamma\) assay it could discriminate between active and latent TB infection in immunocompetent adults with a positive T-SPOT TB result. They found a apparent relationship between active TB and T-SPOT TB with significantly higher IFN-\(\gamma\) levels being associated with active disease. However, there was considerable overlap of the quantitative results of the T-SPOT TB between subjects with TB and those with probable LTBI. T-spot TB had an sensitivity of 82.8% and specificity of 74.2% for distinguishing active from latent disease and thus can not be recommended for the diagnosis of tuberculosis.

Pai et al (2008) evaluated data from several studies on interferon \(\gamma\)-assays and found them to have high specificity even among populations that have received BCG vaccination. The pooled sensitivity was 78% for Quanti FERON TB Gold, 70% for Quanti FERON – TB Gold in tube and 90% for T-spot TB. The pooled specificity for both Quanti FERON was 99% among non-BCG vaccinated and 96% among BCG vaccinated. The pooled
specificity of T-SPOT TB was 93%. TST had a heterogeneous sensitivity but the specificity in non-BCG participants was high i.e. 97%.

**MPB64 skin patch test:**

MPB64 is an immunogenic antigen specific to *M.tuberculosis* complex. It is secreted by *M.tuberculosis*, Mycobacterium bovis and some strains of *M.bovis* (BCG).11 In 1998, Nakamura and colleagues102 demonstrated that a skin patch with MPB64 antigen was capable of eliciting a distinctive response in individuals with active, but not latent, TB. In studies conducted in Japan and the Philippines, the MPB64 patch test was able to distinguish active TB from Latent tuberculous bacterial infection (LTBI) with 98% sensitivity and 100% specificity. In an another study of Philippines, Nakamura and coworkers (2001)103 demonstrated similar results, with a sensitivity of 88% and specificity of 100% for active TB. However, the exact biological mechanism behind the skin response in these studies is still unclear. A skin patch test is currently being developed into a commercial test by Sequella, Inc. (MD, USA) using recombinant rMPT64. The test is being evaluated in trials to establish the accuracy, dose ranges and
other operational characteristics (e.g. ability to read results in dark skinned individuals). Since the patch test is simple, non-invasive and does not require a laboratory or highly skilled personnel, it has the potential to make an impact, especially if it is shown to be accurate, unaffected by anergy (e.g. due to HIV) and capable of distinguishing between LTBI and active disease in high-burden countries.

**Mycobacteriophage based methods:**

Phage-based assays have been evaluated for diagnosis of TB, as well as drug susceptibility tests (DST). They use mycobacteriophages to infect live *Mycobacterium tuberculosis* and detect the bacilli using one of the two methods. In the first method the key principle is amplification of phages (Pha B) after their infection of *M.tuberculosis* followed by detection of progeny phages as plaques on a lawn of M.smegmatis (e.g. FAST plaque – TB and phage Tek MB). For diagnosis, phage assays are directly used on sputum specimens.

In the second method a genetically engineered mycobacteriophage carrying the gene for luciferase may specifically infect only viable *M.tuberculosis*. This phage has been used for detecting viable *M.tuberculosis* directly in clinical
specimens. Luciferase enzyme oxidizes luciferin in the presence of adenosine triphosphate (ATP) and generates light. Viable mycobacteria that are infected with this reporter bacteriophage emit visible light when luciferin (substrate for luciferase) is added. The emitted light may be measured by a luminometer.\textsuperscript{11,105}

Phage based assays are rapid manual tests that use mycobacteriophage for directly detecting \textit{M.tuberculosis} in sputum and have a turnaround time of 48 hours. Fine tuning of this detection method, particularly that associated with phage infection may improve the level of sensitivity (Pai and Kalantri 2005).\textsuperscript{105}

Data from the first meta analysis on diagnosis (13 studies) suggested that phage based assays have high specificity (range 83\% to 100\%), but modest and variable sensitivity (range 21\% to 88\%). Analysis of studies that directly compared phage tests with microscopy against culture as a common reference standard suggested that though overall accuracy of phage based assays is slightly higher than smear microscopy in head to head comparison their diagnostic performance characteristics, therefore are fairly similar to microscopy.\textsuperscript{106}
In a study carried out by Shenai et al (2002) who compared phage assay with LJ & BACTEC cultures for pulmonary and extra pulmonary tuberculosis, found that for pulmonary with respect to BACTEC, sensitivity and specificity of phage assay to be 90.6% and 100% respectively. Where as with respect to LJ culture the sensitivity and specificity was 93.1% and 88.2% respectively. For extrapulmonary specimens the sensitivity and specificity of phase assay with respect to BACTEC was 90.9% and 88.8% respectively. Whereas with respect to LJ sensitivity and specificity of phage assay was 87.5% and 93.8% respectively. They found the phage assay to be simple and inexpensive as it does not require any sophisticated or dedicated equipment and results are available within 48-72 hours. As it detected viable tubercle bacilli it might be used as a sensitive tool for monitoring the treatment success.

Barman and Gadre et al (2007) found phage assay a rapid, reliable and cost effective method in diagnosis of pulmonary tuberculosis. They found a sensitivity and specificity of phage assay with respect to growth on LJ was 92.86% and 97.83% respectively.
Certain phage assays (e.g. FAST plaque TB MDRi) are designed to detect rifampicin resistance in culture isolates. Newer versions of this kit are being developed for the detection of drug resistance directly from sputum specimens (FAST plaque TB Response). Drug resistance is diagnosed when *M. tuberculosis* is detected in samples that contain the drug (e.g. rifampicin). When phage based assays do not detect *M. tuberculosis* in drug containing samples, the strains are classified as drug sensitive (Trollip et al, 2003). As seen in the meta analysis (19 studies) by Pai et al (2005) on rifampicin resistance, when performed on culture isolates phage assays appear to have relatively high sensitivity and specificity. Eleven of 19 (58%) studies reported sensitivity and specificity estimates in ≥95% when applied to isolates. These assays have relatively high sensitivity and specificity. This is expected because of the large numbers of bacilli in the isolates. However, the need for primary isolation reduces the applicability of this assay.

If phage based assays can be directly used on clinical specimens and if they are shown to have high sensitivity and
specificity, they have the potential to improve the diagnosis and management of drug resistant TB.¹¹⁰

**Detection of Protein and enzyme detection:**

The detection of microbial products or components has been used in recent years to diagnose infections caused by *M.tuberculosis*. For example, tuberculostearic acid is a fatty acid that can be extracted from the cell wall of mycobacteria and then detected by gas chromatography / mass spectrometry in clinical samples containing few mycobacteria. Because of the limited number of species that can cause meningitis and the fact that *M.tuberculosis* appears to be the only one of these species that releases tuberculosteric acid into the surrounding environment, the presence of this substance in CSF is thought to be diagnostic of tuberculosis meningitis. Performance of this assay is limited to only a few laboratories.³⁶

Adenosine deaminase (ADA) is a host enzyme whose production is increased in certain infections caused by *M.tuberculosis*. For example, elevated levels of this enzyme were found in the majority of patients with tuberculous pleural effusions (98% sensitive); the test for the enzyme was determined to be highly specific as well (96% specificity).³⁶
In a study by Parandaman et al (2000)\textsuperscript{111} who studied 25 patients with bacteriological, radiological and clinically proved tuberculosis for ADA activity in pleural fluid samples with a positive result in 19 specimens (76%) where as culture was positive in 6 patients proving the usefulness of ADA test to diagnose tuberculous pleuritis. Burgess et al (2001)\textsuperscript{112} assessed the use of ADA test in 18 patients proved to have tuberculous ascitis by bacteriological, clinical evidence, histology and response to treatment. They got a sensitivity and specificity of 94\% and 92\% respectively. They suggested it as a rapid technique for identifying patients with tuberculous ascitis.

**Approaches based on molecular biology:**

During the past decade the advantages of diagnostic molecular techniques have been so widely published that there is increased pressure on clinical microbiology laboratories to either include them in their research activities or risk being left behind in the quality of service that they provide to their clinicians and patients. Before introducing any molecular technique in a diagnostic laboratory, several strategic questions must be addressed.\textsuperscript{113}
Some of these are: (i) Which organism to target, (ii) which clinical specimen to test? (iii) Do molecular tests fulfill the required criteria of high sensitivity and specificity, speed, simplicity and clinical relevance?\textsuperscript{113}

In general molecular techniques are indicated for (i) detection of organisms that cannot be grown in vitro or for which current culture techniques are too insensitive. (ii) Detection of organisms requiring complex culture media and/or prolonged incubation time.\textsuperscript{9,113}

Since arriving at correct diagnosis of tuberculosis meets both the criteria listed above, nucleic acid amplification techniques (NAT) for diagnosis of tuberculosis have attracted considerable interest with the hope of shortening the time required to detect and identify \textit{M.tuberculosis} in respiratory specimens such as sputum or BAL.

The basic principle of any molecular diagnostic test is the detection of a specific sequence in clinical sample by hybridizing the nucleic acid it contains with a complementary sequence (probe) followed by detection of the hybrid. For detecting tuberculosis the sensitivity of this procedure ($10^3$ AFB/ml) is below that of sputum smear microscopy ($10^4$
AFB/ml) after ZN staining. Therefore its only use remaining is rapid identification of mycobacterial species from culture both from conventional agar as well as from radiometric liquid media such as BACTEC. Commercially available nucleic acid probes are available for the *M.tuberculosis* complex, the avium complex, *M.kansasii*, *M.gordonae*. This problem of low sensitivity of DNA hybridization techniques, in detecting target sequences present in the clinical samples have been over come by amplifying the target sequence in the specimens using suitably designed primers before hybridization. Any sequence of nucleic acid can be amplified by using DNA polymerase, if the information on that sequence is available.\textsuperscript{9,113}

**Amplification methods :**

It is virtually impossible to read any medical journal without encountering at least one application of polymerase chain reaction (PCR) technology. The PCR has forever changed the scope of research questions that can be addressed by virtually eliminating the problem of limited sample size. The importance and elegance of the concept has been recognized through awarding of the Nobel Prize in chemistry to its
inventor, Karry Mullis, less than 10 years after publication of the first practical application of PCR (Saiki, 1985).\textsuperscript{114}

The development of PCR by Mullis and colleagues was a milestone in biotechnology that heralded the beginning of molecular diagnostics. Although PCR is the most widely used nucleic acid amplification method, other approaches have been developed and several have unique properties and advantages. Examples of each category will be discussed in the section below. These techniques have analytical sensitivity unparalled in laboratory medicine, creating new opportunities for the clinical laboratory to impact patient care.\textsuperscript{115}

\textbf{Development of PCR }\textsuperscript{:19}

1985 – appeared the first publication of PCR technology in science.

1986 – PCR found application in DNA typing of HLA-DOA locus.

1987 – Introduction of the automated thermocycler.

1988 – Taq polymerase declared molecule of the year by science.
1990 – H. Erlich and K. Mullis received the biochemical analysis award.

1991 – RT-PCR developed using a single thermostable polymerase, facilitating diagnostic tests for RNA viruses.


1993 – Kary Mullis shares Nobel Prize in chemistry for conceiving PCR.

1994 – PCR-based testing for screening donated blood for HIV, HCV and HBV.

1995 – The first automated system for routine diagnostic PCR launched.

1996 – AMPLICOR® Mycobacterium tuberculosis test receives FDA approval.


1999 – HLA C-34 and DRB – 36 HLA typing kit for tissue typing launched.
So simple is the PCR process, at least to molecular microbiologists that its inventor, Kary Mullis, says their universal reaction has always been, “Why did not I think of that?” Among the host of scientific prizes heaped on Mullis, for the very bright idea, he says came to him during a 1983 moonlight drive in the California mountains are the best known, the Japan prize and the Nobel prize, both awarded to him in 1993.19

**Polymerase Chain Reaction (PCR):**

PCR is a simple in vitro chemical reaction that permits the synthesis of essentially limitless quantities of a targeted nucleic acid sequence (DNA or RNA), present even as a single copy in the initial preparation. At its simplest the PCR consists of (i) a DNA preparation containing the desired segment to be amplified (target sequence), (2) a molar excess of two oligonucleotide primers (about 20 bases long) specific, i.e. complementary to the two 3′- borders (the sequence present at or beyond the 3-ends of the two strands) of the desired segment, (3) a heat stable DNA polymerase e.g. Taq (isolated from the bacterium Thermus aquaticus) and (4) an equimolar mixture of deoxyribonucleotide triphosphates vis, dATP (deoxyadenosine
triphosphate), dTTP (deoxythymidine triphosphate), dCTP (deoxycystidine triphosphate) and dGTP (deoxyguanosine triphosphate) and a Tris – HCl buffer, MgCl$_2$ & KCl. This constitutes the reaction mixture.$^{115,116}$

The whole PCR procedure is carried out in completely automated and compact thermal cycler that precisely controls the temperature at which the steps occur, the length of time that the reaction is held at the different temperatures and the number of cycles. A PCR cycle consists of three steps denaturation, annealing and extension. At the end of each cycle, the PCR products theoretically doubles. Thus after n PCR cycles, the target sequence can be amplified 2n-fold. Usually, 20-30 cycles are carried out in most PCR experiments. After 20 cycles of PCR a million fold amplification is achieved and, after 30 cycles, a billion fold. In practice, the amplification may not be completely efficient, because of failure to optimize the reaction conditions or the presence of inhibitors of the DNA polymerase.$^{115,116}$

**Procedure:**

The reaction mixture is placed in small 0.2 to 0.5 ml PCR tubes and placed in the thermal cycler.
To initiate a PCR, the reaction mixture is heated 90-98°C (commonly 94°C) to separate the two strands of target DNA (denaturation), the duration of this step usually is 1-2 minutes at 94°C. The mixture is now cooled to temperature (generally 40-60°C) to permit the primers to anneal to the complementary sequence in the DNA. The step is called annealing. The duration of annealing step is usually 1 minute. The temperature is now so adjusted that the DNA polymerase synthesizes the complementary strands by utilizing 3'-OH of the primers, this reaction is the same as that occurs in vivo during replication of the leading strand of the DNA duplex. This step is called extension. The primers extend towards each other so that the DNA segment lying between the two primers is copied; this is ensured by employing primers complementary to the 3’ ends of the segment to be amplified. The duration of primer extension is usually 1-2 min at 72°C. The completion of the extension step completes the first cycle of amplification, each cycle may take few minutes (ordinary 4-5 minutes). The next cycle of amplification is initiated by denaturation which separates the newly synthesized DNA strands from the old DNA strand (target DNA). Thus at each cycle the newly synthesized
DNA strand as well as old strand anneal to the primers and serve as templates for DNA synthesis. The amount of DNA produced is thus doubled at the end of each cycle.\textsuperscript{115,116}

\textbf{Detection of amplified products :}

The amplification products from a PCR can be studied in a variety of ways to derive information about the target sequence in the original DNA molecule used as template.

1) \textbf{Electrophoretic separation :} The repeating sugar-phosphate backbone of nucleic acids results in a net negative charge evenly distributed over the linear molecules. Therefore, movement of DNA or RNA in response to an electric field will be proportional to the molecular weight or length of the molecule. This property is used to characterize the size of nucleic acid fragments by electrophoretic separation. Electrophoresis is carried out in gel either made of Agarose (usually used in conjunction with submarine electrophoresis) or acrylamide (usually used in vertical format). Multiple samples are applied in separate wells at one end of the gel along with size standards, referred to as DNA or RNA ladders/markers in one or more lanes of the gel. When voltage is applied the samples move towards the positive electrode in a linear fashion, each
sample well forming linear lane of migration. Simplest approach to visualizing the bands separated by electrophoresis is by staining with intercalating dyes (e.g. ethidium bromide) which insert between stacked bases and viewing with ultraviolet transilluminator. In some instances the nucleic acid fragments may be detected by radioactive or fluorescent tag which can increase sensitivity. Comparison of the distance of migration of an unknown sample with the ladder either by eye or by computer assisted instrument allows size determination.¹¹⁷

2) **Hybridization assay**: When the hybridization reaction is used to analyze the nucleic acid content of an unknown sample, the process is known as hybridization assay. The property of complementary base pairing allows fragments of known composition (the probe) to interrogate an unknown for the presence of matching (complementary) sequences. All hybridization assays, therefore, require several basic elements: a probe, a sample, controlled conditions permissive of complementary base pairing and a method for detecting specific probe sample hybrid.¹¹⁷

A wide variety of techniques have been applied to collection and analysis of specific hybrids. Once specific
hybrids have been collected, methods of detection are obviously linked to method of labeling. Many research applications and the first applications of hybridization assays utilized radioactive labels such as phosphorous – 32 (\(^{32}\)P), iodine (\(^{125}\)I), sulphur 35 (\(^{35}\)S), Carbon – 14 (\(^{14}\)C) and tritium -3(\(^{3}\)H) with detection through autoradiography or scintilating counting. Short half life of these probes, hazard to laboratory personnel and cost of radioactive waste disposables contributed to the need for non-radioactive methods.\(^{117}\)

Non-isotopic labeling and detection systems for nucleic acids, are stable systems facilitating standardization of the procedure. In some applications nucleic acids are directly linked to a signal generating compound usually a fluorochrome but occasionally an enzyme. More commonly nucleic acid, are indirectly detected in a multistep assay similar in concept to an indirect antibody reaction. Biotin was the first label to be used and is detected by high affinity interaction with avidin or streptavidin molecule that is in turn complexed or conjugated to signal generating enzyme (colorometric or chemiluminencent), fluorochrome or metallic particles detected by light scattering. Many other functional groups have been
developed as nonisotopic labels such as, bromodeoxyuridine, deoxigenin and sulfone. Detection in these cases is achieved with high affinity antibodies in turn conjugated to enzyme or fluorochrome. Biotin may also be detected with an antibody (antibiotin) rather than an avidin or streptovidin molecule.\textsuperscript{117}

3. **Reverse hybridization**: Reverse hybridization is similar in many ways to the traditional southern blot, where in the amplicon – probe hybridization occurs on a nitrocellulose or similar substrate. In this technology, the multiple probes are immobilized on a nitrocellulose strip and the amplicon is applied to the strip, which is the reverse of southern blot. Lines or dots form at the site of amplicon – probe hybridization. When this pattern is compared with a key, one can interpret the results of this reaction. The advantage over traditional southern blotting is that numerous probes are assayed simultaneously and radioisotopes are not used. The first version of reverse – hybridization assay examined was the LiPA (line probe assay), Mycobacteria assay (Innogenetics). This assay used a broad range PCR directed against the 16S to 23S spacer region and probes for *M.tuberculosis* complex, *M.avium intracellulare* (MAI) complex, *M.avium*, *M.intracellulare*, *M.tuberculosis*, *M.avium intracellulare*.
M. kansasii, M. chelonae group, M. gordonae, M. xenopi and M. scrofulaceum. \textsuperscript{71}

Miller et al (2000)\textsuperscript{118} studied 60 clinical isolates of mycobacteria from 59 patients using this product and found no discrepancies with routine laboratory identification with the majority of the isolates, although some were not identified to the species level. PCR followed by reverse hybridization has also been used on direct clinical specimens, with excellent results from smear positive specimens. The newer version, the INNO-LiPA Mycobacteria v2, has expanded the number of identifiable mycobacterial species to 16. Tortotoli et al (2003)\textsuperscript{119} tested 197 mycobacteria belonging to 81 taxa with this product and found 100% specificity and sensitivity for 20 out of 23 probes.

4. **DNA sequencing**: DNA sequencing for the analysis of an amplified product is now a common method of postamplification analysis. One of the most useful regions of the 16S gene complex for the identification of mycobacteria involves the hypervariable A region. This has been used to achieve rapid and accurate sequence – based identification of the most common clinically relevant mycobacteria. The rpo B
gene, which encodes for DNA-dependent RNA polymerase has been used by several groups for the identification of mycobacteria. Sequencing this genetic target is popular since it not only provides identification information, but also provides information regarding the susceptibility of the isolate to rifampin.  

5. **Microarray analysis**: Microarrays, devices commonly referred to as gene chips, have been used for the identification of mycobacteria and the detection of the genetic determinants of resistance. Toresch et al (1999) \(^\text{120}\) describe the use of a microarray that examined two genetic regions, the 16S rDNA and rpoB gene. They examined 70 mycobacteria representing 27 different species and were able to identify 26 species of them.

**PCR for tuberculosis**: 

For diagnosis of tuberculosis by PCR many different DNA amplification targets have been used such as gene encoding the 65KDa (GroEL), 38 KDa (Pho S, C1E Ag 78 and Pab), MPB 64 (23 KDa), IS 6110, IS 986, IS 990, 2.4 Kb DNA insert, TR C4, GCRS, US Patent 08/997897, 85B, etc. \(^5,49,53,112,121\)
However, the target most frequently amplified is the IS 6110 repetitive element, which is present in multiple copies (upto 20) in most strains of *M.tuberculosis* complex. A significant proportion (40%) of the South Indian isolates have been reported with single copy or lacked this sequence.

As the IS 6110 sequence has been shown to be repetitively present in *M.tuberculosis* genome, it helps to increase the sensitivity of PCR over that obtained in the amplification of single DNA sequence. Also this target has been found to be very specific for *M.tuberculosis* complex in a number of studies. However homologus sequences have been detected in potentially pathogenic mycobacterium strains such as *M.intracellulare, M.fortuitum, M.Kansasii, M.xenopi, M.malmoense* and *M.chelonae* is some studies.

In a study carried out by Chauhan et al (2007) in which *M.tuberculosis* isolated from different part of India were typed by Restriction Frangment Length Polymorphism for IS6110 element of *M.tuberculosis* complex. The study conducted at National JALMA Institute of leprosy and other mycobacterial diseases, Agra included 308 isolates of *M.tuberculosis* from different parts of India. Among these, 185 were from north
India, whereas 24, 57 and 42 were from southern, eastern and western states of India respectively. The range of IS6110 copies among isolates varied from 0-19 with no isolate having copies more than 19. At the national level, 56% of the isolates studied showed high copy number (6-19 copies of IS6110, 13% showed intermediate copy number (3-5 copies), 20% showed low copy (1-2 copies) and 11% showed no copy (lacking IS6110 element). At the regional level, isolates from all four region’s (state’s) showed a similar trend with respect to the copy numbers when compared to the national data. Thus the isolates from South India was shown to have a similar number of copies as in the other states of India. From this study it was found that 11% of the isolates from all the regions were lacking the IS6110 elements bring in into question the use of this target to diagnose tuberculosis.

**DNA Extraction :**

The first very important step before carrying out PCR is DNA extraction from the clinical specimens, and has great impact on the final outcome of the results of PCR. DNA amplification using PCR has allowed great progress to be made in the rapid and accurate diagnosis of tuberculosis. However,
despite numerous reports in the literature, amplification techniques do not yet have an established role in the laboratory for tuberculosis diagnosis, nor have they replaced traditional techniques.\textsuperscript{18,125,126} This is specifically the case in suspected extra-pulmonary tuberculosis for which a rapid and accurate laboratory diagnosis is of prime importance. One of the reasons for PCR to have fallen short of expectations for the diagnosis of tuberculosis is the presence of inhibitors in the specimen and the other achieving of an optimal cell lysis. Various DNA extraction procedures have been described like treatment with proteinase K and non-ionic detergents, boiling with nonionic detergents, freezing and thawing, sonication, NaOH treatment, cetyl trimethylammonium bromide-NaCl treatment, simple boiling, proteinase-K-lysosome-EDTA treatment, STET method (Sucrose, triton x-100, EDTA), cex, resin treatment, caotrophic salts.\textsuperscript{6,8,24,49,112,123,127-130}

Buck et al (1992)\textsuperscript{131} compared four simpler DNA extraction methods (i) Treatment with proteinase K and non-ionic detergents, (ii) Boiling with non-ionic detergents, (iii) Freezing and thawing and (iv) Sonication with a more extensive reference method for extraction described by
Eisenach et al. With the reference method the investigators were able to detect between 10 and 100 cells of *M. tuberculosis* in PCR. First the test was carried out with *M. tuberculosis* ATCC 27294 grown in liquid culture. Sonication produced the most promising results. Between 10 and 100 organisms could be detected by this procedure in PCR and this compared favourably with the reference method. Proteinase K and nonionic detergents produced DNA that could be amplified only if the suspension contained $10^3$ or more organisms. Freezing and thawing with 2% Triton X-100 produced similar results. No amplification occurred when suspension were treated by freezing and thawing in distilled water or boiling with nonionic detergents (Triton X-100 or Tween-20). The sonication procedure was further investigated with actual clinical samples and 24 out of 26 positive specimen (92%) could be detected by PCR. One sample had very few number of bacteria and the other sample had large number of bacteria (4+ growth in culture) but was very viscid in nature which caused inhibition of DNA extraction by the sonication method indicating the limitation of this method in viscous specimens. They concluded sonication to be an easy and effective method for DNA
extraction which could be carried out in almost any laboratory setting and number of specimens can be treated simultaneously. Since the tubes remain closed during the process, dissemination of infectious aerosols is prevented.

Honore-Bouakline et al (2003)\textsuperscript{128} compared two DNA extraction methods, pretreatment with proteinase K (PK-Roche) and complete DNA purification by cetyl-trimethylammonium bromide (CTAB-Roche) on 144 extrapulmonary specimens to evaluate the impact on PCR (Cobas-Amplicor method). Amplification and detection of the amplicons were impaired by a high number of inhibitory specimens (39 to 52\%). CTAB-Roche allowed to detect more culture positive specimens by PCR than PK-Roche. Comparison with the final diagnosis of tuberculosis confirmed that CTAB-Roche produced the best sensitivity (53.8\%) compared to culture (43\%), PK-Roche (16\%) and smear (13\%). The specificities were CTAB-Roche (78.8\%), culture (100\%) and PK-Roche (96.5\%).

PCR for tuberculosis has been reviewed by various workers as follows:

Chia et al (1990)\textsuperscript{51} performed PCR by using a 24 base synthetic oligonucleotides that bracket a 165-base region of a
gene that codes for a 65-kilodalton antigen of *M.tuberculosis* to serve as primers to amplify the *M.tuberculosis* DNA. They tested the specificity of the primers for *M.tuberculosis* complex by carrying out amplification for 10 non-tuberculous mycobacteria, including *M.avium*, *M.bovis*, BCG, *M.chelonae*, *M.fortuitum*, *M.gordonae*, *M.kansasii*, *M.paratuberculosis*, *M.phlei*, *M.smegmatis* and *M.xenopi*. They found the primers to be specific to *M.tuberculosis*. They also tested 284 clinical specimens (236 sputum and 48 pleural effusion specimens) and found 118 (41.5%) to be positive by PCR and only 49 (17.3%) positive by culture. Based on the culture and PCR DNA results PCR had an overall sensitivity of 100% and specificity of 62.6% when compared with culture results. They explained this by saying that some of the clinical specimens were positive by PCR and negative by culture. These culture negative specimens were obtained from patients receiving antituberculous treatment. They suggested that DNA amplification method could detect mycobacteria that are unable to grow in vitro. They found the detection limit of PCR to be much better than the conventional method.
Wit et al (1990)\textsuperscript{132} developed a PCR assay for amplification of 336-bp repetitive fragment of \textit{M.tuberculosis} genome. Both culture and PCR gave a positive result for 14 out of 26 clinical samples (53.84\%). Whereas the sensitivity for acid fast staining was only 11.53\% (3 of 26 samples).

Kolk et al (1992)\textsuperscript{133} developed a PCR for detection of \textit{M.tuberculosis} using primers coding for 245 base pair of IS986. PCR was found positive in all 32 culture positive and ZN positive samples, 10 of 12 culture positive and ZN negative samples (All patients responded to treatment for tuberculosis) and all 04 culture negative and ZN positive samples (All patients responded to anti-tuberculosis treatment). PCR detected \textit{M.tuberculosis} in 35 of 178 culture and ZN-negative samples. These 35 had strong supporting clinical evidence of tuberculosis.

Abe et al (1993)\textsuperscript{134} evaluated PCR in 135 sputum specimens by using two amplification systems. One amplifying IS986 region of \textit{M.tuberculosis} and second amplifying r-RNA using Gen.Probe.MTD test kit. Smear gave a sensitivity of 71.9\%. MB-check system gave a sensitivity of 96.9\%. LJ gave a sensitivity of 75\%. PCR for IS986 give a sensitivity, specificity
and negative and positive predictive values of 81.3, 94.2, 94.2 and 81.3% respectively. Whereas the values for the same with MTD test were 90.6, 95.1, 97 and 85.3% respectively. After discrepancy analysis the sensitivity and specificity for PCR IS986 were 84.2% and 100% and for MTD test were 91.9% and 100% respectively.

Shawar et al (1993) detected the amplification products of PCR targeting IS-6110 (317bp segment) by ethidium bromide stained agarose gel electrophoresis and by hybridization with a probe conjugated with alkaline phosphate. Seventy six clinical specimens were examined. The sensitivity, specificity, positive and negative predictive values for PCR using agarose electrophoresis were 55, 98, 85 and 91% respectively. Whereas the values of sensitivity, specificity, positive and negative predictive values by probe detection were 74, 95, 77, 94% respectively. Probe detection was superior detecting 5 \( M.tuberculosis \) cells as compared to 50 \( M.tuberculosis \) cells detected by agarose electrophorosis method from culture.

Nolte et al (1993) developed PCR for rapid diagnosis of tuberculosis by amplifying a fragment in IS 6110 region of \( M.tuberculosis \) genome in 313 sputum specimens. Of 123
specimens that were culture positive for *M. tuberculosis*, 112 (91%) were positive for *M. tuberculosis* by PCR. Of the 17 culture positive specimens for MOTT, none were positive by PCR. PCR assay detected 105 of 110 (95%) smear positive specimens and 8 of 14 (57%) smear negative specimens.

Claridge et al (1993)\textsuperscript{18} who did the PCR by amplifying a 317-bp segment within the insertion element IS 6110. Culture was done by BACTEC & LJ both, for all the specimens. Five thousand samples were evaluated with 623 (12%) sample being culture positive. Of which 218 (35%) showed growth of *M. tuberculosis*. Of these 218 samples 181 (85%) were positive by PCR. Two thirds (66%) of the specimens which grew *M. tuberculosis* were positive for AFB staining. When only sputum samples were considered 127 (71%) of the *M. tuberculosis* culture positive specimens were smear positive. There were 179 culture positive and 176 smear positive sputum specimens. There were two culture negative / smear positive / PCR negative results for patients on antituberculous medication. PCR results were accurate for 181 specimens, culture positive for *M. tuberculosis* and for 937 specimens which were culture negative for *M. tuberculosis*. Thirty seven
specimens representing 29 patients were negative by PCR but showed growth, while 11 specimens from 10 patients were positive by PCR but were culture negative for \textit{M.tuberculosis}. The 37 false negative results (culture positive and PCR negative) were traced to low number of organisms in the specimen or the presence of inhibitors or might be low number of copies of IS6110 in some strains. The 11 patients whose specimens were culture negative and PCR positive were said to be due to amplicon or bacterial DNA contamination introduced at the time of lysing (n=4), 2 patients were receiving anti-tuberculous drugs than considered true positives and remaining specimens were either positive because of cross reactivity to MOTT or were questionable. The smear positive rates for all specimens were 75\% and 24\% for the PCR positive and PCR negative groups respectively. Of the 145 samples that were positive for \textit{M.tuberculosis} by culture and smear, 136 (94\%) were positive by PCR. Of the 73 samples that were \textit{M.tuberculosis} culture positive and smear negative 45 (62\%) were positive by PCR. For sputum of the 127 smear positive specimens, 121 (95\%) were PCR positive; of the 6 missed by PCR, 3 specimens contained inhibitors, one had no inhibitor and one was not available for
testing. Of the 52 smear-negative but *M. tuberculosis* culture–positive sputum specimens, 33 (63%) were positive by PCR. The uncorrected PCR sensitivity was 83.5% with a specificity of 99.0% and a positive predictive value of 94.2%. After corrections were made the sensitivity, specificity and positive predictive values become 86, 99.7% and 98.4% respectively.

Kox et al (1994)\textsuperscript{137} studied 218 clinical specimens from patients suspected of having tuberculosis by PCR, culture and smear. PCR was done using primers within the IS6110 insertion sequence of *M. tuberculosis*. The PCR results corresponded well with the culture results, except for one CSF, one tissue biopsy sample, and one blood sample that were positive in PCR but negative in ZN staining and culture. These samples were obtained from patients who had responded to treatment for tuberculosis. Twelve samples contained mycobacteria other than *M. tuberculosis*. None of these were positive in the PCR, which demonstrated the specificity of the PCR using IS6110 sequence.

D'Amato et al (1995)\textsuperscript{138} evaluated the AMPLICOR MTB and culture results on 985 specimens from 372 patients of pulmonary tuberculosis. Overall the sensitivity and specificity
of AMPLICOR MTB were 61.8% and 98.5% respectively. The sensitivity, specificity, positive predictive value and negative predictive value for all specimens after culture plus clinical correlation were 66.7%, 96.6%, 91.74% and 97.7% respectively. There was no statistical difference between AMPLICOR MTB and culture results ($p \geq 0.1$).

Wobeser et al (1996)\textsuperscript{139} assessed the role of Roche Amplicor \textit{Mycobacterium tuberculosis} PCR test (RMtb-PCR) was compared with mycobacterial culture, with the adjusted gold standard incorporating clinical diagnosis. A total of 1480 clinical specimens were assessed. The sensitivity, specificity and positive and negative predictive values of RMtb-PCR were 79, 99, 93 and 98% respectively.

Rodrigues et al (1997)\textsuperscript{8} used a specific 123 base pair fragment of the insertion element IS6110 in \textit{M.tuberculosis} to analyse 101 pulmonary and extrapulmonary samples by PCR and compare it with culture on Loweinstein – Jensen medium and Mycobacterium growth indicator tube and the clinical findings of the patient. Of the total 101 samples 43 samples were positive by PCR, however only 10 grew in culture. Two samples negative by PCR were positive by culture. The
sensitivity and specificity for culture with DNA amplification was 83.8% and 62.9% respectively. The sensitivity and specificity of PCR compared with clinical feature was found to be 94.7% and 88.8% respectively. Of the 18 sputum samples 8 were PCR positive with disease while only 5 of these grew in culture giving a culture sensitivity of 63%. Of the total 41 CSF sample 13 were positive for PCR and were proven cases of tubercular meningitis. Culture was positive only in 3 cases (21%). Sensitivity of PCR for CSF when clinical picture was taken into consideration was 93% and specificity was 81.5%.

Cohen et al (1998) assessed the diagnostic yield of PCR in 605 patients of pulmonary tuberculosis by two PCR methods: Roche Amplicor PCR assay targeting 16S ribosomal RNA genes and in-house assay targeting IS6110 sequence of *M. tuberculosis*. PCR was carried out on all the samples within 24 hours of hospital admission. A positive PCR on at least one of two specimens collected in the 1st 24 hours was 85% and 74% sensitive and 88% and 93% specific for tuberculosis by the in-house and Roche techniques respectively.

Banavalikar et al (1998) asserted the role of PCR in diagnosis by testing 85 samples of clinically diagnosed cases of
tuberculosis. They amplified a 169 bp DNA segment belonging to the insertion sequence IS6110. In this study smear for acid fast bacilli were positive in 26 (30.6%) and culture for *M. tuberculosis* is 5 additional specimens, yielding a total positivity of 36.6% (31/85). The PCR assay identified mycobacterial DNA in all the 31 (100%) smear and culture positive and in 28 (51.8%) culture negative specimens with a total yield of 59 (69.4%) positives.

In a study for pulmonary tuberculosis by Beerbal et al (1999)\(^6\), 35 cases were evaluated for PCR. Amplification of DNA was done by the method and primers described by Khandekar et al. In this study microscopy was positive in 17 (48.57%) cases and all of them were PCR positive. In remaining 18 smear negative cases PCR was positive in 15 (83.33%) cases. In this study 27 (77.14%) of the cases were positive by culture. All the culture positive cases were positive by PCR. Out of the 8 culture negative cases PCR was positive in 5 (62.5%). Three cases who were PCR negative were also smear and culture negative. The authors found PCR to be 100% specific and highly sensitive (100% in smear and culture positive cases and
83.33% in smear negative cases and 62.5% in culture negative cases).

In another study by Parandaman et al (2000)\textsuperscript{111} who tested 50 patients having pleuritis using two sets of primers, IS6110 and TRC4 (an insertion element – like repetitive sequence which they have cloned and sequenced). Both probes gave a sensitivity of 100% and specificity of 85%. Among the 50 samples 36 were positive by PCR (72%) and 6 were positive by culture. All these six were positive by PCR. Three patients positive by PCR were having no clinical evidence of tuberculosis and were false positives and that reduced the specificity to 85%. They found PCR to be useful as an additional test for diagnosing patients with tuberculous pleuritis.

Mitarai et al (2001)\textsuperscript{141} evaluated the amplicor Mycobacterium detection kit for diagnosis of pulmonary tuberculosis. The sensitivity and specificity of conventional culture method was 60.2% and 99.8% respectively, whereas the figures for amplicor assay was 61.8% and 97.4% respectively. There was no statistical significant difference between these methods. They found amplicor PCR assay to be specific and rapid for the diagnosis of pulmonary tuberculosis.
Prasad et al (2001) used primers derived from 1282 bp DNA fragments of *M.tuberculosis* (US patent 08/997897) to test 255 sputum specimens of which 65 were from clinically diagnosed cases and 110 from controls. The positive rate of PCR was found to be higher (83%) than that of culture (70%) and substantially greater than of smear (61.5%). All smear positive cases were positive by both culture and PCR. In respect of smear negative cases, positivity rates of PCR and culture were 54% and 24% respectively. Sensitivity of PCR, culture and smear microscopy was 83%, 70.7% and 61.5% while specificities of these tests were 100%. Positive predictive values of PCR, culture and smear microscopy were 100% for all the three methods and negative predictive values were 94.5%, 90.9% and 83.3% respectively.

Borun et al (2001) compared two sets of primers IS6110 and IS990. They studied 265 specimens by culture (LJ & BACTEC 12B) and PCR by the 2 sets of primers. A total of 123 samples were positive (culture, BACTEC & DIG – PCR ELISA positive) and 132 were negative. Six were culture negative but PCR positive and they were from patients who had clinical manifestation of TB or were suspected of having a TB
reactivation and were under antituberculous therapy. Four specimens were PCR negative but culture positive and was explained as presence of inhibitors in the specimens or lack of sensitivity of PCR compared to culture. Sensitivity of the assay in comparison to conventional diagnosis (culture) was shown to be 96.5% and the specificity 95.3%. They also tested these primers for specificity using 16 non-tuberculous mycobacterial strains but only \textit{M.tuberculosis} complex tested positive.

In a study by Pasricha et al (2001)\textsuperscript{142} a total of 279 clinical samples including 79 pulmonary, 118 extrapulmonary and 82 ocular were tested by conventional methods and PCR using IS6110 based primer. Two \textit{M.tuberculosis} strains (H37RV and 437RA) and 6 MOTT strains (\textit{M.avium} complex, \textit{M.kansasii}, \textit{M.chelonei}, \textit{M.smegmatis}, \textit{M.xenopi} and \textit{M.fortuitum}) were also tested for specificity of the primers and the primers were found to be specific for \textit{M.tuberculosis} strains only. Among 79 pulmonary specimen 24 specimens were positive for acid fast bacilli (AFB) by bacteriological methods, while PCR was positive in 5 specimens. Among the above 24 specimens, 3 were identified as \textit{M.tuberculosis} by culture and PCR was positive in only one of them. While among 118 extra-pulmonary
specimens 20 specimens were positive for AFB and only 3 positive by PCR. PCR was positive in only 2 out of the 7 specimens which were proved to be *M.tuberculosis* by culture. Among 82 ocular specimens, only 4 were positive for AFB and PCR was positive only in 2. PCR was negative for the only specimen that was identified by culture as *M.tuberculosis*. All the six specimens in which MOTT strains were isolated were negative by PCR. When compared with culture gold standard for detection of *M.tuberculosis*, the sensitivity and specificity of PCR was found to be 30% and 95.6% respectively. The low sensitivity of PCR was attributed to (i) the presence of low copies or no copy of the IS6110 in the genome of *M.tuberculosis* strains isolated from South India as this study was carried out in South India, Chennai, (ii) to inhibitors present in the samples and (iii) to selection of inappropriate region of IS6110 in the assay.

In another study by Michael et al (2002) to evaluate PCR for rapid diagnosis of tuberculosis meningitis. Primers used were that detected IS6110 element of M.tuberculous complex genome. In all 102 subjects were studied of whom 17 belonged to definite tuberculous meningitis group, 17 to
possible tuberculous meningitis group and remaining 68 to non-TB group. Taking into consideration microscopy, culture, PCR and response to antituberculous treatment final diagnosis was made for 34 patients. PCR was positive in 24 (71%) cases, culture in 17 cases (50%) and microscopy in one case (3%) and the specificity was PCR-100, culture-100 and microscopy 97% respectively.

Kesarwani et al (2004) assessed PCR for diagnosis of extrapulmonary tuberculosis in 65 clinical specimens. Forty seven specimens were diagnosed finally as tuberculous on the basis of clinical suspicion, AFB staining, culture for \textit{M.tuberculosis}, histopathology and PCR results. This included 9 fluid samples of which only 1 (11.11%) was AFB smear positive, 3 (33.3%) were culture positive and only 4 (44.4%) were cytologically suggestive of tuberculosis while all were positive by PCR. PCR had 100% specificity and sensitivity for fluid tubercular samples. Of the 38 tissue specimens diagnosed as tuberculous 6 (15.8%) were AFB smear positive, 35 (92.1%) were histopathologically positive and 37 (97.36%) were PCR positive. Culture was not done for the tissue samples. There was one sample which was PCR negative but
histologically positive for tuberculosis (False negative) as the patient responded to anti-tubercular treatment. PCR had a sensitivity and specificity of 97.36% and 100% respectively on tissue samples. The overall sensitivity, specificity, positive predictive value and negative predictive value of PCR was 97.87%, 100%, 100% and 94.73% respectively. The primers used in this study detected the IS6110 region of *M.tuberculosis* genome.

In a study by Mauricio and Julia (2004) who analysed 4 below mentioned primers IS6110 (2 sets : one promoting the amplification of 123 bp fragment and another that promotes amplification of a 541 bp fragment), 65-KDa, 38-KDa and MPB64. All the primers were found only to be specific for *M.tuberculosis* as they did not amplify DNA extracted from *M.fortuitum*, *M.avium-intracellulare*, *S.sonnei*, *S.paratyphi A*, *E.coli* and *S.aureus*. Eighty one clinical samples were tested by all the primers and conventional methods. Smear and culture were negative in 24 samples as was the PCR. The 19 samples testing smear positive, as well as the isolated strains (culture positive) were 100% positive for all PCR’s with the exception of 89.4% results for the PCR with MPB64 primers. The percent
positivity for the amplification of the other primer were IS6110 (123bp) 92.1%, IS6110 (541bp) 86.8%, 65KDα-76.3%, 38KDα – 86.8% and MPB64 – 60.5%. The primers specific for amplifying the 123 bp IS6110 fragment gave the highest positivity (92.1%), diagnostic agreement (0.9143), co-positivity (94.7%) and co-negativity (100%).

In a study by Goessens et al (2005) who compared the performances of BD probe Tec ET (Becton Dickinson) and COBAS AMPLICOR MTB (Roche) for Mycobacterium tuberculosis complex in 824 respiratory specimens from 580 patients. Out of 824 specimens 109 specimens from 43 patients were culture positive for \textit{M.tuberculosis}. Of these 109 specimens, 67 (61.5%) were smear positive (Auramine staining), 85 (78%) were positive by the COBAS AMPLICOR test and 94 (86.2%) were positive by the BD probe test. Of the 715 culture negative specimens, 17 were positive by smear, 11 were positive by the COBAS AMPLICOR test and 12 were positive by the BD probe test. After discrepancy analysis and review of the patients' clinical data, 130 specimens from 50 patients were considered true positive specimens. This resulted in the following sensitivities: microscopy, 61.5%; COBAS AMPLICOR MTB test 78% & BD
probe ET 86.2%. The specificities of each system, based on the clinical diagnosis were 99.7% for microscopy, 99.9% for COBAS AMPLICOR MTB test, and 99.9% for BD probe Tec ET.

Diagnosis of extrapulmonary tuberculosis by smear culture and PCR using universal sample processing technology was done by Soumitesh Chakrovarti et al (2005). The highest sensitivity in PCR was achieved when two gene sequences i.e. dev R (Rv 3133c) and IS6110 were targeted. The sensitivity and specificity values ranged between 66.7% and 83% and 75% and 100% respectively.

A comparison was made by Negi et al (2005) between smear by ZN staining, radiometric BACTEC culture (BACTEC-460 system) and PCR test amplifying 65 KDa antigen coding region of *M. tuberculosis* genome in the diagnosis of osteoarticular tuberculosis. Forty six clinical samples of synovial fluid and tissue were included in the study. PCR test was found to be much more sensitive than the ZN smear examination and BACTEC culture (p<0.05). The positivities of the three tests were 78.2% for PCR, 21.7% for smear and 43.3% for BACTEC. Further sensitivity and specificity for PCR employing BACTEC culture as the “gold standard” was 100%.
Negi et al (2005)\textsuperscript{146} evaluated the performance of 65 KDa antigen based PCR assay in clinical samples. The PCR test was compared with ZN staining, LJ and BACTEC culture. Of the 145 samples tested, PCR gave a highest sensitivity of 74.4% followed by BACTEC 55.86%, LJ – 48.9% and ZN smear – 33.79%. The sensitivity of PCR in smear positive cases was 100%, whereas in smear negative cases was 62.5%. Similarly a 98.33% sensitivity rate was observed by PCR test in clinical samples which were positive for \textit{M.tuberculosis} by both the culture methods. PCR was positive in 45.24% and 34.37% LJ negative and BACTEC negative specimens respectively. For pulmonary specimens the sensitivity of various tests were smear 50%, LJ – 51.5%, BACTEC – 54.5% and PCR – 72.7%. For the extrapulmonary group the sensitivities were smear – 20.25%, LJ – 46.83%, BACTEC – 55.6% and PCR - 75.9%. The mean detection time for \textit{M.tuberculosis} was 24.03 days by LJ, 12.89 days by BACTEC and less than one day by PCR test.

Negi et al (2006)\textsuperscript{147} evaluated 159 clinical specimens of pulmonary and extrapulmonary by PCR based on DNA sequence coding for a 38 KDa protein antigen b (pab), specific for \textit{M.tuberculosis} and compared it with AFB smears, LJ
medium growth and growth in BACTEC 460 system. The resulting sensitivity for various tests were; smear 35.2%, LJ culture 47.1% and for BACTEC 53.4% respectively. For the pulmonary TB specimens sensitivity for smear, LJ culture, BACTEC and PCR were 49.2%, 52.11%, 56.75%, 74.3% respectively. For the extrapulmonary specimens the sensitivity of smear, LJ culture, BACTEC & PCR were 23.86%, 43.18%, 48.86% and 71.59 respectively. On comparing statistically PCR test was found to be more sensitive than the other three tests for diagnosis of TB (p<0.05). The mean detection time for *M.tuberculosis* was 20 days by LJ culture, 12.8 days by BACTEC culture and less than 1 day by smear examination and PCR test.

In a study by Dil-Afroze et al (2006) who targeted MPB64 gene of *M.tuberculosis* complex to study 82 patients with tubercular pleural effusion found smear microscopy by ZN staining to be positive in 15 (20%) patients. Culture was not done. PCR was positive for 32 out of 48 in the tubercular group and none positive in 34 samples constituting the non-tubercular group. Thus sensitivity of PCR for detection of tubercular pleurisy worked out to be 67% while specificity for
the same was 100%. There were negative PCR results in 16 patients with pleural tuberculosis and this was said to be due to inhibitors that are particularly high in pleural fluids or small amount of sample available for PCR. This study reported a low sensitivity of 67% as compared to some other studies which had sensitivity of 100%, so the authors have suggested the use of multiplex PCR using MPB64 and IS6110 targets to accomplish 100% sensitivity.

Diagnostic potential of IS6110, 38KDA, 65KDA and 85B (RT-PCR) sequence base polymerase chain resection in the diagnosis of 172 pulmonary and extra pulmonary samples by Negi et al (2007). For every sample smear microscopy culture on both LJ and BACTEC 12B media were done, contaminated culture samples were not included in the study and only a total 159 samples were included. PCR test was found to have higher positivity than conventional test and BACTEC culture (p<0.5), though positivity differed in each PCR. PCR targeting IS 6110 showed sensitivity of 83% followed by 65KDa (81.1%), 38-KDa (74.21%) and 85B -Rt-PCR (70.4%). Of the 71 pulmonary samples, positivity was 35 (24 sputum and 11 BAL – 49.29%) for microscopy, 37 (25 sputum and 12 BAL – 57.11%) for
growth on LJ and 42 (33 sputum and 9 BAL – 56.75%) by BACTEC system. PCR targeting IS6110 gene showed highest positivity 64 samples (90.14%, 50 sputum and 14 BAL), followed by 65 KDa 63 samples (88.73%, 50 sputum and 13 BAL), 38 KDa 55 samples (77.46%, 45 sputum and 10 BAL) and the least sensitive 85B 48 samples only (67.6%, 41 sputum and 7 BAL). Of the 88 extrapulmonary samples the number positive for microscopy, LJ, BACTEC system was 21 (23.86%), 38 (42.18%) and 43 (48.63%) respectively. PCR targeting IS6110 showed the highest sensitivity of 77.27% followed by 65KDa, mRNA, 38KDa showing a sensitivity of 75, 72.7 and 71.59% respectively, though the difference was not found to be statistically significant (p<0.05). In smear positive samples all PCR tests approached positivity of 100%. In smear negative cases PCR targeting 65 KDa showed a highest positivity of 63.1%. IS6110 based PCR showed the positivity of 100% in clinical samples positive in LJ medium. Similarly PCR based on IS6110 and 65KDa showed a highest positivity of 97.65%. All four PCR showed 100% positivity for clinical samples which were positive by all the other three methods used (ZN, LJ and BACTEC). Even in 43 samples negative by all the other three
tests used, IS6110 PCR test was able to detect 11 positives (25.58%) and there were not labeled as false positives as these cases responded to antituberculous therapy. The author concluded by mentioning PCR to have a potential important role in strengthening the diagnosis of TB especially targeting IS6110.

**Real-time PCR:**

PCR technology has advanced from detection at the end point of the reaction in conventional to real time PCR where detection is done while the reaction is occurring. Real time chemistries allow for the detection of PCR amplification during the early phases of the reaction, and measuring the kinetic of the reaction in the early phases of PCR provides distinct advantages over traditional PCR detection. The power of real time PCR had been expanded into application for mycobacteria such as monitoring the efficacy of drug therapy, genotyping for drug resistance and identification of species.\(^{148}\)

A variety of fluorescence formats / detection chemistries are available for correlation of the amount of PCR product with fluorescence signals. There are SYBR Green I dye, minor groove
binder (MGB) DNA probes, hybridization probes, taq man probes, molecular beacons and scorpions.\textsuperscript{115,148}

**Applications in mycobacterial research :**

(i) **Detection of mycobacteria :**

Real time PCR has been used for rapid and specific detection of *M.tuberculosis* from acid fast bacilli smear positive respiratory specimens by using flurogenic hybridization probes specific for *M.tuberculosis*. Similarly real time PCR has been reported to be useful for the rapid diagnosis of lymphadenitis using fine needle aspiration and tissue biopsy where primers and probes were designed on the basis of the internal transcribed spacer sequence, enabling the recognition of the genus Mycobacterium and the species *M.avium* and *M.tuberculosis*. This study reported the technique to be more sensitive (71.6\%) than conventional staining (46.3\%) and culture (41.8\%). This test had a specificity of 100\% for the detection of atypical mycobacteria.\textsuperscript{149}

A real time PCR to detect 16S rDNA of mycobacteria using Pan-Mycobacterial primers and a pair of FRET (Fluorescence resonance energy transfer) probes specific for *M.tuberculosis*
complex gave a comparable result in detecting mycobacteria from clinical samples with Roche Amplicor PCR.\textsuperscript{150} Halder et al (2007)\textsuperscript{151} studied 148 sputum specimens by dev PCR assay using two molecular beacon probes for visual or flurometric end point detection of \textit{M.tuberculosis}. The assay detected 25 fg \textit{M.tuberculosis} DNA. Dev PCR (Beacon assay) had a sensitivity of 92.5\% and specificity of 92.9\%. It was compared with PCR for IS 6110 region and both assays performed at almost equivalent level.

(ii) Detection of mycobacterial species :

Identification of mycobacteria by real time PCR using reference strains and clinical mycobacterial isolates has been studied.\textsuperscript{152,153} Differentiation of \textit{M.tuberculosis} complex, \textit{M.avium} and other non-tuberculous mycobacteria (NTM) has been done using hybridization probes.\textsuperscript{152} Targeting the 16S rRNA gene, 3 different probes specific for mycobacteria, \textit{M.tuberculosis} complex and \textit{M.avium} were constructed. The thermal melting temperature (Tm) for the different mycobacteria was as follows. \textit{M.tuberculosis} 64.35\textdegree{}C, M.Kansasii 58.81\textdegree{}C, \textit{M.avium} 57.82\textdegree{}C, \textit{M.intracellulare} 54.46\textdegree{}C, \textit{M.marinum} 58.91\textdegree{}C, and rapidly growing mycobacteria 53.09\textdegree{}C, or
43.19°C. This assay with melting curve analysis consistently accurately detected and differentiated *M. tuberculosis* from NTM. For identification of *M. abscessus* (type I and type II) and *M. chelonae* using light cycler based analysis of the hsp65 gene had been published. Results from 36 clinical strains were compared with hsp65 gene restriction analysis and biochemical profiles of bacilli. As all the three methods yielded identical results for each isolate, this procedure offered an alternative to previously established nucleic acid amplification based technique for the differentiation of mycobacterial species.154

(iii) **Quantification of mycobacterial load**

For detection and quantitation of mycobacteria taq man format has been used. Real time quantitative PCR has been developed which measures accumulation of PCR product through a dual labeled Taq man probe and provided very accurate and reproducible quantitation of gene copies.155 Using two real time PCR Taq Man assays, reduced levels of *M. tuberculosis* DNA load were found in those patients subjected to successful therapy, suggesting potential use of this assay for monitoring treatment efficacy.156 However, in another study by Desjardin et al (1998),157 the rate of disappearance of both AFB
and *M. tuberculosis* DNA did not correlate with the decline in cultivable bacilli in the specimen suggesting that these tests are not reliable for monitoring the treatment efficacy. Rondini et al (2003)\(^{158}\) demonstrated that the IS2404 (amplification using real time PCR) using the Taq Man system is suitable for the quantitative assessment of the dissemination of the mycobacteria in Buruli ulcer lesion.

The drawback of using real time PCR in monitoring treatment is that PCR amplifies DNA from dead bacilli and it is well known that treated patients can harbor dead bacilli. There is thus a scope of using RNA as a target molecule for this purpose. As far now for the physician the best method to judge the efficacy of treatment remains the improvement of the clinical symptoms and negative culture report.\(^{148}\)

**Transcription based amplification :**

Kwoh and coworkers (1989)\(^{159}\) described a method called transcription based amplification. This includes transcription mediated amplification (TMA) and nucleic acid sequence based amplification (NASBA) or 3SR (for self sustaining sequence amplification). TMA and NASBA are isothermal nucleic acid amplification techniques that, although slightly different in
practice, are identical in concept. TMA and NASBA are the intellectual properties of Gene, Probe, San Diego, CA and BioMerieux, Durham, NC, respectively. These techniques essentially recapitulate retroviral replication in vitro, converting RNA into DNA and then using the DNA as a template for transcription of multiple copies of RNA.\textsuperscript{39,115}

The process begins with an RNA target (ribosomal) which exists as a single stranded entity, removing the need for thermal denaturation. A sequence-specific DNA primer binds to the RNA target. Reverse transcriptase (RT, Purified from avian myeloblastosis virus) then extends the primer, creating a DNA-RNA heteroduplex. The 5' end of the sequence specific primer contains the promoter for a T7 bacteriophage polymerase. The presence of this T7 promoter in the 5' end of the primer results in the synthesis of a DNA strand complementary to the initial RNA target containing the T7 promoter at its 5' end. In the case of TMA, the reverse transcriptase enzyme itself degrades the initial RNA template as it synthesizes its complementary DNA. In NASBA, a separate enzyme, RNase H, degrades the initial RNA template. RNase H selectively cleaves RNA, which is heteroduplexed to DNA, but
not RNA alone. In either case, the resultant single stranded, complementary DNA with the T7 promoter sequence binds to a second primer at its 3’ end. The RT enzyme binds to the second primer and extends the primer using the DNA as a template. The result is a ds DNA copy of the target sequence. After this sequence of events, both strands of the DNA copy are flanked by T7 RNA polymerase promoter regions and both strands can serve as templates for this enzyme. Consequently several antisense RNA copies of the target sequence are produced from this ds DNA molecule by the action of T7 enzyme. These RNA can then act as templates for RT, once again resulting in ds DNA molecule, both strands of which can be transcribed by T7 RNA polymerase to produce several copies of the RNA target.

These techniques are best suited for detection of ssRNA targets and do not require a thermal cycler. Other advantage over PCR is that no denaturation is required for the amplification to occur. Hence ds DNA sequences are not denatured and thus are incapable of binding to the primers in the reaction. Combing this techniques with molecular beacons or other sequence specific probes that can be added directly to the
amplification mixture creates a close tube Real Time System which aids in preventing contamination.\textsuperscript{39,115}

In the amplified\textit{ M.tuberculosis} direct (AMTD) test, the mycobacteria after concentration with standard NaOH method are lysed by sonication. Ribosomal RNA is amplified by copying rRNA target sequence onto a transcription complex using reverse transcriptase. RNA polymerase is then used to make numerous copies of the target sequence from the transcription complex. The process is then repeated autocatalyticaly. The amplified sequences are detected by an acridium ester-labelled DNA probe specific for\textit{ M.tuberculosis}. This is a one tube test which minimize laboratory introduced contamination. Shenai et al (2004)\textsuperscript{54} assessed 94 respiratory and 45 non-respiratory specimens (total 139) smears positive for acid fast bacilli by LJ, BACTEC, phage assay and transcription mediated amplification (TMA) to amplify the r-RNA of\textit{ M.tuberculosis}. Only 84 out of 139 were analysed by phage assay (Fast plaque TB TH kit, Biotec laboratories UK). When compared with LJ, TMA had a overall sensitivity, specificity, positive predictive value, negative predictive value and concordance of 93.8\%, 50\%, 80\% and 78.9\% and 80\% respectively for respiratory specimens, whereas
in case of non-respiratory specimens the values were 92.9%, 82.4%, 89.7%, 87.5% and 88.8% respectively. LJ was positive in 64 (68%) respiratory specimens and 28 (62%) non-respiratory specimens. TMA was found to be the most rapid and reliable method as compared to other methods. There were 15 TMA positive LJ negative patients in case of respiratory specimens, when clinically correlated, they had clinical sings of tuberculosis, past history of TB or history of exposure to TB and/or clinical response to anti-TB treatment. The author concluded by saying TMA as highly sensitive for detecting *M.tuberculosis* complex from respiratory, non-respiratory as well as smear negative cases. It was easy to perform, technically less demanding and does not require expensive thermal cycler. As the RNA product of the amplification system is more labile outside the reaction tube, chances of cross contamination and false positive results are reduced. Another advantage of TMA is that dead cells have no transcription machinery, hence only viable cells are picked up and amplified.

F.Franco et al (2006) evaluated genotype mycobacteria direct (GTMD) commercial assay based on NASBA technology allowing 23S rRNA amplification based detection of
*M. tuberculosis* complex, *M. avium*, *M. intracellulare*, M. kansasii and M. malmonese directly from clinical specimens. A total 134 respiratory and extra-pulmonary specimens were processed with a sensitivity, specificity, positive predictive and negative predictive values of 92%, 100%, 100% and 72% respectively. GTMD had an added advantage of being able to detect various species of mycobacterial pathogens in one assay and from the same clinical sample.

**The strand displacement amplification (SDA) technique:**

It is an isothermal template amplification system. SDA exploits the fact that, following ‘nicking’ of a single strand of dsDNA by a site specific restriction, DNA polymerase can bind and synthesise a complementary copy of the ssDNA; the nicked strand becomes displaced from the strand being copied during the process of DNA synthesis. Incorporation of alpha-thio substituted nucleotides (For example: de-oxyadenoside5’-[alpha-thio] triphosphate) into the reaction mixture renders the newly synthesized strands resistant to nicking by the endonuclease enzyme. Double stranded molecule having single strand with incorporated alpha-thio substitution can only be cut by the restriction enzymes in the native strand, so the
ssDNA that is displaced during the copying process can subsequently act as template for binding of the primer and extension of the nucleotide strands. Single-strand nicking and subsequently polymerization and strand displacement continue to occur because of the continual regeneration of unaltered, single strand ‘nick-able’ site in the duplex molecules. The amplification product is detected with probe.\textsuperscript{39} 

These single single-stranded products also bind to detector probe for real time detection. The diagnostic applications of SDA include the direct detection of \textit{M. tuberculosis} in clinical specimens.\textsuperscript{39,115} 

SDA has a reported sensitivity high enough to detect as few as 10-50 copies of a target molecule. By using a primer set designed to amplify a repetitive sequence with 10 copies in the \textit{M. tuberculosis} genome, the assay is sensitive enough to detect 1-5 genome copies of the bacterium.\textsuperscript{115} The main advantage of SDA is that unlike PCR, it can be performed at a single temperature after initial target denaturation. Recently SDA has been adapted to quantify RNA by adding a reverse-transcriptase step. This modification is used to determine HIV virus load.\textsuperscript{115}
Hellyer et al (1999)\textsuperscript{161} found detection of \textit{M. tuberculosis} and antigen (85B protein) in RNA by reverse transcriptase-SDA to be useful method for monitoring therapeutic efficacy and for rapid invitro determination of drug resistance.

Walker & Linn (1996)\textsuperscript{162} have combined SDA with fluorescence polarization with fluorescence labeled detector probe for detection of \textit{Mycobacterium tuberculosis} DNA by using the IS6110 insertion element as the target sequence. The assay was done using known quantities of \textit{M. tuberculosis} DNA. They achieved extremely sensitive detection of \textit{M. tuberculosis} DNA with a simple protocol of only 15 minutes of SDA at 60\degree C. Their sensitivity was consistently between 10 and 1 \textit{M. tuberculosis} genomes, which corresponds to 100–10 copies of IS6110 target sequence. In addition positive SDA / polarization results were obtained for processed sputum samples supplemented with $10^4$ \textit{M. tuberculosis} genomes.

Dawn et al (1996)\textsuperscript{163} tested a total of 294 respiratory specimens including 75 with culture positive results for presence of \textit{M. tuberculosis} DNA by SDA. A region of IS6110 insertion element was amplified and then detected by chemiluminescence assay. SDA chemiluminescence results
were converted to theoretical numbers of *M.tuberculosis* organisms. A positive threshold (PT) value, above which 95% of the SDA results were judged to be *M.tuberculosis* positive (sensitivity 95%), was found to be 2.4 *M.tuberculosis* organisms per SDA reaction. The analogous PT value for 95% sensitivity on smear positive specimen was 3.6 *M.tuberculosis* organisms per reaction. The PT of 2.4 *M.tuberculosis* organisms per reaction detected 100% of culture positive, smear positive specimens (sensitivity=100%), while 95% sensitivity was achieved with a PT of 15.5 *M.tuberculosis* organisms per reaction. Specificities, which were calculated with respect to culture and smear negative specimen ranged from 96% at a PT of 15.5 *M.tuberculosis* organisms to 84% at a PT of 2.4 *M.tuberculosis* organisms per reaction. SDA specificity ranged from 90% (PT=2.4 organisms) to 98% (PT=15.5 organisms) for the *M.tuberculosis* negative specimen from patients who had not received chemotherapy. SDA specificity in the *M.tuberculosis* negative specimens from patients who receive chemotherapy was lower (85 to 94%).
**Probe amplification methods:**

Probe amplification methods differ from target amplification in that the amplification products contain a sequence only present in the initial probes. The two types of amplification methods using this approach are: Qbeta replicase amplification and the ligase chain reaction (LCR).\(^\text{39}\)

The Qbeta replicase method makes use of a ‘replicase’ enzyme that is able to synthesize the genomic RNA of bacteriophage Qbeta. The RNA genome of phage Qbeta has several ssRNA ‘loops’ and partially double stranded regions (like molecule of tRNA). In the amplification test, a naturally occurring variant of the phage particle called MDV-1 is used; this variant behaves as a substrate for Qbeta replicase enzyme and can be manipulated such that an oligonucleotide probe sequence can be inserted into one of the loops. The MDV-1 ‘probe’ is added to the reaction mixture and binds to the target sequence (If the target is in DNA, heat is used to denature the DNA, enabling the probe to hybridize). Once the probe region in the loop of the molecule anneals to its recognition sequence, it becomes resistant to hydrolysis by RNase; RNase treatment hydrolysis the unbound probe and wash step removes these
molecules from the reaction mixture. Addition of Qbeta replicase enzyme to the probe target complex and subsequent incubation results in specific amplification of the probe. The Qbeta replicase amplification method can be used to detect either DNA or RNA targets. When a suitable combination of capture and detector probes is used, *M. tuberculosis* is detected with a sensitivity of up to one colony forming unit. The inhibitors of PCR do not seem to affect this assay.

Shah et al (1950)\textsuperscript{164} found manual Qbeta replicase assay a rapid, sensitive, semiquantitative and specific for the direct detection of *M. tuberculosis* from clinical specimens. The detector probe sequence was complementary to a region of *M. tuberculosis* 23S r-RNA target. In comparison with culture the overall assay sensitivity and specificity was 97% and 96.5% respectively. After analysis of samples with discrepant results, the assay sensitivity and specificity was 97.3% and 97.8% respectively.

Smith et al (1997)\textsuperscript{165} used a automated Qbeta replicase assay for direct detection of *M. tuberculosis* in sputum. Seventy one of 90 (78.9%) of culture positive samples were positive by Qbeta assay, while 7% of culture negative samples were assay
positive, corresponding to a sensitivity of 79% and specificity of 93%. Following discrepancy analysis the sensitivity of the assay was 84% and 97% respectively.

**Ligase chain reaction (LCR):**

LCR is another probe amplification technique. Single stranded target DNA is incubated with oligonucleotide probes that bind to the target in an end-to-end fashion. A thermostable DNA ligase then ligates or joins the two probes together. The resulting duplex is heated, causing denaturation and separation of the target ssDNA and the ligated probes. The ligated probes and the target again bind probe sequences in an end to end fashion, followed by ligation to form another duplex. These steps are repeated several times. Geometric accumulation of the ligation products results in probe amplification. Attachment of functional groups (biotin, enzymes) to the probe results in labeling of the ligated probe products, enabling them to be detected.  

LCR is potentially useful for screening persons at high risk for developing tuberculosis and extrapulmonary tuberculosis. The LCx *M. tuberculosis* assay (Abbot), primarily makes use of the respiratory specimens. It has a high
sensitivity and specificity for smear positive and negative specimens.\textsuperscript{115}

Ribeiro et al (2004)\textsuperscript{166} found ligase chain reaction (Abbott LCx MTB) to be a rapid, simple and valuable technique as a complementary tool for the diagnosis of tuberculosis. He tested a total of 297 respiratory specimens and found LCx test to have a sensitivity, specificity, positive predictive value and negative predictive value of 92.7\%, 93\%, 67.8\% and 98.7\% respectively. When compared to clinical final diagnosis, the sensitivity, specificity, positive predictive value and negative predictive value for LCx was 88.9\%, 96.8\%, 86.5\% and 97.4\% respectively.