CHAPTER – I

GENERAL INTRODUCTION
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Tuberculosis is a necrotizing bacterial infection with protein manifestation and wide distribution. The lungs are most commonly affected but lesions may occur also in the kidney, bone, lymphnode or meninges or be disseminated throughout the body (Harrison and Tinsley 12th ed. 1999). Tuberculosis is a disease of world – wide prevalence affecting almost 60 million people world – wide and is responsible for more serious illness than any other of the infectious disease (Stewart, and Beswick 1977). It is a specific infectious disease caused by mycobacterium tuberculosi predominately and occasionally by M. bovis and M. africanum. Mycobacterium tuberculosis is transmitted almost exclusively in the sputum and cough spray of open case of pulmonary tuberculosis (Toplay and Wilson 8th ed. vol.3, 1983) on 23rd April 1993 WHO declared a global T.B emergency at a crowded press conference in London. Tuberculosis kills more adults each year than any other infectious disease more than A.I.D.S., Diarrhoea, Malaria, and other Tropical disease combined (klauda, 1994).

In India it is estimated that about 1.5% of India’s population (14 million persons) could be affected with pulmonary tuberculosis. Nearly half of the country’s population is infected with tubercle bacilli that form the reservoir of those who would breaks down into active cases in the future. The chain of infection in the community is maintained by smear positive patients, estimated to be about 3 to 3.5 million (Mukherjee, 1995).

It is estimated that about 140 lakh people are suffering from tuberculosis in our country, of whom about 35 lakh are sputum positive and highly infectious. About 22 lakh cases of tuberculosis are added every year out of which about 10
lakh are sputum positive. About 5 lakh people in India die due to tuberculosis every year i.e. One patient with tuberculosis dies every minute, more than 1000 people die every day. Unless treated effectively one sputum positive patient can infect 10-15 individuals in one year (Module for laboratory tech. Sep.1997).

Robert Koch (1882) isolated *Mycobacterium tuberculosis* and proved its causative role in tuberculosis (Lock, w.1983) The mycobacteria are usually straight curved road shaped organism with parallel sides and rounded end hardly 1-10 μm long and 0.2 –0.6 μm wide in size which frequently form small clumps (plate 5 A). Cells of *M. tuberculosis* are often filamentous with occasionally branching and aerial hyphen. those of *M. kansas* are often elongated with a distinct banded or beaded appearance while those of *M. avium* are after almost coccoid (Runyon 1974).

In 1969, Deland and Wagner developed a technique for automated detection of metabolism of bacteria by measuring the $^{14}$CO$_2$ liberated during the decarboxylation of 14C labeled substrates present in the medium. This technique has been applied successfully to blood culturing detection of antibiotic effect on bacterial growth. *Neisseria species*. Cummings and co-workers in 1975 carried out preliminary work that showed the same principal could be applied to detect growth of *M. tuberculosis* (Cummings, et. al., 1975).

Middlebrook further developed the technique and introduced 7H$_{12}$ liquid medium containing a 14C labeled substrate specific for mycobacterial growth (Middlebrook. G. et. al., 1977).

The approved list of Bacterial Names (Skerman et. al. 1980) includes 40 mycobacterial species and several others have been reintroduced subsequently are shown in Table-1.
*Table-1: Alphabetical list of approved mycobacterial names

<table>
<thead>
<tr>
<th><em>M. africanum</em></th>
<th><em>M. leprae</em> (&lt;i&gt;lepraemurium&lt;/i&gt; (the leprosy bacillies))</th>
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<tbody>
<tr>
<td><em>M. asiaticum</em></td>
<td><em>M. malnoense</em></td>
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<tr>
<td><em>M. aurum</em></td>
<td><em>M. marinum</em></td>
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<tr>
<td><em>M. avium</em> (&lt;i&gt;The avian tubercle bacilli&lt;/i&gt;)</td>
<td><em>M. microti</em></td>
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<tr>
<td><em>M. bovis</em></td>
<td><em>M. neoaurum</em></td>
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<tr>
<td><em>M. chelonei</em> (&lt;i&gt;orm. Chelonei&lt;/i&gt;)</td>
<td><em>M. nonchromogenicum</em></td>
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<tr>
<td><em>M. chitae</em></td>
<td><em>M. parafortuitum</em></td>
</tr>
<tr>
<td><em>M. duvattii</em></td>
<td><em>M. paratuberculosis</em> (&lt;i&gt;rohne’s bacillies&lt;/i&gt;)</td>
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<tr>
<td><em>M. farcinogenes</em></td>
<td><em>M. phlei</em></td>
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<tr>
<td><em>M. flavescens</em></td>
<td><em>M. scrafulaceum</em></td>
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<tr>
<td><em>M. fortuitum</em></td>
<td><em>M. senegalense</em></td>
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<tr>
<td><em>M. gadium</em></td>
<td><em>M. simiae</em></td>
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<td><em>M. gastri</em></td>
<td><em>M. smegmatis</em></td>
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<tr>
<td><em>M. gilvum</em></td>
<td><em>M. szulgai</em></td>
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<tr>
<td><em>M. gordonae</em></td>
<td><em>M. terrae</em></td>
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<tr>
<td><em>M. haemophilum</em></td>
<td><em>M. Thermoresistibile</em></td>
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<tr>
<td><em>M. intracellular</em></td>
<td><em>M. triviale</em></td>
</tr>
<tr>
<td><em>M. kansasii</em></td>
<td><em>M. tuberculosis</em> (&lt;i&gt;the tubercle bacillies&lt;/i&gt;)</td>
</tr>
<tr>
<td><em>M. konossoense</em></td>
<td><em>M. ulcerance</em></td>
</tr>
<tr>
<td><em>M. leprae</em> (&lt;i&gt;the leprosy bacillies&lt;/i&gt;)</td>
<td><em>M. xenopi</em></td>
</tr>
</tbody>
</table>

*From Skerman et al. (1980) and Hill et al. (1984)*
The mycobacteria are included in section 16 of volume 2 of Bergey manual of systematic Bacteriology (Wayne, and Kubica 1896). *Mycobacterium* is the only genus of the family mycobacteriacea. The distinguishing characteristic of this genus include acid fastness, the presence of mycolic acid and the appropriate DNA G+C content 66% - 72% mol except mycobacterium lepra has 55% mol. (Levy-Frebault and Portaels 1992).

With few exceptions numerical or Adansonian taxonomy correlates closely with that obtained by antigenic analysis and DNA relatedness.

Difficulties arise in the case of the *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. intracellur* are *M. Paratuberculosis* and *M. Lepraemurium*) which could like wise be regarded similarly (Mac Intyre and Stanford 1986 a, b.).

The first step in any staining technique is proper preparation of the smear (Smithwick, R. W. 1976). A direct microscopy is the easiest, cheapest and most rapid procedure (Rouillon et. al.1976) has estimated there must be 5,000 to 10,000 bacilli l ml of sputum to be detected microscopically.

In contrast to microscopy, culture techniques have been estimated to detect 10 to100 viable mycobacteria per ml of sample however microscopy less sensitive then culture for detecting mycobacteria. The most widely used staining technique is that of Ziehl and Neelsen (Z-N). It is a hot acid fast stain (Bishop P.J. and Neuman G. 1970). Kinyou is a cold acid-fast stain (Smithwick R.W., 1976) (David H.L., 1976). The brilliance of acid fastness depends on the trapped fuchsin, which is ensured by the fuchsin - mycolin acid bounding of the cell wall (Barksdale, L. and Kim K.S., 1977) (Goren. 1972).
Hagemann (1937) who originally used berdeine sulphate as the dye introduced fluorescence microscopy but later recommended auramine (Truant et. al.1962) used 2 aryl methanedyes, auramine 0 and rhodamine B together (plate 5 B). This combined staining method detected acid fast bacilli in 358 of 3000 sample of sputum as against only 274 by the Ziehl – Neelsen method (Somlo et.al., 1969).

The conventional acid fast staining methods. Ziehl-Neelsen and Kinyon, utilize carbolfuchsin solution for the primary stain acid alcohol as a decolorizing agent, and a methylene blue counterstain. The auramine or auramine – rhodamine fluorochrome stains, considered more sensitive then the carbolfuchsin stains, have proved to be very reliable and specific (Sommers et.al. 1983), 15% to 18% of all culture – positive specimens had smears that were positive on auramine rhodamine stain but negative on Kinyon or Ziehl – Neelsen stain.

Examination of uncentrifuged material gave positive results in 30% of patients subsequently found to be positive on culture (Berean and Roberts, 1988).

Demonstration of acid-fast bacteria (AFB) in smears plays a key role in the diagnosis of tuberculosis and can supplemented by isolation of mycobacteria.

Robert Koch originally grew the tubercle bacillus on heat-coagulated bovine or sleep serum a culture medium invented by the Irish physicist John Tyndall (Topley & wilsond 8th ed. vol. 3, 1983).

Egg base media such as Lowenstein Jensen (L-J) (plate 3 A), have been used to cultivate mycobacteria. In 1958, Middle brook and Cohn devised an agar base medium to permit more rapid detection of mycobacterial growth (Middlebrook et. al. 1977). However it is still required an average of three weeks to recover mycobacteria from clinical specimens.
of *M. tuberculosis* was increased by 4.1% and that of other mycobacteria by 10.5%, except for *M. malmoense* whose rate was increased by 70% (Ispahani and Baker 1988).

Once mycobacteria are recovered from a specimen it is necessary to identify these acid-fast bacilli because of the prevalence of mycobacteria other than tuberculosis (MOTT bacilli or atypical or non-tuberculous mycobacteria). An early differentiation help the physician to manage the patient and potential contacts appropriately, thus considerable cost and labor may be saved if definite information is available early (Alexander et al. 1983).

In 1969 Deland and Wagner developed a technique for automated detection of metabolism of bacteria by us measuring the $^{14}$CO$_2$ liberated during the carboxylation of $^{14}$C labeled substrates present in the medium. This technique has been applied successfully to blood culturing detection of antibiotic effect on bacterial growth. Neisseria species Cummings and co-worker in 1975 carried out preliminary work that showed the same principle could be applied to detect the growth of *M. tuberculosis* (Cumming et al. 1975) Middlebrook further developed the technique and introduced 7H$_{12}$ liquid medium containing a $^{14}$C labeled substrate specific for mycobacterial growth (Middlebrook, G. et al. 1977).

A radiometric method using "Bactec 460 TB" apparatus (plate 2) now enables rapid obtaining of drug susceptibility data and differentiation of clinical mycobacterial specimens into those belonging to the mycobacterium TB complex (comprised of *M. tuberculosis, M. bovis, M. bovis BCG, M. africanum* and non-tuberculosis mycobacteria (MOTT) (Rastogi 1991).
In India it is estimated that about 1.5% of India's population (14 million persons) could be affected with pulmonary tuberculosis. Nearly half of the country's population is infected with tubercle bacilli that form the reservoir of those who would break down in to active case in the future. The chain of infection in the community is maintained by smear positive patients estimated to be about 3 to 3.5 million (Mukherjee A.K., 1995).

Clearly, the rapid and accurate detection of *M. tuberculosis* has a strong impact on the care treatment of the infected patient and on the control of disease in the population. The goals for the diagnostic mycobacteriology laboratory that should be met as we enter in the year 1990 include as follows;

(i) Smear reports to the physician within 24 hrs

(ii) Detection of acid fast bacilli (AFB) within 14 days

(iii) Identification to the species level within 17 to 21 days

Availability of antimicrobial susceptibility test results within 28 days (Michael A.Pfaller 1993).

The radiometric methods has been adopted for rapid detection of the growth of mycobacteria in sputum and other clinical material homogenised it necessary is added Middle brook 7H12 broth contain an antibiotic cocktail.

The most widely used of these is the ‘Mitchison cocktail’ containing carbenicilline, trimethroprim, polymyxin B and amphotericin (Mitchison. et.al. 1972, 1983).

Mycobacteria utilise a 14C labelled substrate (fatty acid) present in the 7H12 medium and release $^{14}$CO$_2$ into the atmosphere above the liquid medium vials with growth are tested on the Bactec 460 T.B. instrument the $^{14}$CO$_2$ aspirated from the vials this $^{14}$CO$_2$ analysed into the ion chamber and its radioactivity is
determined quantitatively in terms of number on the scale from 0-999 these
to number are designated as the growth index (GI). A value of 100 corresponds to
approximately 0.025 μCi. A growth index reading of 10 or more is indication of
metabolism. The Bactec system has also been adapted for drug susceptibility
testing of mycobacteria. This method agrees very well with conventional results
(Bailey and Scotts 1994).

In India today, there is a population of approximately 950 million people
of these approximately 300 million are infected with tuberculosis (TB). 12 million
have active tuberculosis and 3 million on are highly infections. There are half a
million deaths each year caused by T.B. not only this is the number of cases in our
country as well as drug resistance in tuberculosis is on an increase. Present
conventional methods for tuberculosis take 3 to 4 months for reporting the final
diagnosis of species and drug susceptibility pattern. So there is a need for the
institution to adopt alternative some other diagnostic methods, which is not only
rapid but also sensitive and specific. This is conveniently achieved by use of
commercially available automated equipment. Although giving rapid results, the
isolation rate is no higher then by conventional culture techniques and the high cost
of the equipment and media precludes its use in most laboratories (Laszlo and
Siddiqi, 1982) and (Roberts et. al. 1983).

Although there are reports from the western countries stating that the
BACTEC is found to be good in both sensitivity and specifically (Roberts et. al.
1983) (Gross and Hawkins 1985). So for there are only few reports available from
India of the utility of Bactec for rapid diagnosis and susceptibility testing. That is
why this present study is being undertaken where are attempt will be made to
compare the conventional and the Bactec methods for isolation. Identification and drug susceptibility testing of mycobacteria.

All types of clinical specimens, pulmonary as well as extra pulmonary can be processed for BACTEC primary isolation in a manner similar to conventional isolation procedures. Details are provided in the CDC Manual "Procedures for the isolation and identification of mycobacteria" (Kent and Kubica 1985, Vestal, 1975).

Sputum specimens must be digested (liquefaction) and decontaminated (selected elimination of bacteria other than mycobacteria). Other specimens, which may contain organic material and contaminating bacteria, should be liquefied and decontaminated in the same way as sputum. Specimens which contain little organic matter and are collected aseptically do not require the liquefaction / decontamination procedure. Clinical specimens collected in large volume require a centrifugation step to concentrate the mycobacteria present in the specimen.

BACTEC 12B is a liquid medium (plate 3 B) that does not allow observation of colonial morphology. However, the GI in 12B medium can be characteristic of certain types of Mycobacterial growth.

With conventional techniques, once a culture is positive, definitive identification is carried out by performing a battery of biochemical test using growth obtained the primary isolation medium, or on a subculture if the original growth is not sufficient. The time required for isolation is usually 3 to 6 weeks, and may be longer when subculturing is necessary. Clinically, as well as epidemiological, early differentiation of M. tuberculosis (TB) from mycobacteria other than M. tuberculosis (MOTT) is more important than complete identifications of species of the isolated culture. Therefore, the BACTEC approach
has been directed towards a rapid and reliable differentiation of TB from other mycobacteria.

The BACTEC procedure for drug susceptibility testing of mycobacteria is based on the same basic principle employed in the conventional method. The only difference is that a liquid medium is used and instead of counting colonies after about 3 weeks, the growth is monitored radiometrically and the results are reportable within 4 to 5 days. BACTEC drug susceptibility is determined by following the modified version of the conventional proportion method.

The critical proportion for resistance is taken as 1% for all antituberculosis drugs. This means that if 1% or more of the test mycobacterial population is resistant, the culture is considered resistant for laboratory reporting purposes. Resistance is determined by comparing the rate of growth in the control and the 12B vials containing test drug. Several published studies have reported that results obtained by the BACTEC method compared well with the conventional proportion method (employing 7H10/7H111 media) or the resistance ratio method employing L- egg medium (Bannister et al. 1985; Roberts et. al. 1983; Siddiqi et. al. 1981; Siddiqi 1985; Steadham 1985; Vincke et al 1982). The accuracy and reproducibility of the BACTEC method has also been evaluated, with excellent results (Good, 1984; Siddiqi et al 1983; Terrand et al. 1985; Hawkins et al 1988).

Initially, it was recommended that the BACTEC drug susceptibility test should be read daily including the weekend. However, later studies indicate that a non-weekend protocol can also be adopted successfully (Hawkins 1986).

Direct drug susceptibility testing of AFB smear-positive specimens can be successfully carried out using the BACTEC method. In the first studies, reportable susceptibility results were obtained from more than 67% of the AFB smear-
positive and more than 86% of the culture positive specimens with an average reporting time of 10.7 days for *M. tuberculosis* (Broman 1982). There was excellent agreement when the BACTEC direct susceptibility test results were compared with results obtained by the conventional plate method.

At the time the first direct susceptibility study was carried out, there was no BACTEC test available for the identification of mycobacteria. With the introduction of the BACTEC NAP TB differentiation test, a new approach has been adopted. Now, complete information about isolation and identification (TB or not TB) is available by the time direct susceptibility results are reported. Field trials of the direct susceptibility test together with identification indicated good agreement with the conventional results. This allows significantly earlier reporting (Libonati, 1984; Stager, 1984).

The BACTEC drug susceptibility testing procedure is well established for *M. tuberculosis*. However, if an appropriate inoculum is used, susceptibility testing of MOTT bacilli can also be performed with reproducible results. Good correlation of BACTEC and conventional drug (S.I.R.E) susceptibility test results has been reported for *M. Kansasi* (except streptomycin) and *M. marinum* (Hawkins, 1984). On the other hand, results for some other MOTT bacilli do not correlate as well as those for *M. tuberculosis* (Canetti et al 1969; Steadham 1985). In general, in 12B medium, MOTT bacilli show greater susceptibility to streptomycin, rifampin and ethambutol, and are almost always resistant to INH. It is possible that more drug-cell contact in the liquid medium and a more rapid result (4 days as compared to 2-3 weeks) may influence the overall susceptibility test results.

The difference in the susceptibility-testing pattern of MOTT bacilli provided by BACTEC and conventional methods is of great interest and deserves
further investigation. At present, there is no accepted method for susceptibility testing of nontuberculous mycobacteria. Susceptibility testing is generally carried out following the conventional methods recommended for *M. tuberculosis*. However, susceptibility testing of MOTT bacilli has been reported by several investigators with known antituberculosis drugs as well as newer antimicrobials (Steadham 1985; Heifets et al 1988; Heifets 1991; Lee and Heifets 1987). A recent multi-center study established a standardized procedure to carry out susceptibility testing of *M. avium* with the radiometric method and reported good inter-laboratory reproducibility (Siddiqi 1993).