COMPARISON OF LOWENSTEIN-JENSEN MEDIUM, MIDDLEBROOK 7H10 MEDIUM AND BACT/ALERT 3D FOR ISOLATION OF MYCOBACTERIUM TUBERCULOSIS FROM CLINICAL SPECIMENS

By

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M. D.

In

MICROBIOLOGY

Under the guidance of

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Date: **Dr. NAVEEN.G**

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LIST OF ABBREVIATIONS USED

FU	-	Fungi
GNB	-	Gram Negative Bacilli
GPC	-	Gram Positive Cocci
L J medium	-	Lowenstein Jensen Medium
MB 7H10	-	Middlebrook 7h10 Medium
MDR-TB	-	Multidrug Resistant Tuberculosis
MTB	-	Mycobacterium Tuberculosis
NTB	-	Non Tuberculous Mycobacteria
OADC	-	Oleic Acid, Albumin, Dextrose, Catalase
PCR	-	Polymerase Chain Reaction
SDA	-	Strand Displacement Amplification
PCR	-	Polymerase Chain Reaction

ABSTRACT

Introduction – Tuberculosis is the most common cause of death due to single infectious agent worldwide in adults. India alone accounts for 30% of global tuberculosis burden. There is manifest need for method of cultivation of mycobacteria that is reliable, economical and has short turnaround time.

Objective – Present study was attempted to assess the feasibility of using MB bact and middlebrook7H10 as primary isolation medium for mycobacteria. It has been compared with LJ medium, the gold standard.

Materials and Methods – Various clinical specimens from total of 230 clinically suspected cases of TB were studied. All isolates were decontaminated using modified Petroff's method. Each sample was subjected to ZN staining and simultaneously inoculated onto LJ medium, middlebrook7H10 medium and MB bact. Growth from the culture were confirmed by ZN staining and speciated using biochemical reactions.

Results – Out of 230 samples screened, 116 isolates were obtained. Of the 116 all of them were isolated from MB bact, 82 were isolated by LJ medium and 62 were isolated by middlebrook7H10. 82 isolates were obtained by MB bact and LJ medium, 62 were obtained by middlebrook7H10 and MB bact, 58 by LJ and middlebrook7H10 and 58 by LJ medium, middlebrook7H10 and MB bact. Neither L J medium nor middlebrook 7H 1O medium could isolate mycobacteria exclusively. The average isolation time by L J, middlebrook 7H10 medium and MB BACT was 30.81 days,31.06 days and 18.70 days.

Interpretation and conclusion- MB BACT is a better medium compared to L J medium and middlebrook 7H10 medium both in terms of number of isolates obtained and isolation rate.

MB BACT method proved to be a very speedy method and could isolate mycobacteria 7-10 days earlier compared to L J medium and middlebrook 7 H10 medium.

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Tuberculosis has been for many centuries the most important of human infections, in its global prevalence, devastating morbidity and massive mortality.

Despite many advances in its diagnosis and treatment, problem of tuberculosis is on its rise, both globally and in India. At present global incidence of this disease is increasing at the rate of 0.4 % per year[1].

It is estimated that a third of world's population, about two billion people are infected with the tubercle bacilli. Every year between eight and nine million new cases of tuberculosis appears and three million persons die from the disease[2]. The large majority of the cases and deaths are from the poor nations. India is one of the worst affected countries. More than 40 % of the population is infected and some fifteen million suffer from tuberculosis in the country, of which over three million are highly infectious open cases. In 2009, out of the estimated global incidence of 9.4 million TB cases, two million were estimated to have occurred in India[3.]

With the progress of the AIDS pandemic, tuberculosis has become a problem for the rich nations also. A close relation has emerged between tuberculosis and HIV.

Worldwide spread of multi drug resistant tuberculosis (MDRTB) has added new troubles to already existing problem. At present 3.2 % of world's new cases of TB are Multi drug resistant[4]. In India incidence of MDRTB ranges from 1.3 % to 3 %[5].

The most effective control measure to check the spread of TB is to detect it early and to treat optimally to the earliest. Although ZN stain smear Microscopy is the most

commonly employed for early detection, it is rather insensitive and fails to detect large number of cases[6].

Under these circumstances, cultivation of Mycobacterium provides a sensitive and specific means for diagnosis of TB. Conventional culture methods such as Lowenstein Jensen medium requires 3 to 6 weeks for isolation plus additional 1 to 2 weeks for speciation. Such a prolonged turnaround time in the diagnosis is unacceptable as rapid detection and identification of MTB is essential for both medical and epidemiological purpose[7].

Thus, there is manifest need of a culture method that is reliable and has a short turnaround time. Each method has its own advantages and disadvantages, starting from LJ medium and middlebrook 7H 10 medium to the present trendy and speedy automated methods like MB Bact 3D device[8, 9, 10,11,12,13].

In the present study, an attempt has been made to access the feasibility of using middlebrook 7H10 medium and MB Bact 3D device as primary isolation medium for MTB. It has been compared with LJ medium, the gold standard.

AIMS AND OBJECTIVES OF THE STUDY

-To assess the feasibility of using MB bact and middlebrook7H10 as primary isolation medium for mycobacteria.

- To compare L J medium, middlebrook 7H10 medium and MB BACT automated system with respect to number of isolates, rate of isolation, type of isolates and contamination rates.

REVIEW OF LITERATURE

INTRODUCTION

As we enter into millennium, the incidence of tuberculosis in the world is expected to increase from 8.8 million cases in 2005 to 9.27 million cases in 2010.It is estimated that about 1/3rd of the population is infected and another 300 million will be infected in the next decade[14].Thus,Mycobacterium tuberculosis remains the leading cause of infectious disease in man. It causes mainly pulmonary infections and can also affect other organs like intestine, meninges, bones, joints, skin, endometrium etc.

Developed countries like USA were successful in controlling tuberculosis as shown by the decline in the death rate from 199 per lakh in 1980 to 0.5% per lakh in 1990[17].But,the emergence of AIDS and multiple drug resistant tuberculosis have again caused spurt in the cases of tuberculosis. The high rate of drug resistant tuberculosis currently reported from many countries is alarming[18]. This problem of multidrug resistance in tuberculosis cases is very acute in developing countries, including India.

HISTORY:

Tuberculosis may have emerged during Neolithic times, when human population increased and aggregated and cattle were domesticated[19]. Examination of a skeleton dating from the four thousand BC, excavated from Arene Candida Cave in Liguria, Italy demonstrated evidence of spinal tuberculosis[20].

Laennaec, in the beginning of nineteenth century laid the foundation of our present day knowledge of pathology of tuberculosis. He demonstrated nodules in the lungs of

diseased patients to which he called as “tubercles” and gave the name tuberculosis to the disease.

In 1882, Robert Koch first demonstrated the tubercle bacilli.

Tuberculosis was present in America prior to the arrival of Columbus[21,22]. The earliest evidence of tuberculosis in Britain comes from Roman times[23]. Tuberculosis was certainly well described by the time of Hippocrates and Aristotle in the fifth century BC when it was described a phthisis” which was translated into English as consumption[24].

In 1882, Robert Koch reported the isolation of the tubercle bacillus in the Berlin physiological society[25]. Ehrlich introduced the staining technique using hot solution of Carbol fuschin[26]. Then Ziehl-Neelson modified it further, which is named after them[27]. French bacteriologist calmette together with Guerin used specific culture media to lower the virulence of bovine tubercle bacteria creating the basis of our BCG vaccine[28].

EPIDEMIOLOGY

Today, tuberculosis is a major global public problem. In 1997,it was estimated that worldwide there were 7.96 million new cases of tuberculosis, in addition to the 8.22 million existing cases and that 1.87 million people died of tuberculosis. In India, there are an estimated 3.5 million people who are sputum positive with about 2.2 million new cases every year and 0.5 million people dying from TB every[29]. These death rates only partially depict the global TB threat as more than 80% of TB patients are in the economically productive age group of 15-49 years[30].

The WHO and IUATLD launched, along with several partners , a global Drug Resistance Programme, 1994. The first global report on DRS was released in 1997 and included data from 35 countries/geographical areas. median prevalence of resistance to at least one drug among new tuberculosis cases was 9.9% while prevalence of acquired resistance ranged from 5.3% to 100% with a median value of 36%[31].

A second global report followed in 2000 and included data from 58 countries/geographical sites. Prevalence of resistance to at least one drug among previously treated cases, the prevalence of resistance to at least one drug was 23%.There were several "hot spots" around the world where MDR-TB is high and could threaten control programmes. These include Estonia, Latvia, Russians oblasts(territories) of Ivanovo in Europe, Argentina and the Dominican Republic in the Americas and Cote d' Ivoire' in Africa[31].

Bacteriology:

Taxonomy:

Common name: tuberculosis bacterium

Kingdom: prokaryote(bacteria)

Phylum: firmicutes

Class: Actinobacteria

Order: Actinomycetales

Family: mycobacteriaceae

Genus: mycobacterium

Species: Tuberculosis[35]

Morphology:-

Mycobacteria are aerobic ,acid-alcohol fast; slightly curved or straight rods (0.2-0.6 × 1-10μm) which occasionally branch.(extensively branched filaments may occur but they readily fragment into rods and coccoid elements). The organisms are non-motile , non-sporing and non capsulated. They are usually considered to be gram positive but are not readily stained by grams method.

They have guanine-cytosine(G+C) DNA base ratio in the range of 62-70 mol%.mycobacterial mycolic acids are relatively complex and have high molecular weight (60 to 90 carbons).They lack components having more than two points of unsaturation in the molecule. On pyrolysis,they release c22 to c26 straight chains of saturated long chain fatty acids[36].

Pigment production, rate of growth, serological tests.

Pigment production, rate of growth, serological tests,bacteriophage typing, animal pathogenicity, special biochemical tests including enzyme tests and lipid analysis and resistance to thiosemicarbazone are the various tests used for the differentiation of mycobacterium species.

According to Runyon- atypical mycobacterium can be divided into four group[37].

GROUP	PIGMENT	EXAMPLE
GROUP I	Photochromogen, Yellow pigment on brief exposure to light.	M.Kansasii pulmonary infections and cervical lymphadenitis
GROUP II	Scotochromogen , Yellow-orange pigment both in light and dark.	M.Scrofulacium Cervical lymphadenitis in children.
GROUP III	Non photochromogen No pigment even on exposure to light.	M.avium-cervical lymphadenitis in children,lung disease in elderly. M.intracellulare-chronic pulmonary disease indistinguishable from TB.
GROUP IV	Rapid growers	M.fortutium M.chelonei Chronic abscess in human beings.

PATHOGENESIS OF TUBERCULOSIS:-

M.tuberculosis is transmitted by inhalation of infective droplets coughed or sneezed into air by a patient of pulmonary tuberculosis. This droplet nucleus contains ,one or two viable tubercle bacilli which after inhalation, are deposited on alveolar surface where the bacilli begin to multiply. M.tuberculosis pathogenicity is related to its ability to escape killing by macrophages and induce delayed type of hypersensitivity[38] . This has been attributed to several components of M.tuberculosis cell wall , namely cord factor , lipoarabinomannan(LAM), complement and highly immunogenic 65-KDM tuberculosis heat shock protein[36].

Mycobacteria activated T cells interact with macrophages in three ways:-

1. CD4 helper T cells secrete interferon γ which activates macrophages to kill intracellular mycobacteria, through reactive nitrogen intermediates including NO,NO2 and HNO3 .
2. CD8 suppressor T cells lyse macrophages infected with mycobacteria through a Fas-independent,granule dependent reaction and kill mycobacteria[39].
3. CD4 –CD8 –T cells lyse macrophages in a Fas-dependent manner without killing mycobacteria . Mycobacteria cannot grow in the acidic extracellular environment, lacking in oxygen and so the mycobacterial infection is controlled.

Simultaneously large number of monocytes migrates into the infected area. Within few weeks , the number of bacilli increase and spread to secondary sites i.e to the regional lymph nodes. The lymph node enlarges as bacilli multiply intracellularly. The term progressive primary tuberculosis is often used to describe this stage. The bacilli, after entering blood stream may be trapped in the tissue ingested by blood monocytes. When CMI response is suppressed ,tubercle bacilli multiply and when it is restored ,there is infiltration ,which on contact with antigen produces cytokines which are responsible for extensive caseation, liquefaction and cavity formation . Necrosis may rupture into blood vessels, spreading mycobacteria throughout the body leading to tuberculosis of bone,abdomen,reproductive system, kidneys etc or break into airways ,releasing infectious mycobacteria in aerosols[40].

Immunity :-

The host response to infection with M tuberculosis has two dimensions ,one mediating protective response and other causing inflammatory illness, tissue destruction ,mediated by products elaborated by host cells while trying to contain the bacilli[40].

Development of protective immunity correlates with type 1 response in which TH1 and TH2 cells act in concert with CD8 cells and with other cells like macrophages,B cells and some stromal cells. These interactions cause release of cytokines IL-2,IL-12 and IFNγ[41].

The other response is type 2,in which cytokines released are IL4,5,6,10 and 13 which help delayed hypersensitivity and antibody production[41].

CD4-CD8-recognise antigens in association with CD1 cells.These cells appear to be cytotoxic and secrete the TH1 cytokine pattern suggesting that they contribute to development of immunity[41].

Pathology of pulmonary tuberculosis:

Respiratory tract is the primary target organ for tubercle bacilli, where it establishes and causes tissue destruction. The tubercle bacilli may then spread via lymphatics or blood vessels to other systems involving various organs as brain, meninges, bones, joints, kidneys or genitals. The infection is of two main types[42].

a) Primary Tuberculosis:

The infection to an individual who has not been previously infected or immunized is called primary tuberculosis or ghons complex or childhood tuberculosis.

Primary complex or ghons complex is the lesion produced at the portal of entry with foci in the draining lymphatic vessels and lymph nodes. The most commonly involved tissue for primary complex are lungs and hilar lymph nodes[42].

Fate: The lesions may heal by fibrosis, may undergo calcification and even ossification. It may disseminate to other parts of same or opposite lung. The organism may spread through the circulation and may cause primary military tuberculosis[42].

Secondary tuberculosis:

The infection of an individual who has been previously infected or sensitized is called secondary tuberculosis or post primary or reinfection. The adult type is generally due to reactivation of primary infection(endogenous) or it is of the exogenous origin. It affects usually the apices of the lungs[42].

Fate : The lesions may heal with scarring and calcification , it may lead to fibrocaseous tuberculosis , tuberculosis caseous pneumonia or miliary tuberculosis[42].

Antituberculosis chemotherapy:-

Earlier empirical measures such as blood letting ,horse riding ,sea voyages , graded exercise,absolute bed rest,calcium,injections of extracts of gold or other heavy metals,artificial pneumothorax or peritoneum ,thoracoplasty and various other exotic remedies were practiced usually in the setting of a sanatorium for the treatment of tuberculosis,but they were,without much success[43].

Specific treatment aimed at the causative agent became available for the first time only in 1944 with the discovery of streptomycin by Selman Waksman[44,45]. Soon after the discovery of streptomycin,para amino salicylic acid (PAS) in 1949 and isoniazid in 1952 became available,heralding the era of effective antituberculous chemotherapy.This required treatment for a prolonged duration of 18 to 24 months . The discovery of Rifamicin in the late 1960s and the rediscovery of the antimycobacterial activity of Pyrazinamide soon after, were major breakthrough in the treatment of tuberculosis that made it feasible to shorten the duration of treatment considerably[15].

Although antituberculous drugs have been known and used extensively for the last few decades, the mechanism of their action is not completely understood. So, understanding of proper use of older as well as the newer drugs, is essential for the eventual worldwide control of this disease. Clinically chemotherapy leads to

1. Loss of toxemia and
2. Destruction or slowing of multiplication of bacilli in the tissue of the host.[46]

The goals of antituberculous chemotherapy were:-

1. To convert sputum in to negative state in the shortest time.
2. Prevent the emergence of drug resistance.
3. Assure a complete cure without relapse[47].

The introduction of short course chemotherapy in the early 1980s added to the continuous and gradual decline of tuberculosis, resulted in great hopes for the complete eradication of the disease. This optimism was short lived, partly as a result of the Acquired Immune Deficiency Syndrome (AIDS) pandemic and partly due to the multidrug resistance among M. tuberculosis. Although the AIDS epidemic has a deleterious effect on the control of tuberculosis, it has also stimulated further research in to chemotherapy and immunotherapy against the disease. Now antituberculosis drugs and / or new derivatives of old drugs have been studied [47].

According to the govt of India, under RNTCP, the guidelines for categorisation of patient before initiation of antituberculous chemotherapy and different treatment regimes for different categories are[48]:-

Category of treatment	Type of patient	Regimen	Period
Category 1	New sputum smear positive seriously ill,new sputum smear negative Seriously ill,new extrapulmonary	$2H_3R_3Z_3E_3+4H_3R_3$	6 months
Category 2	Sputum smear positive A) relapse B) failure C) treatment after default, others	$2H_3R_3Z_3E_3S_3+$ $1H_3R_3Z_3E_3+5H_3R_3E_3$	8 months
Category 3	New sputum smear negative, not seriously ill New extrapulmonary,not seriously ill	$2H_3R_3Z_3+4H_3R_3$	6 months

Here :- R-rifampicin S-streptomycin
H-isoniazid Z-Pyrazinamide

E-ethambutol

The prefix indicates the duration of drug administered in months. The subscript indicates the number of doses per week.

Antituberculosis drugs:-

In the last two decades many drugs have been tried for the treatment of tuberculosis . To assess the efficiency of any drug as an antituberculosis drug , in vitro and in vivo tests are usually carried out. In –vitro tests are done by using the culture media or using new molecular techniques which have been discussed later. In-vivo tests are usually done on mouse model , but the results in species differ from those in other species

. In-vitro tests have limitations, as there is a discrepancy between in-vitro and in-vivo results[46].

After obtaining the results from these in-vivo and in-vitro assessment of the drugs and from clinical trials, certain drugs are accepted as antituberculous drugs.

These drugs are classified as follows[49]:-

First line drugs	Isoniazid,pyrazinamide,rifampicin,ethambutol and streptomycin.
Second line drugs	Thiacetazone, p-aminosalicylic acid , cycloserine , ethionamide, capreomycin, kanamycin, amikacin.
Third line drugs	Quinolones, rifamycin, clofazimine, macrolide, β lactams, folate antagonists.

On the basis of animal experiments and clinical trials , Mitchison(1985) classified and graded antituberculosis drugs into 3 categories[47,50].

Grade	Prevalence of resistance activity	Early bactericidal activity	Sterilizing activity
High	Isoniazid , rifampicin	Isoniazid	Rifampicin Pyrazinamide
Medium	Ethambutol Streptomycin	Ethambutol Rifampicin	Isoniazid
Low	Pyrazinamide Thiacetazone	Streptomycin Pyrazinamide Thiacetazone	Streptomycin Thiacetazone ethambutol

- The agents which are mentioned under prevention of resistance activity, when combined with others , can prevent the emergence of resistant mutants to the companion drugs.
- Drugs with early bactericidal activity ,induce a rapid decrease in the number of living bacilli in the sputum at the beginning of treatment.
- Thus, reducing the bacillary load in the patient and quickly convert the sputum positive to negative , thus reducing the risk of transmission.
- Drugs with sterilizing activity have the ability to kill all the tubercle bacilli in the lesions of experimental tuberculosis in animals and probably also in humans. They reduce the relapse rate to a minimum within a short period[47].

Drugs effective against mycobacteria can be also divided into 2 categories[52]:-

Broad spectrum agents:- these display antibacterial activity against mycobacteria and against other bacterial species also.

Narrow spectrum agents:-whose activity is primarily restricted against mycobacteria or even to individual mycobacterial species.

Drugs used or under investigation for the therapy of mycobacterial infections.

Spectrum	Antimycobacterial drugs
Broad spectrum agents	Cycloserine, fluoroquinolones, macrolides, rifamycins, streptomycin
Narrow spectrum agents	Capreomycin, clofazamine, dapsone, ethambutol,ethionamide, isoniazid, isoxyl/thio p-aminosalicylic acid, pyrazinamide, thiacetazone.

Mechanism of action , pharmacokinetics, adverse effects and drug resistance of antitubercular drugs.

1) Isoniazid (H) (INH, Isonicotinic acid hydrazide)

Isoniazid (H) is the hydrazide of isonicotinic acid. INH was first reported to be effective in the treatment of tuberculosis in 1952[52]. It is the oldest synthetic antituberculous drug. Isoniazid is bactericidal and most commonly prescribed drug for active infection and prophylaxis[53].

Although this drug has widespread use since the 1960[s], the mechanism of action are becoming known now[51]. INH is a prodrug which is activated by Kat G gene into the active form. But, recent studies have demonstrated that catalase-peroxidases can oxidize INH to an electrophilic substance, the presumed activated form of INH[54].

Concerning the target of activated INH, winder et al in the early 1970s, established that INH had a profound and specific effect on mycolic acid synthesis[55]. Takayama et al demonstrates that INH inhibits the desaturation of C_{24} and C_{26} acids leading to the accumulation of saturated fatty acids of corresponding chain length and the molecular target was most likely an enzyme in the production of an unsaturated C_{24} fatty acid [56,57]. Mdluli et al supported this possibility when they observed that in M tuberculosis treated with INH,there was up regulation of protein with all the characteristics of a long chain acyl ACP, leading to speculation that the target in M tuberculosis was a tetracosanoyl ACP requiring desaturase and the immediate accumulating product was the c_{24} – tetracosanoyl ACP. The conclusion from these studies is that the catalase-peroxidase activated INH binds to the enoyl ACP reductase NADH binary complex.

Isoniazid is well absorbed in the gastrointestinal tract and readily distributed to all body fluids and tissues. It is metabolized by acetylation in the liver and excreted mainly by kidneys.

Adverse reactions of isoniazid include hepatitis,peripheral neuropathy, skin rashes and neurological disturbances.

Mutations in the catalase-peroxidase system encoded by Kat G lead to high level INH resistance[59]. Similarly mutations in the protein coded by inh A gene lead to low level INH resistance. A third protein associated with INH resistance is encoded by aph C . aph C is an alkyl-hydroxyperoxidase reductase involved in the cellular response to oxidative stress. Mutations in the Kat G and the inh A genes are associated with approximately 70-80% of isoniazid resistant mycobacterium tuberculosis[30].

Resistance pattern to INH [31].

The range of Initial Drug Resistance (IDR) for INH has increased from 1.51 -31.7 % in 1994-97 to 0.0-42.6% in 1999-2002, while that of Acquired Drug Resistance (ADR) has increased from 5.3 - 69.7 % in 1994-1997 to 0.0 - 71 %, in 1999-2002 [32].

As frequency of drug resistance varies from country to country. In 1989, resistance to INH in single drug resistance strain in USA was reported to be 4% where as in countries like Philippines, Mexico & Korea it was lower i.e. 2%[60].

In India, Initial resistance, as reported in Kolar (1989) is 32.9% and 17.3% at Bangalore [33]. In 1992, Jain et al have reported Initial resistance to INH as 18.5% [34] .Recently, the Initial resistance, to INH as low as 3.2% at Pune[61]. Malhotra B et al have

reported similar resistance rates from Jaipur. They have reported Initial and Acquired resistance to INH as 13.6% and 39.70% respectively [62].

Acquired resistance to INH ranges from 47% at Wardha (1982-89) to 81% at Gujarat (1983-86) (64,28). Jain et al (1992) have reported Acquired resistance to INH as 50.7% [34].

2) Rifamycins (R) :-

Rifamycins are a group of structurally similar complex macrocyclic antibiotics. [64] The therapeutically useful Rifamycins are semi synthetic derivatives of Rifamycin B, obtained from culture filtrates of Streptomyces mediterraneae. Rifamycins (Rifampicin and Rifabutin)are included in broad spectrum agents, as are effective against tuberculosis, leprosy, mycobacteria other than tuberculosis infections, most gram positive and gram negative bacteria. They are active against both extra and intracellular tubercle bacilli.[47]

Rifampicin has rapid bactericidal activity against tubercle bacilli. Rifapentine which has tenfold greater activity against Mycobacterium tuberculosis than Rifampicin, is currently in Phase II trials as a new component of multiple drug regimes for treatment of tuberculosis. [51]

Rifamycins act by binding to the β subunit of bacterial DNA dependent RNA polymerase to prevent initiation of transcription.[65,66]

Rifampicin is well absorbed from the gastrointestinal tract and is distributed .In effective concentrations in the liver, bones, lung, urine, saliva and ascitic fluid but not in CSF. The drug is deacetylated in liver into an equally active metabolite, both are excreted

in the bile, the excreted nonmetabolised drug is reabsorbed and enterohepatic cycle is established. 60% of its oral dose is excreted in the faeces and 30% in the urine[47].

Adverse reactions of Rifampicins include nausea, vomiting, abdominal cramps, diarrhoea, headache, drowsiness, skin rashes, red discolouration of urine & tears, flu like syndrome, renal failure, thrombocytopaenia, hemolytic anaemia and liver dysfunctions [47].

Rifampicins primary action is to block the elongation step in DNA dependent transcription by mycobacterial RNA polymerase [59], Nearly all mutations resulting in RMP resistance occur within a central 27 codon portion of the gene encoding the beta subunit (rpo B). Alterations in codons for Ser 531 or His 526 seem to make up most mutations found in resistant clinical isolates . A very small percentage of Rifampicin resistant clinical Mycobacterium tuberculosis isolates do not map to the 511-533 region of RNA polymerase, but may be present in the carboxy terminal region of the protein.[53]

Resistance pattern to Rifampicin :

Although the IDR to Rifampicin has not changed considerably from 1994 to 2002 (up to 16.8% in 1994 to 15.6 % in 2002) , the ADR to Rifampicin has decreased from 57.9%, in 1997 to 50% in 1999 with an increase up to 61.4% in 2002 [32].

In India, Rifampicin resistance is a major cause for concern. Reports from N Arcot and Bangalore during 1983 to 1990 show primary resistance to Rifampicin was same i.e. 2.3% where as it increased from 1990 to 2000, as evidenced by report from Tamilnadu (4.4%) and Jaipur (6.8%) [33,62]. But Acquired resistance to this drug was higher in 1983 to 1990 as reports from N Arcot, Raichur, Delhi and Jaipur show it as 12%, 17.1%, 33.3%

and 28.2% respectively[34,62]. In contrast, recent report from Waradha shows Initial resistance to be only 0.5% .

3) Rifabutin (Ansamycin) :-

Rifabutin is a spiropiperidyl rifamycin derivative, has been found to be active against Mycobacterium tuberculosis in vitro[47]. It is more active than Rifampicin against M avium complex with lower MIC and a lower natural resistance rate. For Rifampicin resistant strains, the MIC's of Rifabutin were found to be much higher indicating cross resistance between the two compounds. The data concerning in vivo activity of Rifabutin against M tuberculosis have been somewhat conflicting .Rifabutin was found less active or inactive than Rifampicin in pulmonary This may be due to the low plasma concentration, which are not fully compensated for by the slightly greater antituberculous activity of Rifabutin in – vitro.

41 Ethambutol (E) :-

Ethambutol is a synthetic agent active only against mycobacteria[47]. It has a specific bactericidal action and is used in most modern combinations of antitubercular therapies[53].

The primary mechanism of action of ethambutol is the inhibition of arabinogalactan synthesis, which is critical in the development of cell wall structure [(51).] Takayama & Kilburn demonstrated that the incorporation of ^{14}C from glucose into cell wall arabinan was immediately inhibited upon addition of ethambulol to young cultures [(51)].

Ethambutol is well absorbed following oral administration and is distributed rapidly and widely to most cells, tissues and body fluids.The drug is dialysable and is metabolised by oxidation in the liver, within 24hrs of administration, 50% of ingested dose is excreted unchanged and 15% is excreted as metabolites in the urine [47].

The most important adverse reaction is optic neuritis with decrease in visual acuity, constriction of visual fields and loss of red / green discrimination [47].

Telenti et al have now demonstrated that natural resistance to EMB results from an accumulation of genetic events determining over expression of the Emb proteins (of which three Emb A, B, C were described) structural mutations in Emb B or both [67].

During the period 1994-97, the Initial and Acquired drug resistance to Ethambutol was in the range of 0.0-9.9%&0.0-29.6% respectively, which increased slightly in time period 1996-1999, when they were in the range of 0.0%-11.1%&0.0-32.1% respectively. Across the world, Initial resistance to Ethambutol during 1999-2002 ranged from 0.0% to 24.8% while Acquired drug resistance in the same period was 0.0% to 54.2% [32].

5) Aminoglycosides :

Consists of two or more amino sugars joined in glycosidic linkage to a hexose or aminocyclitol [68].

a) Streptomycin (S):

Streptomycin is produced by Streptomyces griseus and is a member of the aminoglycoside aminocyclitol group of antibiotics [51,71]. It has a broad spectrum of

antibacterial activity, mostly against gram positive and gram negative bacteria and mycobacterial species [51].

Streptomycin acts by binding irreversibly to a single site in the bacteria 30s ribosomal subunit, preventing initiation of protein synthesis and causing misreading of proteins whose translation is already under way [51,66].

Streptomycin is not absorbed by the gastrointestinal tract and must be given parenterally. It is distributed widely in tissues and body fluids at low concentrations but can accumulate in tissues by binding strongly to the cells, including those of the inner ear and kidney. It is eliminated almost entirely by glomerular filtration [47].

Adverse reactions of Streptomycin include vestibular and auditory toxicity, nephrotoxicity, transient giddiness and skin rashes [47].

Resistance to Streptomycin is attributed to two distinct classes of mutations i.e.

1. Point mutation in S_{12} ribosomal protein, encoded by the rpsl gene, resulting in single amino acid replacement and
2. Mutation occurring on the 16s RNA, encoded in the rrs locus and is thought to interact with the ribosomal S_{12} protein [53].

Resistance pattern to Streptomycin :

The range for Initial drug resistance to Streptomycin although increased slightly from 1994-1997 to 1996-1999, it increased considerably during 1999 - 2002 i.e. 0.3 to 51.5%. ADR to Streptomycin showed an initial decline from 0.0 - 82.6 % in 1994 - 1997 to 0.0 - 52.4 % in 1996-99 but later on there was increase in the ADR to 0.0 - 73. 1 % in 1999-2002 [32].

As per Indian Council of Medical Research, prevalence of Initial and Acquired drug resistance to Streptomycin were 12.5% and 22.9% respectively [16].

b) Capreomycin :

Capreomycin is a macrocyclic polypeptide antibiotic that is produced by Streptomyces capreolus [51]. It is no more effective than Streptomycin against tubercle bacilli and is used either as a primary or secondary drug in combination with other agents [47].There is no direct information on the mode of action of capreomycin but because it is structurally similar to viomycin, it is likely that Capreomycin also inhibits protein synthesis [51].

c) Kanamycin:

Kanamycin is also produced by Streptomyces species and is active against tubercle bacilli and some Gram-negative and Gram-positive bacteria [47]. It appears to be more toxic to the vestibulocochlear nerve than streptomycin and is used mostly as a secondary agent against resistant tubercle bacilli [47].

Initial and Acquired resistance to Kanamycin as reported in Japan in 1992 were 1% &2.8% respectively [70].

d) Amikacin :

Amikacin is effective against tubercle bacilli and it has also been used with Ethambutol, Rifampicin and ciprofloxacin for the treatment of Mycobacterium avium disease in AIDS patients [47]. Amikacin has a complete cross resistance with Kanamycin.

The drug is no more effective then Streptomycin or Capreomycin against tubercle bacilli but is much more expensive [47].

6) Pyrazinamide (PZA):

Pyrazinamide is a synthetic pyrazine analogue of nicotinamide. It is particularly active against tubercle bacilli in an acidic environment both intracellularly and extracellularly in highly inflammed tissue [47]. It is effective against other mycobacteria also [53]. As it has a synergistic and accelerating effect in combination with Isoniazid and Rifampicin, these three drugs are used to shorten the time period of treatment from 12/18 months to 6 months. Thus, this forms the basis of current WHO DOTS (directly observed therapy, short course) [51,71].

Like INH, Pyrazinamide is also a pro-drug, transported or diffused through the mycobacterial cell wall and converted into pyrazinoic acid (the active drug form) [47].

The drug is well absorbed from the gastrointestinal tract. Despite being 50% bound to plasma proteins, pyrazinamide diffuses readily into all body fluids and tissues .The concentration of pyrazinamide in CSF is equal to that in plasma the drug particularly useful in tuberculous meningitis. Pyrazinamide is hydrolysed by the liver to pyrazinoic acid, an active metabolite. 70% of oral dose of Pyrazinamide is eliminated by the kidney[47].

Adverse effects of the drug, include hepatitis, skin rashes, arthralgia gout hyperuricaemia [47].

Resistance to pyrazinamide may be due to mutations in the gene pyrazinamidase (PNCA) so that there is reduction or elimination of activity of amidase [51].

In literature very few reports are available about the prevalence of resistance of pyrazinamide among Mycobacterium tuberculosis.

Rosha et al had reported Initial resistance to pyrazinamide as 6.6% at New Delhi in 2001 [72].

7) P-Aminosalicyclic Acid (PAS) :-

PAS is a highly specific bacteriostatic agent [47] against M. tuberculosis, so it is included in narrow spectrum antituberculosis agents group. Following the introduction of modern short course anti tuberculosis regimens, PAS is rarely used today as a chemotherapeutic agent. Today, it is used specifically in the treatment of Multidrug resistant M. tuberculosis infections [51,73].

The mode of action of PAS is unclear. Some inconclusive evidence suggests that, PAS competes with p-aminobenzoic acid (PABA) or that PAS interferes with the salicylate dependent biosynthesis of the iron-chelating mycobactins involved in iron assimilation [51,74].

PAS is available as the acid and its sodium salt, both of which are readily absorbed from the gastrointestinal tract. It is distributed well to body fluids and tissues but poorly into the brain and CSF. It is excreted by glomerular filtration and tubular secretion [47].

Adverse effects of PAS include anorexia, nausea, vomiting, diarrhea, hepatitis, high sodium load and skin rashes. [47]

Initial resistance to PAS has been reported as 28% during 1964-1965 at Chandigarh [75].

8) Ethionamide :-

Ethionamide is a synthetic derivative of isonicotinic acid, highly specific against mycobacteria [47]. The action of ethionamide is similar to that of isoniazid; i.e. inhibition of the biosynthesis of mycolic acid. It has been reported that a low degree of isoniazid resistance is often associated with resistance to ethionamide [47, 76].

It is rapidly absorbed after a single oral dose and is widely distributed into tissues and body fluids including CSF [47].

In South Africa, Initial resistance to Ethionamide has decreased from 20.8%in 1960 to 6.1% in 1970 to 2.5% in 1980 [77].

Adverse effects of Ethionamide include gastrointestinal irritation, metallic taste, nausea, anorexia, vomiting, diarrhea, hepatitis, stomatitis, skin rashes, photosensitivity, goiter, acne, impotence, peripheral neuropathy and arthralgia [47].

9) Thiacetazone :-

Thiacetazone is a thiosemicarbazone derivative [47]. It was introduced to the antituberculosis pharmacopoeia as a cheap and effective substitute for aminosalicyclic acid in combination chemotherapy and is used mostly in developing countries where the cost of medication is a significant factor in the control of tuberculosis. It has a bacteriostatic action on Mycobacterium tuberculosis [47]. The mode of action of this drug is unknown [51].

Thiacetazone is well absorbed after oral administration. About 20% of oral dosage is excreted in the urine unchanged and some metabolites of the drug are also excreted by the kidney [47].

Skin rashes, nausea, vomiting, diarrhea, hepatitis, bone marrow suppression, agranulocytosis, dizziness, ataxia, vertigo and tinnitus are the adverse effects of thiacetazone [47]. In patients suffering from HIV, the risk of severe and potentially fatal cutaneous hypersensitivity reaction increases with thiacetazone treatment. Hence, this drug is avoided in known or suspected case of HIV infection. Initial resistance to Thiacetazone has been reported as 3.7% at Cameroon in 1995 [78].

10) Cycloserine :-

Cycloserine is a structural analogue of the amino acid D-alanine produced by Streptomyces spp. which can also be produced synthetically. It is active against M-tuberculosis and other bacteria by competing with D-alanine which is essential for the synthesis of cell walls. This results in cell death by lysis. Usually, it is used as a second line agent in combination therapy of drug resistant tuberculosis.

The drug is well absorbed after oral dose and is distributed to all body fluids tissues including CSF. 70% of the oral dose is excreted unchanged in urine within 72 hours [47].

Adverse effects of the drug include drowsiness, lethargy, headache, dizziness, confusion, disorientation, loss of memory, convulsion, coma suicidal tendencies, personality changes and hyperirritability [47].

Cycloserine resistance has been attributed to alr A gene by a recent genetic analysis of a recombinant clone[51, 79].

11) Macrolides :-

The macrolides comprise a family of antibiotics ranging from erythromycin to roxithromycin, azithromycin and spiramycin. [51] Roxithromycin (RU-28965) seems promising in the treatment of tuberculosis. [47] Other macrolides have been used in clinical trials for treatment of M avium disease in AIDS patients. [47] Clarithromycin and azithromycin hold promise by causing a reduction in the bacillary load as measured with serial blood cultures. Results with Clarithromycin alone were better than the combination of Rifampicin, Isonazid Ethambutol and Clofazimine in the treatment of M avium disease in AIDS patients [47].

The molecular basis of macrolide action has been elucidated in non-mycobacterial species. These antibiotics bind to a single high affinity site in the peptidyl tRNA binding region of the bacterial 50s ribosome subunit, causing dissociation of peptidyl -tRNA from ribosomes and hence inhibition of bacterial protein synthesis. The binding site contains 235 ribosomal RNA with which macrolides interact at the 2058-2062 nucleotide region of the molecule. Resistance in the mycobacteria is associated with single point mutations at nucleotides 2058 and 2059 in 23s ribosomal unit [51].

- Lactam Antibiotics :-

- Lactam Antibiotics are extensively used against both gram positive and gram negative bacteria and have been evaluated against M tuberculosis also, but mycobacteria produce lactamase and are resistant to lactam antibiotics, penicillinase resistant

penicillins such as Methicillin, Oxacillin and Dicloxacillin are not hydrolysed and act as competitive inhibitors of lactamase but they do not have sufficient intrinsic activity against M. tuberculosis. Among the cephalosporin family , ceforanide appeared to be a promising antituberculous drug. [47, 80]

A new group of lactam compounds with little or no intrinsic antimicrobial activity appeared to be potent lactamase inhibitors and enhanced the antimycobacterial activity of associated penicillins. Clavulanic acid and Amoxicillin (Augmentin 375mg) produced growth inhibition in 25 of 30 strains of M. Tuberculosis at a concentration of 8mg/L. The activity of Augmentin is enhanced by changing the molar ratio of ampicillin, clavulanic acid from 1:1 to 2:1[47].

12) Fluoroquinolones :-

These are 4 Quinolones that contain a carboxylic acid moiety in the 3rd position of the basic ring structure. Newer quinolones contain a fluorine substituent at position 6, and many of these compounds contain a piperazine moiety at position 7. [70]

The quinolone antibiotics and the newer fluoroquinolones are synthetic derivatives of nalidixic acid [51]. These compounds are bactericidal against M. tuberculosis. There does not appear to be any synergism between fluoroquinolones and other antitubercular drugs and their activity is independent of resistance to other antitubercular drugs [52]. Fluoroquinolone therapy for tuberculosis is predominantly used in patients infected with multidrug resistant organisms. In spite of these precautions, in the use of fluoroquinolones as antituberculars, fluoroquinolone resistance is emerging via primary resistance

mechanisms as well as nosocomial infection with fluoroquinolone resistant organisms [53].

The target of fluoroquinolone action is the bacterial DNA gyrase, an ATP dependent type II DNA topoisomerase that catalyzes the negative supercoiling of DNA. The enzyme is a heterotetramer composed of two A and two B subunits (A_2B_2) encoded by the gyr A and gyr B genes respectively. Fluoroquinolones bind to the gyrase, inhibiting supercoiling and subsequent process such as replication and transcription [53].

Fluoroquinolone resistance in M tuberculosis is associated with point mutations within a part of gyr A (encoding the A subunit of DNA gyrase) termed the quinolone - resistance determining region (QRDR) [51]. Other mechanisms of resistance include changes in cell wall permeability, active quinolone efflux pumping [53].

a) Ciprofloxacin :-

Marinis and Legakis reported that ciprofloxacin was active against all strains of M tuberculosis which are sensitive to primary antituberculosis drugs and also active against almost all strains showing intermediate sensitivity or resistance to one or more of the primary antituberculosis agents. Almost all isolates were inhibited at a concentration of 3.2mg/lit (3.2ug/ml). Following a single oral dose of Cipro 250, 500 mg, mean peak plasma concentrations of 0.76 to 2.9 mg/lit are achieved within 0.5 to 2.3hrs. The drug may achieve levels in the pulmonary tissue in excess of those in plasma. So, the achievable Ciprofloxacin level will be able to inhibit almost all of the clinically important species of mycobacteria including those showing resistance to one or more of the primary antimycobacterial agents [82].

Gay et al reported that Ciprofloxacin is active against 90% of M. tuberculosis with MIC of 1ug/ml (1mg/L) [83]. In vitro studies showed that its activity against M tuberculosis is independent of that of Streptomycin, isoniazid, Ethambutol and Pyrazinamide and that there is antagonism between Ciprofloxacin and Rifampicin. In the mouse model, Ciprofloxacin plus Rifampicin appeared to be more effective than Isoniazid plus Rifampicin [84]. There are no controlled study reports on the clinical use of Ciprofloxacin for the treatment of human tuberculosis even though it is being used with other second line antituberculous drugs for the treatment of multidrug resistant disease.

The drug is generally well tolerated. It is distributed widely into most body fluids and tissues at concentrations exceeding those in plasma, CSF concentration is low. It is partially metabolised in liver and the metabolites are excreted in urine and faeces. Adverse reaction include nausea, vomiting, abdominal pain, headache restlessness, dizziness, insomnia, hallucination manic reaction etc. [47].

Various workers have evaluated the role of Ciprofloxacin in the treatment of tuberculosis. During 1990-2000, the MIC values of Ciprofloxacin were as follows.

Sukesh Rao in 1993 reported that when Ciprofloxacin was used in the retreatment of 16 patients, 13 showed favorable response [85].

K. Karak (Paul) &P K De in1995, showed MIC to Ciprofloxacin as 0.15 -1.2 mg/l (97). In 1993 P Venkatraman observed the geometric mean for MIC of ciprofloxacin was 2.00ug/ml for sensitive strains and 2.17 ug/ml for resistant strains [87].

But by 2001, Deepak Rosha et al reported a very high resistance to ciprofloxacin, 22.6% making quinolones increasingly ineffective as a second line drug in 2001 [72].

b) Ofloxacin (DL 8280)

Ofloxacin is used in the treatment of respiratory tract and urinary tract infections and has also been evaluated now for the treatment of tuberculosis.

In 1983, Tsukamura showed that ofloxacin was active against M. tuberculosis as well as against potentially pathogenic non-tubercular mycobacteria by in-vitro testing [88].

Tsukamura, in 1985, during in-vitro testing reported that ofloxacin might be beneficial in the treatment of drug resistant tuberculosis because no cross resistance between ofloxacin and other antituberculosis drugs was seen [89].

A few clinical studies have been conducted with ofloxacin. Tsukamura and colleagues in 1985 reported the first study of ofloxacin in the treatment of patients with chronic cavitary pulmonary tuberculosis whose sputum remained positive for M tuberculosis after a prolonged treatment with antituberculosis drugs. Sputum cultures in 5 of the 19 patients were converted to negative but resistance to ofloxacin emerged in patients whose sputum did not convert [47,89]

In a study conducted by Hong Kong Chest Service / BMRC, 17 patients with pulmonary tuberculosis who had shown resistance to SHR and subsequently retreated with ofloxacin, 10 showed the response and in 3, disease remained quiescent. Ofloxacin appeared to be better drug of choice in combination with any companion drug available [90].

P .Venkatraman et al in 1993 noted that the geometric mean of MIC for Ofloxacin was 2.00ug/m! for sensitive strains and 2.05 ug/ml for resistant strains, but K Karak & P K De showed that MIC to Ofloxacin was lower 0.15 -0.6mg/l in 1995[86,87].

As a last choice ofloxacin may be given to patients of tuberculosis where bacilli show resistance to many antituberculous agents but this will lead to the selection of ofloxacin resistant mutants. These patients will no longer respond to other potentially available newer quinolones as cross resistance is well known phenomenon among these compounds [91].

Quinolone Resistance Around The World :

Retrospective analysis of drug susceptibility tests on M, tuberculosis isolated in Philippines from 1995-2000 showed resistance to ciprofloxacin as 26.8% and ofloxacin (35.3%) of the MDR strains, more than half (51.4%) were resistant to ciprofloxacin and ofloxacin [92,93]. In Spain a rising trend of minimum inhibitory concentration for ofloxacin has been observed in strains both susceptible and resistant to primary antituberculosis drugs [94]

P. Venkatraman et al have reported the MIC for ciprofloxacin and ofloxacin in a narrow range of 1-4ug/ml for most of the M. tuberculosis strains listed. However, their use in patients with multiple drug resistant organisms may lead to selection of drug resistant mutant as shown by increase in MIC for drug resistant mutants. They therefore, suggested that these drugs should be used only in judicious combination with other drugs [87].

c) Sparfloxacin :- (AT 4140)

It is a new difluorinated quinolone with claims of invitro activity and in vivo efficacy equal to or better than Ofloxacin and Ciprofloxacin. In a study conducted by Rastogi and Goh, comparison of bactericidal action with reported plasma peak concentration has shown that sparfloxacin has a potential use against tubercle bacilli [(95)]. Among fluoroquinolones, it was first to display an efficacy similar to that of Isoniazid in preventing multi drug resistant tuberculosis infections. Truffort, et al reported that Sparfloxacin (AT-4140) was six to eight fold more effective against experimental M tuberculosis in mice than Ofloxacin [(96)]

d) Moxifloxacin (BAY 12-8039):-

Moxifloxacin (BAY 12-8039) is a new 8-methoxyquinolone with broad spectrum activity against gram-positive, gram negative and anaerobic bacteria. Moxifloxacin has also in vitro activity comparable to that of Rifampicin against susceptible, resistant strains of M tuberculosis to one or more of the commonly used antimycobacterial agents [(97)]

Miyazaki E et al reported that Moxifloxacin was active against highly virulent clinical strain of M tuberculosis (CSU 93) in mice and MIC was 0.25 ugm/ml, which is below that of Ciprofloxacin and Ofloxacin. These results are comparable to the in vitro activity of Moxifloxacin against CSU 93 strain or M tuberculosis. The drug activity was also comparable to that seen with INH used in the same animal model. A combination of Moxifloxacin and INH was also highly effective in reducing M tuberculosis loads and sterilizing the lungs of infected mice, indicating that Moxifloxacin may prove to be useful in combination therapy against active tuberculosis especially of drug resistant tuberculosis. [(98)]

Newer drugs :-

These new drugs are under trial for their utility.

1) Oxazolidinones are a new class of specific bacterial protein synthesis inhibitors which exert activity against M tuberculosis, both methicillin susceptible and resistant staphylococcus aureus and other gram positive bacteria. U-97456 is one of a series of five indolinyl derivatives which has a MIC of 1 mg/L against M tuberculosis, however it has no activity against M avium. [99, 100, 101]
2) Gangamicin - is a new synthetic antibiotic which exerts its activity by virtue of being an analogue of coenzyme Q and Vit.K and thereby inhibits cell-wall synthesis. It is active against both M. tuberculosis and M avium (MICs 1-20mg/L and 2.0-8.0 mg/L respectively) The activity also encompasses drug resistant M tuberculosis strains [102].
3) Acridinones are a recent introduction and their mode of action may involve RNA synthesis inhibition. [103]
4) The 2'2' bipyridyl analogs, VUF 8514 and VUF 8842 have bactericidal activity M tuberculosis, including resistant strains [104]
5) K-130 is a dihydrofolate reductase inhibitor which although being targeted towards M leprae does possess activity against M tuberculosis [105]
6) BCH 950 is an inhibitor of ceil wall synthesis similar to isoniazid but with fewer adverse effects. It is in Phase 1 clinical trials [105].
7) Isoxy (thiocarlide). A number of diacyl thiureas have shown considerable activity in experimental infections with M tuberculosis. One, of these 4,4' diisoamaloxydiphenylthiourea (isoxyl, ISO thiocartide) has proved clinically useful. It has

been shown that like INH, ISO strongly inhibited mycolic acid synthesis in M bovis during a 6 hr exposure to 10 ug / ml [51].

8) Recently, a series of compounds containing a nitromidazopyran nucleus that possess antituberculous activity have been described. After activation by a mechanism dependent on M tuberculosis F420 cofactor, nitromidazopyran inhibited synthesis of protein & cell wall lipid. They exhibit bactericidal activity against both replicating and dormant M tuberculosis [31].
9) Lead compound PA-824 showed bactericidal actively against multidrug resistant M. tuberculosis and promising oral activity in animal models. [31]

Drug Resistance in Mycobacterium tuberculosis :

Phenomenon of acquiring resistance to antitubercular drugs by Mycobacterium tuberculosis is known since very early days of chemotherapeutic era. The problem has become more acute in the last two decades. It is now increasingly becoming known that drug resistant tuberculosis is an impediment in effective control of tuberculosis.

The important factors that are responsible for this phenomenon are treatment with single drug, inadequate dose or duration of therapy, contact with a drug resistant case etc. Of late, HIV/ AIDS has been regarded as one of the important risk factors. In fact, coinfection of HIV and TB was largely responsible for re-emergence of the later in developed countries.

Classification of Drug Resistance – Changing Concepts

Traditionally drug resistance in MTB was classified as Primary (if patient has not taken treatment) and acquired (if he has taken treatment). But for the purpose of

drug resistance surveillance WHO and IUATLD have revised these as **resistance among new cases** and **resistance among previously treated cases** respectively.

Resistance among new cases is defined as "Presence of resistant strains of MTB in a patient who, in response to direct questioning denies having had any prior anti-TB treatment for more than 1 month and in countries where adequate documentation is available for whom there is no evidence of such history."

Resistance in a previously treated patient is defined as "Presence of resistant strains of MTB in a patient who, in response to direct questioning admits having been treated for tuberculosis for one month or more or in countries where adequate documentation is available, in a patient for whom there is evidence of such history."[138]

MDR – TB at present is defined as, "The disease due to MTB that is resistant to two frontline anti-tubercular drugs viz. Isoniazid and Rifampicin, with or without resistance to other drugs."[138]

Prevalence of Drug – Resistant Tuberculosis :

a. Global Scenario :

The magnitude of this problem worldwide is not exactly known. The studies done in various parts to estimate the prevalence have certain drawbacks such as, inclusion of small or non-representative samples, failure to distinguish between the patient who had anti-tubercular treatment and those who had not, lack of uniformity of the method, lack of standardization of laboratory procedures etc. Lack of facilities for culture and anti-mycobacterial drug susceptibility testing in all parts of world was also a major hurdle in the exact assessment.

In 1994, WHO and IUATLD have initiated a global project on anti-tubercular

drug resistance surveillance. It has published the report on prevalence of resistance to four frontline drugs in 35 countries between 1994 to 1997. Resistance was found in all the countries and it was most common for INH and Streptomycin.

In American continent, drug resistance is highest in Dominican Republic. Elsewhere is low including in USA and Brazil.

Despite high rates of HIV coinfection, level of drug resistance is low in African countries. This is mainly due to lack of access to anti-tubercular drugs and relatively late introduction of Rifampicin.[139]

In Western European countries the rate of prevalence is extremely low i.e. less than 1 %. But in East European countries rising trends have been observed. This is due to irregular supply of drugs, which has resulted due to economic down growth seen in post-communist era.[138]

The prevalence of drug resistance including multi-drug resistant tuberculosis is exceptionally high in almost all former Soviet Union countries. This is once again attributed to diminishing standards of health care facilities in these countries.[138]

In Asia, the level of drug resistance is high in Chinese provinces of Henan and Leioning, and in Israel. Similar trends of high level resistance has been seen in South East Asian countries such as India, Thailand, Vietnam. However, level of drug resistance was low in Korea.[138]

b. Indian Scenario :

In India too, magnitude of problem of drug resistance varies from area to area.

In the decade of 1960s ICMR conducted two nationwide surveys at nine urban chest clinics in different parts of India. The results showed resistance level of 8.2% to

INH alone, 5.8% to Streptomycin alone and 6.5 % to both of them. Studies conducted a decade later to assess the prevalence in new cases showed that they had not changed significantly.

Resistance to Rifampicin was reported first in the decade of 1980s from North Arcot, Pondichery, Bangalore and Jaipur.

A retrospective study at Delhi by Jain, Chopra and Prasad showed high level of resistance among new cases to INH (18 5 %) and low level of resistance to Rifampicin.[140]

The rate of MDR – TB in India is very low and ranged from 0 to 6%. MDR – TB in new cases was found to be less than or equal to 3.2% and its level in previously treated cases was less than or equal to 6%,[138] except in Gujarat where it was in the range of 11.4 to 18.5%.[141]

Venkatakumari and Jagannath have reported prevalence of MDR – TB in the state of Tamil Nadu to be 20. 3%. They observed that majority of their patients had irregular and interrupted treatment owing to non-availability of drugs.[142]

Need for Antimycobacterial Drug Susceptibility Testing :

Reliable anti-mycobacterial drug susceptibility testing is an integral component of tuberculosis control programme of any country. Its need arises,

1. To guideline the anti-tubercular treatment in a patient.
2. To confirming drug resistance in patients who are demonstrating poor response to anti-tubercular treatment.
3. To evaluate trends in primary and acquired resistance within the community i.e. for the

purpose of surveillance.

The rates of drug resistance are considered as the indicators of quality of tuberculosis control programme in that country.[138] Acquired drug resistance develops in patients who do not receive appropriate treatment or who fail to take their medication.

Types :

Over the last 40 years many methods have been developed for performing anti-mycobacterial susceptibility testing. Earlier efforts were directed towards development of a test which is simple, reliable, reproducible and the one which can be employed in resource poor settings. Of late much emphasize has been given to reduce the turn – around time required for testing. Semi automated and fully automated methods are particularly helpful in this regard.

All the available techniques regardless of their underlying principle can be categorised into two broad groups viz. **direct testing** and **indirect testing** depending upon the inoculum used.[143]

Direct Method :

Here clinical sample, sputum in a case of pulmonary tuberculosis, is "directly" inoculated on drug containing and control media.

The main advantages of this method are shorter turnover time and better representation of patient's original bacterial population. But it suffers from important disadvantages such as high contamination rate, lack of standardized inoculum.[143]

It is not so popular in comparison to indirect method.

Indirect Method :

Here pure growth is used as inoculum instead of clinical sample.

The method has lesser contamination rate and the inoculum size is amendable for standardisation.[143]

Most of the methods described here employ indirect susceptibility testing.

Methods of Antimycobacterial Drug Susceptibility Testing :

1. Absolute Concentration Method :

The method involves the use of graded concentrations of drugs incorporated in the medium, usually LJ. A standardized inoculum is inoculated on them. Several concentrations are tested and results are expressed in terms of MIC.

This method is greatly affected by viability of organism. It is not commonly used in India.

2. Resistant – Ratio Method :

Ratio of MIC of test strain and that of standard laboratory strain H37Rv gives the measure of resistance of test strain.

The test is also affected by inoculum size and viability of organisms. Ganga dharan et. al. Have recommended this method for routine drug sensitivity. They found it reliable if bacteriological techniques are standardized.[110]

3. Proportion Method :

This method attempts to demonstrate a clinical observation that if more than 1 % of patient's tubercle bacilli are resistant to a drug, the therapy with that drug is unsuccessful.

Several dilutions of inoculum are seeded in both control and drug-containing media so that at least one of them yields isolated countable colonies. If the number of

colonies grown on drug containing medium are equal to or more than 1 % of the colonies grown on control medium then the strain is said to be resistant for that drug.

There is much controversy regarding the medium to be used for performing the test. NCCLS discourages the use of LJ medium on the grounds that potency of drug is unreliable after inspissation and recommends Middlebrook 7H10 or Middlebrook 7H11 media. However, National Tuberculosis Institute, Bangalore recommends the use of LJ medium.[144, 145]

Currently, this method is the method of choice for performing drug susceptibility testing.

4. E Test :

This method involves use of a plastic strip to which drug is coated in graded manner. Such strip is applied to Middlebrook 7H11 agar plate and incubated for 7 to10 days. MIC are read at the point where ellipse formed by inhibited growth crosses the strip.

Reports regarding its utility in susceptibility testing for M. tuberculosis are variable. Hausdorfer and colleagues have reported high rate of false resistance with this method in comparison to BACTEC and conventional LJ proportional method.[146]

But several other studies report no significant differences in the results obtained by E test and proportion method.[147, 148]

5. BACTEC – 460 :

This method, which was initially used for detection of Mycobacteria, has now been successfully adapted for susceptibility testing. It is based on principle of proportion method.

A multicentric study by Glenn Robert and colleagues have shown high levels of correlation between the results obtained by BACTEC and conventional proportion method using LJ. At least 20 days were saved reporting the results by BACTEC in comparison of conventional method.[149]

Both qualitative and quantitative methods are available, the later being more advantageous as MIC determined can be correlated with the drug concentrations attainable in humans.[109]

6. MGIT :

Several studies regarding the successful use of MGIT for susceptibility testing have been published.

M. Palaci and colleagues have demonstrated excellent correlation among the results obtained by MGIT and conventional indirect proportion method on LJ slant. The mean time for reporting the results by MGIT and conventional method was 5 days and 16 days respectively.[150] Similar studies conducted in other parts of world agree with these finding.[151]

A multicentric study involving 441 specimens has indicated that this system is as efficient as BACTEC 460 TB method for susceptibility testing and holds the potential to replace the later one.[152]

However, Enrico Tortoli and collegues have argued that this method does not allow the use of universally accepted basic concept of proportion method and hence the data generated is not reliable.[153]

7. MGIT – 960 :

This fully automated method has now been used for susceptibility testing. E.Tortoli

and colleagues have evaluated the performance of MGIT – 960 method and radiometric method and agar proportion method. There was 96.7 % agreement between these methods. The time needed for test completion was on average 2.5 days shorter with BACTEC method. However the MGIT – 960 system showed tendency to overestimate the resistance and also contamination rate was high.[153]

8. ESP – II System :

Few studies are available regarding evaluation of this system for susceptibility testing.

Bergmann and Woods have compared it with conventional proportion method. Their data indicates that ESP–II is a reliable and rapid method for testing susceptibilities of MTB isolates to INH and Rifampicin. Data generated regarding its performance with Ethambutol and Streptomycin was suboptimal as very few isolates were resistant to these drugs.[154]

9. Microscopic observation Drug Susceptibility Assay : (MODS)

This technique originally developed as a rapid technique of tuberculosis culture has now been adapted for susceptibility testing.

Walter Park and colleagues have compared MODS and agar proportion method. Their results suggest high degree of correlation (>96%). MODS detected resistant isolates in 7 days while proportion method did it in 28 days.[155] Studies by David Moore and colleagues have confirmed these findings.[132]

The method is relatively inexpensive and has immense scope of application in resource poor setting like ours.

10. Nitrate Reduction Assay :

This method originally developed at Central Tuberculosis Research Center, Moscow by Griess J.P., is known for its simplicity, rapidity, reliability and inexpensiveness and has potential to be an alternative test for drug susceptibility in resource poor setting.

In a study by Kristian Angeby and colleagues comparing Nitrate Reduction assay and BACTEC 460, an agreement of 94 % was seen between them.[156]

Sunil Sethi and colleagues have compared Nitrate reduction Assay with Proportion Method on LJ slant. They reported overall agreement of more than 99%. The results were available in 7 to 14 days by Nitrate Reduction Assay as compared to Proportion Method which took 4 to 6 week.[157]

Non – Culture Techniques :

1. Colorimetric Methods :

Two such techniques are available at present, viz. Microwell Almar Blue Assay (MABA) and Tetrazolium Microplate Reduction Assay. (TEMA). They are based on oxidation and reduction of dye Alamar Blue and Tetrazolium Bromide respectively.

Both these methods are reliable, give faster results and do not require sophisticated instruments for interpretation.

Luz Cavidez and colleagues have compared these two methods and concluded that TEMA performs the task of drug susceptibility as quickly and as reliably as more expensive MABA.[158]

2 . Phage based assays :

At present three different techniques are available which use mycobacteriophages. Among them most novel one is Luciferase reporter Phage assay. In this technique, viable Mycobacteria are infected with reporter phages expressing luciferase gene of firefly Pnotinus pysalis. Upon infection with viable Mycobacteria it produces detectable signals, which is not possible in the Mycobacterial cells treated with active antimycobacterials. Though this assay has good sensitivity and reproducibility, they are not very well established in routine Mycobacteriology laboratory practice.

Another phage based technique **Pha B assay** (Phage amplified biologically) has been successfully applied for antimycobacterial susceptibility testing.[143]

Recently, in India, a phage based assay – **Fast plaque Assay** has been introduced for detection of Rifampicin resistance. It has been reported to be reliable.

LAB DIAGNOSIS

The key to the diagnosis of tuberculosis is a high index of suspicion. Often, the diagnosis is first entertained when the chest radiograph of a patient being evaluated for respiratory symptoms is abnormal. The longer the delay between the onset of symptoms and the diagnosis, the more likely is the finding of cavitary disease. In contrast, immunosuppressed patients, including those with HIV infection, may have "atypical" findings on chest radiography e.g., lower-zone infiltrates without cavity formation.

PPD Skin Testing

Skin testing with PPD is most widely used in screening for *M. tuberculosis* infection. The test is of limited value in the diagnosis of active tuberculosis because of its low sensitivity and specificity. False-negative reactions are common in immunosuppressed patients and in those with overwhelming tuberculosis. Positive reactions are obtained when patients have been infected with *M. tuberculosis* but do not have active disease and when persons have been sensitized by nontuberculous mycobacteria or bacille Calmette- Gue´rin (BCG) vaccination.[166]

A presumptive diagnosis is commonly based on the finding of AFB on microscopic examination of a diagnostic specimen such as a smear of expectorated sputum or of tissue

Diagnosis of Latent Tuberculosis Infection

QuantiFERON®-TB gold (The Whole Blood IFN-gamma Test Measuring Responses to ESAT-6 & CFP-10 Peptide Antigens) QuantiFERON®-TB Gold is an indirect test for *M. tuberculosis* infection (including disease) and is intended for use in conjunction with risk assessment, radiography and other medical and diagnostic evaluations.

The QuantiFERON®-TB Gold is a test for Cell Mediated Immune (CMI) responses to peptide antigen cocktail simulating the mycobacterial proteins ESAT-6 and CFP-10. These proteins are absent from all BCG strains and from most non-tuberculosis mycobacteria with the exception of *M. kansasii*, *M. szulgai* and *M. marinum*. Individuals infected with *M. tuberculosis* complex organisms usually have

lymphocytes that recognize these mycobacterial antigens. This recognition process involves the generation and secretion of the cytokine, IFN-γ. The detection and subsequent quantification of IFN forms the basis of this test.

The QuantiFERON®-TB Gold test is both a test for LTBI and a helpful aid for diagnosing *M. tuberculosis* complex infection in sick patients. A positive result supports the diagnosis of tuberculosis disease; however, infections by other mycobacteria (e.g., *M. kansasii*) could also lead to false positive results.[167]

SAMPLE COLLECTION[168]

The number of specimen required for diagnosis of smear positive pulmonary TB is two, with one of them being a morning sputum specimen.

Two sputum specimens are collected over one, or two consecutive days. Of the two sputum specimens, one is collected on the spot and the other is an early morning specimen collected at home by the patient. One specimen positive out of the two is enough to declare a patient as smear positive TB. Smear positive TB is further classified as a new or re treatment case based on their previous treatment history, and appropriate therapy is prescribed.

Other respiratory specimens include induced sputum,tracheal aspirate, bronchoalveolar lavage, pleural fluid and lung biopsy tissue.

AFB microscopy

Most modern laboratories processing large numbers of diagnostic specimens use auramine-rhodamine staining and fluorescence microscopy. The more traditional

method light microscopy of specimens stained with Kinyoun or Ziehl-Neelsen basic fuchsin dyes- is satisfactory, although more time-consuming**.**

Grading of AFB smears by Z-N microscopy[169]

No of acid-fast bacilli (AFB)	Fields	Report
No AFB	In 100 immersion fields	Negative
1-9 AFB	In 100 immersion fields*	Record exact figure (1-9 AFB/ 100 fields)
10 to 99 AFB	In 100 immersion fields	1+
1 to 10 AFB	Per field (examine 50 fields)	2+
More than 10 AFB	Per field (examine 20 fields)	3+

DETECTION OF MTB

Processing of sample - concentration methods

The most commonly used decontamination and concentration method for sputum samples are Modified Petroff's method and NALC (N Acetyl L cysteine) method.

Conventional culture method

For detection of Mycobacteria in clinical specimens the current "gold standard" consists of a combination of solid and liquid media. In detecting as few as 10^1 – 10^2 viable organisms per ml of specimen in the optimal case, culture is more sensitive than smear. Also it is the only reliable means to monitor effectiveness of therapy in TB patients.[170]

Specimens may be inoculated onto egg- or agar-based medium (e.g., Lowenstein-Jensen or Middlebrook 7H10) and incubated at 37ºC under 5% CO2. Because most

species of mycobacteria, including *M. tuberculosis*, grow slowly, 4 to 8 weeks may be required before growth is detected. Although *M. tuberculosis* may be presumptively identified on the basis of growth time and colony pigmentation and morphology, a variety of biochemical tests have traditionally been used to speciate mycobacterial isolates. In today's laboratories, the use of liquid media for isolation and speciation by nucleic acid probes or high-pressure liquid chromatography of mycolic acids (HPLC) has replaced the traditional methods of isolation on solid media and identification by biochemical tests. These new methods have decreased the time required for bacteriologic confirmation to 2 to 3 weeks.[162]

Rapid and sensitive detection of *Mycobacterium tuberculosis* is of clinical importance for the treatment, control, and prevention of tuberculosis. Despite new nucleic acid amplification assays, unequivocal diagnosis of tuberculosis continues to rely on cultivation of *M. tuberculosis*.

Automated culture method[171]

The fully automated systems that allows continuous monitoring of mycobacterial cultures are

1) BACTEC 460
2) MB/BacT
3) ESP culture system II

The **BACTEC 460 system** (Becton Dickinson, Sparks, Md.) has been marketed since 1977. The Middle brook 12B medium for this system contains ^{14}C labeled

palmitic acid as the substrate. During mycobacterial growth, ^{14}C -labeled $^{14}CO_2$ is produced and released into the headspace air of the vials. A $^{14}CO_2$ detection device allows early determination of mycobacterial growth. However, this system requires radioactive reagents, causing waste problems, and vials have to be handled and punctured for readings at least eight times during 6 weeks of incubation, requiring a considerable amount of work and increasing the risk of cross contamination.

The recently developed **MB/BacT system** (Organon Teknika, Turnhout, Belgium) relies on a continuous colorimetric CO_2detection device to indicate mycobacterial growth in a closed system. A solid-state sensor at the base of each vial contains the colorimetric indicator, which changes from green to yellow when CO_2 is produced in the vial. Each compartment of the instrument where the vials are incubated contains a reflectometer and a detection unit.

ESP culture system II is based on the detection of pressure changes in the head space above the broth medium resulting from gas production or consumption due to growth of micro organisms

Other phenotypic detection methods

The fast plaque TB (Biotech Labs Ltd., Ipswich, UK) is a rapid manual test for the detection of *M. tuberculosis* from clinical specimens within 48 h.[172] This test utilizes specific mycobacteriophages (Actiphage™) to reflect the presence of viable *M. tuberculosis*. Mycobacteriophages are added to a clinical specimen and allowed to incubate for one hour to allow phage infection of target tubercle bacilli. After the incubation period, a virucidal solution (Virusol™) is added, which destroys all

phages that have not infected the bacilli. The remaining phages replicate in the infected bacilli until new progeny phages are released as the cells lyse. The progeny phages are amplified by the addition of a non- pathogenic rapidly growing mycobacterial host *M. smegmatis* (Sensor™ cell), which is also able to support phage replication. This is visualized as plaques, which are clear areas in a lawn of Sensor™ cell growth. The number of plaques visualized is directly related to the number of viable tubercle bacilli in the original sample.

Mycolic acid analysis (HPLC)

Mycolic acids are present in all mycobacteria. Their composition is constant for all strains of a given species and varies from species to species. An HPLC method for analysis of mycolic acid esters, has been standardized and demonstrated to be a rapid and reliable method for identification of many mycobacterium species[173]

In this procedure, mycolic acids are extracted from saponified mycobacteria, converted to p-bromophenacyl esters, and analysed by HPLC. The mycolic acid esters are separated on a reversed-phase C18 column by a methanol-methylene chloride gradient elution and detected by UV or fluorescence detection spectrophotometry.

The standardized method recommends a visual comparison of a sample HPLC pattern on an atlas of reference strain patterns in combination with the use of peak height ratios.

GENOTYPING METHODS FOR DETECTION OF M.TUBERCULOSIS [174]

DNA probes:

Based on information about specific gene sequences well defined oligonucleotide probes for identification of various clinically relevant mycobacteria have been developed and are readily available. These probes are being used in several countries for rapid confirmation of the identity of mycobacterial isolates. When used along with newer methods of detection of the early growth (such as BACTEC, Septi-Chek, MGIT) these are of great help in rapidly confirming the diagnosis as identity of isolate can be established within 1 to 2 days with gene probes as compared to much longer time required with classical biochemical tests

INNO LIPA

The LiPA assay is based on the principle of reverse hybridization. Biotinylated DNA material, obtained by means of a PCR amplification of the 16S-23S ribosomal RNA spacer region, is hybridized with 14 specific oligonucleotide probes immobilized as parallel lines on membrane strips. The addition of streptavidin labeled with alkaline phosphatase and of a chromogenic substrate results in a purple-brown precipitate on hybridized lines**.**

Gene amplification methods for direct detection of *M.tuberculosis* sequences from clinical specimens:

Gene amplification techniques have made a major impact on the diagnosis of mycobacterial diseases. These methods may be classified as those based on polymerase chain reaction (PCR) and others based on isothermal amplification reactions. Gene amplification techniques are highly sensitive and under optimum

conditions may detect 1-10 organisms. If systems are adequately standardized, evaluated and precautions for avoiding the contamination are taken, these assays can play a very useful role in early confirmation of diagnosis in paucibacillary extra-pulmonary forms of tuberculosis.

PCR methods

A variety of PCR methods have been developed for detection of specific sequences of *M.tuberculosis* targeting either DNA or rRNA/and these could be based on conventional DNA based PCR, nested PCR and RT-PCR. Targets include insertion and repetitive elements, various protein encoding genes, ribosomal rRNA etc. A large number of PCR assays targeting different gene stretches of *M. tuberculosis* have been described. Different Indian investigators have used separate gene targets like MPB 6433, repetitive sequences , GC repeats , devR, 38kD, TRC^{160}, IS 1081 and a system patented by Central Drug Research Institute (CDRI), Lucknow. Some of these assays (CDRI) have been repeatedly found to be reproducible, highly sensitive and specific in double blind evaluations. IS-1081 based system has been further modified and a new nested PCR target of this gene has been developed at Centre for DNA Fingerprinting and Diagnostics (CDFD), Hyderabad. This strategy can be used for confirming the diagnosis and also monitoring the progress.

Isothermal amplification techniques:

These techniques represent a major step forward in the application of gene amplification technology without thermal cycler. The important methods are:

(i**) Strand displacement amplification (SDA):**

It is an isothermal in vitro nucleic acid amplification technique which is based upon

the ability of HincII to nick the unmodified strand of hemiphosphorothiote form of its recognition site and the ability of exonuclease deficient klenow to extend 3' end at the nick and displace the downstream DNA strand. Exponential amplification results from coupling sense and antisense reactions.

(ii**) Gene probe amplified *Mycobacterium tuberculosis* direct test:**

This assay employs the isothermal amplification of *M. tuberculosis* complex rRNA followed by detection of amplicon with acridinium ester-labeled DNA probe. Reports about the application of this system are encouraging.

(iii) **Q-beta (QB) replicase based gene amplification**:

This approach involves production of RNA in the amplification reaction using QB replicase as the enzyme and reaction at fixed temperature (for example 37°C). Using a suitable combination of capture and detector probes, sensitivity up to one colony forming unit has been reported for *M. tuberculosis*. Further, the inhibitors of PCR were observed to have no effect on this assay.

Gene amplification methods for identification and speciation of *M.tuberculosis*

Different strategies to identify the isolates from cultures and directly from the clinical specimens have been described. These include amplification of specific gene regions followed by hybridization with species specific probes, sequencing and RFLP analysis such as hsp 65 kDa gene 21, katG22 and rRNA genes (23-25, a CJIL system under publication) have been described. These PCR-RFLP assays help in quick identification of pathogenic mycobacteria including *M. tuberculosis* from the culture isolates as well as directly from the clinical specimens. While PCR-sequencing approach can be applied by reference laboratories the hybridization and RFLP

approaches are easily practicable in clinical mycobacteriology laboratories.

Cultural Characteristics[161]

The bacilli grow slowly, the generation time in vitro being 14-15 hours. Colonies appear in about two weeks and may sometimes take up to eight weeks. Optimum temperature is 37ºC and Optimum pH is 6.4-7.0. *M. tuberculosis* is an obligate aerobe. It grows luxuriantly in culture as compared to *Mycobacterium bovis* which grows sparsely. They are termed as eugenic and dysgenic respectively. The addition of 0.5% glycerol improves the growth of *M.tuberculosis.*

Tubercle bacilli do not have exacting growth requirements but are highly susceptible even to traces of toxic substances like fatty acids in culture media. The toxicity is neutralized by serum albumin (or) charcoal. Koch originally grew the bacillus on heat coagulated bovine serum. Several media, both solid and liquid media have been described for the cultivation of tubercle bacilli. The solid media contain egg (Lowenstein-Jensen, Petragnini, Dorset), Blood (Tarshis), serum (Loeffler) or potato (Pawlowsky). The solid medium most widely employed for routine culture is Lowenstein-Jensen (LJ) medium without starch, as recommended by the International Union Against Tuberculosis (IUAT). Among the several liquid media described, Dubo's, Middle brook's, Proskauer and beck's, Sula's and Sauton's media are the more common.

On solid media, *M.tuberculosis* forms dry, rough, raised, irregular colonies with a wrinkled surface. They are creamy white, becoming buff coloured on further incubation. In liquid media without dispersing agents the growth begins at the bottom, creeps up the sides and forms a prominent surface pellicle which may extended along

the sides above the medium.

Identification of Bacteria

Once an isolate has been recovered in the mycobacteriology laboratory, certain characteristics may be used to classify the isolate before performing biochemical tests.

The first step is to confirm that the isolate recovered in broth or solid media culture is an acid-fast organism by performing an acid-fast stain. Then once the organisms are growing on solid media, phenotypic characteristics such as colony morphology, growth rate, optimum temperature and photo reactivity helps to speciate Mycobacteria. These characteristic do not allow for definitive identification but are presumptive and help in the selection of others, more definitive tests.

Colony Morphology

Colonies of Mycobacteria are generally distinguished as having either a smooth and soft or a rough and friable appearance. Colonies of *M. tuberculosis* that are rough often also exhibit a prominent patterned texture in liquid cultures referred to as cording (curved strands of bacilli)

Growth Rate

Growth rate and recovery time depend on the species of Mycobacteria but are also influenced by media, the incubation temperature, and the initial inoculum size. Mycobacteria are generally categorized as having visible growth within or more than 7 days. Rapid growers are able to produce colonies in fewer than 7 days.

Temperature

The optimum temperature and range at which a Mycobacterial species may grow may be extremely narrow, especially at the time of initial incubation. The growth of *M.tuberculosis* occurs at 37 °C.

Photo-reactivity

Mycobacterium species have traditionally been categorized in three groups according to their photo reactivity c Photochromogens - that produce carotene pigment upon exposure to light (color ranges from pale yellow to orange). Scotochromogens - that produce pigment in the light or the dark.

Non Chromogenic Or Nonphotochromogenic- colonies are buff colored and are nonphotoreactive i.e. on exposure to light does not produce pigment e.g., *M. Tuberculosis*[161] characteristics.

Cultivation of TB bacilli :

Robert Koch successfully grew TB bacilli for the first time by using heat coagulated bovine serum.

In 1907 A. S. Griffith and F. Griffith, working for Royal commission of Tuberculosis, reported dorset egg as the most satisfactory medium for primary cultivation of MTB. In 1930 Lowenstein modified the dorset egg medium. In 1946 Copper and Cohn suggested use of malachite green for suppression of contaminants.

Jensen in 1955 further modified it by using glycerol as carbon source instead of starch. This modification called as Lowenstein-Jensen medium is the most popular medium for cultivation of tubercle bacilli today.

First agar based medium, containing bovine serum, oleic acid was described by

Middlebrook and Dubos in 1947. During 1950s Middlebrook and Cohen developed a series of defined culture media with agar as solidifying agent. Middlebrook 7H10 and 7H11 are widely used agar based media today.

First liquid medium for cultivation of tuberculosis bacilli was described by Von Schweinitz in 1883. In 1884 Proskaur and Beck described a liquid medium containing asparagines, glycerol and mineral salt. Soutan in 1912 modified it. Dubos in 1947, advocated the use of enzymatic digest of casein.

In 1975 Cummings and co-workers were first to utilize ^{14}C-labelled substrates viz. glycerol and acetate to detect growth of MTB. Later, Middlebrook and Co-workers formulated Middlebrook 7H12 broth, a liquid medium with ^{14}C-labeled palmitic acid as substrate.[108] This medium has revolutionized the isolation at TB bacilli.

Methods To Culture Mycobacterium tuberculosis :

Culture of the tubercle bacilli is important in many ways. It not only provides a more sensitive and specific means of diagnosis of disease, especially in smear negative cases, but also provides pure growth of the organism which subsequently can be used for identification of organism by various phenotypic and genotypic tests. The pure growth is also helpful for performing drug susceptibility testing indirectly.

An ideal culture medium for isolation of MTB should have following characters :

1. Should give early and luxuriant growth from a small inocula.
2. Should enable preliminary separation of mycobacterium based on pigment production and characteristic colony morphology.

3. Should be able to suppress the growth of contaminants effectively.
4. Should be user friendly i.e. should be easy to prepare, should have longer shelf life.
5. Should be economical.

However, such an ideal medium at present is for from reality as all contemporarily used media have some or other inherent drawbacks.

Almost all the currently used media are variations of 3 general types i.e. egg based, agar based and liquid media. Within each general type there are non-selective and selective formulations. The later contain various antibacterial and antifungal agents to suppress their growth and impart some degree of selectivity to medium for Mycobacteria.

Following is the classification of media that are in common use currently for cultivation of M. tuberculosis.

I. Solid Media :

A. Egg based :

- Lowenstein-Jensen (LJ) medium.
- Gruft modification of LJ medium.
- American Thoracic Society (ATS) Medium. B. Agar Based :
- Middlebrook 7H10 medium.
- Middlebrook 7H10 selective medium.
- Middlebrook 7H11 medium.
- Middlebrook 7H11 selective medium.

II. Liquid Media :

A. Radiometric :

- Middlebrook 7H12 medium. (BACTEC

460). B. Non- Radiometric :

- Middlebrook 7H9 Broth.
- Septi –Chek AFB system.
- Mycobacterial Growth Indicator Tube (MGIT).
- MB-Redox Tube.

C. Continuous Growth Monitoring Systems :

- ESP Culture system- II
- BACTEC 9000 MB.
- BACTEC MGIT- 960.
- MB/BacT.

III. Miscellaneous :

- Slide Culture.

In the following pages some of the more commonly used culture media and commercial systems have been briefly reviewed with special reference to rate of isolation duration of isolation and rate of contamination.

1. Lowenstein – Jensen Medium :

This is the most commonly used medium for cultivation of tuberculosis bacilli. The medium, in its commonly used form consists of coagulated whole egg, defined salts, glycerol, asparagine etc. Addition of malachite green in the concentration of 0.025 gm% makes it somewhat selective.[111]

Over the years many modifications of this medium have been described. Gruft modification, which in addition to previously described ingredients contains RNA (5

mg %) and antibiotics Penicillin (50u/ml), and Nalidixic acid (35mg/ml), is more selective than original LJ. It is more popular in European countries.[111] Petran and Vera have suggested use of Cycloheximide (400mglml), Lincomycin (2mg/ml) and Nalidixic acid (35 mg/ml instead of Penicillin and Nalidixic acid only. This modification gives both antibacterial and antifungal coverage to medium.[112] Recently Hemvani and Chitins have recommended the use of 3 % human blood in the LJ medium for enhanced and earlier growth.[113]

The reported rate of isolation of tubercle bacilli an LJ medium ranges from 70% to 85%.[114, 115] It is affected by various factors such as quality of specimen, time lag between collection of specimen and its inoculation on medium, concentration of malachite green in medium, use of antitubercular chemotherapy by patient and number of slants used. The rates are also affected by AFB smear results.

Stager and colleagues have shown that LJ was substantially inferior to BACTEC in recovery of MTB, but only marginally so in comparison to Middlebrook 7H11. The isolation rates of LJ were at par with Middlebrook 7H11 medium if 2 LJ slopes were incocluated.[116] But Wilson ML and collegues, in their comparison of LJ and Middlebrook 7H11 have reported recovery rates of 40 % and 81 % respectively.[117]

The average time required to detect growth on LJ medium differs in smear positive case and smear negative case. In smear positive cases it is 18 to 22 days and in smear negative cases it is 28 to 31 days. Its is substantially longer compared to BACTEC radiometric system which detects growth as early as in 7 to 8 days in for smear positive cases and up to 14 to 28 days in smear negative cases.[108] In case of

Middlebrook 7H11 agar the duration required for recovery is on an average 22 days i.e. almost same as that of LJ.[116]

The rate of contamination on LJ medium ranges from 0 to 9%.[118, 119, 108] It mainly depends upon the method of decontamination used.

Because of these drawbacks, LJ medium is gradually being phased out from routine use by many Mycobacteriology laboratories of developed countries.[107] But in the country like ours, because of cost effectiveness and simplicity in its preparation and use, LJ medium will continued to be used extensively at least in near future.

2. Middlebrook 7H10 and 7H11 Media :

Both these media are prepared from basal medium of defined salts, vitamins, co-factors, oleic acid, albumen. Catalase, glycerol, dextrose. Middlebrook 7H11, in addition to these contains 0.1 % casein hydrolysate which improves recovery of isoniazid resistant strains.[107, 120]

Middlebrook 7H10 is made selective by adding Cycloheximide, Lincomysin and Nalidixic acid.[22,31] Mitchison made Middlebrook 7H11 selective by addition of carbeneillin, AmphotericinB, Polymyxin B and Trimethoprim.[111, 120]

Stager et. al. have shown that there is not much difference in the recovery rate of Middlebrook 7H11 medium in comparison of LJ medium (79 % and 76 % respectively). The mean recovery times in smear positive patients for Middlebrook 7H11 and LJ were 18.1 days and 22.3 days respectively. In smear negative patients these figures were 32.4 and 31.8 days respectively.[116]

Micro colony method, originally described by Runyon in 1970 and later modified by Weltch and colleagues is based on observing the microscopic morphologic features of young colonies of Mycobacteria grown on thin layer of Middlebrook 7H11 medium.[121] Though this method is less expensive and requires about half the time needed for conventional culture, the recovery of MTB is less efficient.[122]

Both these media show high contamination rates in comparison to LJ medium, probably because they contain malachite green in 10 times smaller quantities than LJ.[111]

3. BACTEC 460 TB :

This technique utilizes ^{14}C labelled palmitic acid in Middlebrook 7H12 liquid medium. Mycobacteria, if present utilize this radio-labelled substrate for metabolism and release $^{14}CO_2$, which is detected by BACTEC system and reported in terms of growth index. The bottles which yield growth index more than 10 are considered positive.[120]

Mycobacterium tuberculosis complex can be differentiated from MOTT by incorporating P-nitro-a-acetylamino-b-hydroxy propiophenone (NAP).[108, 123] The compound inhibits Mycobacterium tuberculosis complex but not MOTT. Further and MTB and M.bovis can be differentiated by incorporating thiophene-2-carboxylate hydrazide (TCH).[123]

Choong Park and colleagues in their study comparing BACTEC system with LJ observed that BACTEC system detected 93 % cases while LJ could detect 82 %

cases. BACTEC required an average of 7 days for detecting MTB while LJ required 18 days. Contamination rates for BACTEC and LJ were 6.2 % and 9.1 % respectively.[108] Similar observations have been made by P Venkatraman and Co- workers in a study conducted at Tuberculosis Research Center, Chennai.[124]

Thus BACTEC system in comparison to LJ medium can detect significantly higher number of cases at an earlier date and with minimal contamination rate. These advantages along with greater safety to laboratory personnel have made this system very popular in developed countries.

Despite these advantages, the system has certain limitations, such as inability to observe colony morphology, high cost, use of radioactive materials, use of needles and cross contamination.[120, 124, 107]

4. Septi – Chek AFB system :

This system consists of a capped tube containing Middlebrook 7H9 broth with enriching substances and antibiotics. The solid phase is in the form of a paddle. One side of paddle is covered with Middlebrook 7H11 agar. The reverse side is divided into two halves, one containing LJ medium with NAP and other contains chocolate agar. This arrangement enables the system to detect M. tuberculosis, non-tuberculosis Mycobacteria and contamination simultaneously.

The overall isolation rate of this medium is 80-95%.[125, 126] This means it is far more superior than LJ medium for recovery of the organism. This may be partially due to greater inoculum size used for inoculating Septi-Chek for recovery of MTB.

With regard to time for detection, Septi-Chek appears to be equivalent or

slightly better than LJ medium but inferior to BACTEC.[107, 125, 126] The average time taken by Septi-Chek to detect growth of M.tuberculosis is 20 days.[107, 123]

Conflicting reports are available regarding rate of contamination of septi-chek in comparison to solid medium. Hoffner SE co-workers[125] and Tortoli E and Co-workers[126] have reported lesser rate of contamination than LJ but Isenberg and colleagues have reported higher contamination rate with septi-chek (4.5%) than LJ (2%).[127]

5. Mycobacteria Growth Indicator Tube :

It contains Middlebrook 7H9 broth along with nutritional supplement and antibiotic cocktail and a fluorescent compound which is embedded in a silicon sensor.

Oxygen diminishes the fluorescent output of the sensor. As the actively growing and respiring Mycobacteria consume the dissolved oxygen, the sensor glows indicating Mycobacterial growth. The fluorescence is observed by UV light of wavelength 365mm.

It appears from various studies that the system has better recovery rate than LJ medium but less than BACTEC method.[128, 129, 130]

Akos Somoskovi and Pal Magyar have reported much shorter mean recovery time for smear positive and negative patients are 7.2 days and 19.1 days respectively, 20.4 days and 25.8 days respectively than conventional LJ medium.[131]

Rate of contamination appears to differ with the method of decontamination used. In one study when Trisodium phosphate was used for decontamination the

contamination rates for MGIT and LJ medium were 4 % and 1.2 % respectively but when Na0H-NALC was used there was no significant difference in contamination rate.[130]

6. Microscopic Observation of Broth Culture :

This simple technique is based on the fact that morphology of M.tuberculosis in liquid culture is characteristic and recognizable, consisting of tangles or cords of the organism. When broth culture of the organism is viewed with inverted microscope characteristic Mycobacterial growth can be detected long before it becomes visible to naked eye.

A 24 well plate containing middlebrook 7H9 broth with OADC supplement and PANTA antibiotic cocktail is inoculated with decontaminated sputum sample. The plates are examined daily from days 5 to 15, on alternate days from days 16 to 25 and twice weekly from days 26 to 40, under on inverted light microscope at x 40 magnification.

A Peruvian study has shown that, this technique could detect 94 % cases while conventional LJ medium could detect 87 % cases. The mean time required for culture positivity by microscopic observation of broth culture in pretreatment and on treatment cases was 8.2 days and 10 days respectively. Similar figurer for LJ medium were 17.3 days and 23.6 days respectively.[132]

This technique is relatively inexpensive, rapid, compares well with standard methods of culture and hence is especially useful in disease endemic resource poor countries. [132]

7. Continuous Monitoring systems :

a. MGIT- 960 :

It is fully automated system for growth and detection of Mycobacteria with a capacity to incubate and continuously monitor 960 MGIT tubes every 60 minutes for increase in fluorescence. Growth detection is based upon the metabolic utilization of oxygen by Mycobacteria.

An Italian multicentric study by Enrico Tortoli et.al. comparing this system with BACTEC 460 and LJ has shown that yield obtained by MGIT 960 was slightly lesser than radiometric method but significantly more than LJ. This system could detect growth 1.5 days and 12 days earlier than BACTEC and LJ respectively. The contamination rates of MGIT 960, BACTEC and LJ were 10 %, 3.7 % and 17% respectively.[133]

b. MB/BacT System :

This method relies on continuous colorimetric CO_2 detection device to indicate Mycobacterial growth in a closed system. A solid sensor at the base of each vial contains the colorimetric indicator, which changes from green to yellow when CO_2 is produced in the vial. Each compartment of the instrument where vials are incubated contains a reflectometer and a detection unit. The measured values are transmitted every 10 minutes to a computer which indicates vials with Mycobacterial growth based on sophisticated algorithm.

A Swiss study comparing this method with BACTEC 460 and LJ medium revealed that MB/BacT could recover MTB in 86.3% cases, BACTEC in 91.8% and LJ in 79.5% cases. Time taken for culture positivity were 17.5 days, 14.3 days and

24.3 days for MB/BacT, BACTEC and LJ respectively. Rates of contamination

on MB/BacT and BACTEC were 9% and 2.7 % respectively. Despite its inferior performance in comparison to BACTEC, authors have recommended MB/BacT as potential replacement for BACTEC on the grounds of lesser work load and minimized exposure to lab worker.[134]

c. BACTEC – 9000 MB System :

It is a fluorescence based continuously monitoring detection system which uses a modified Middlebrook 7H9 broth along with a supplement and antibiotics to suppress the growth of contaminating organisms. The growth of organism is detected by oxygen specific sensors.

A large multicentre study has shown that this system was much efficient in recovering MTB than LJ medium but less so in comparison with radiometric method. The mean recovery time in smear positive cases was 12.2 days and 9.3 days with BACTEC 9000MB and BACTEC460 respectively. The rate of contamination was significantly high with BACTEC 9000MB i.e. 6.8 % in comparison to 1.6 % of BACTEC 460. Despite these drawbacks authors have recommended BACTEC9000MB as alternative to radiometric method due to comparable recovery rate and total elimination of problems associated with radiometric method.[135]

d. ESP – II Culture System :

This fully automated continuously monitoring system is based on the detection of pressure changes within the headspace above the broth culture medium in a sealed bottle i.e. either gas production or gas consumption due to Mycobacterial growth.

Enrico Tortoli et. al. have compared this system against BACTEC, ESP –II and LJ were 89 %, 79 % and 64 % respectively, the differences being statistically significant. With regard to time needed for detection of positive culture, it was significantly longer with LJ (28 days on average) than remaining two systems , between which there was no difference (average 18 days). Authors concluded that ESP system when used in combination with a solid medium performs as well as radiometric method. It offers the advantage of full automation and absence of radio isotopes. Woods and colleagues also found this system to be reliable and less labour intensive alternative to radiometric method when used in conjugation with middlebrook 7H11 agar.

MATERIALS AND METHODS

Study Centre :

Present study was carried out at Mycobacteriology division of Department of Microbiology of BLDEU's Shri B M Patil medical college, Bijapur.

Study Period :

Over a period of one and a half years, from October 2009 to may 2011.

Study Type :

Cross sectional study.

Sample Size :

230, suspected cases of tuberculosis attending the OPDs of Medicine, Paediatrics, Respiratory Medicine, surgery and admitted to the wards of these departments were included in the study.

Selection of Cases :

Selection of cases was based on certain inclusion and exclusion criteria, which were as follows

INCLUSION CRITERIA

1. All age groups and both sexes having suspected tuberculosis were included in present study.
2. Only those cases yielding growth of mycobacterium tuberculosis from cultured sample were included in the study and were further identified.

EXCLUSION CRITERIA

1. Cases of tuberculosis, which do not yield growth of mycobacterium tuberculosis but yield growth of other bacteria, were excluded.

A. **SAMPLE COLLECTION**

1. **SPUTUM:**

-Early morning sputum sample was collected in a sterile leak proof, wide mouthed, screw capped container.

-The patient was instructed to;″rinse his mouth with water, stand facing a wall, away from wind, keeping both hands on his hips, cough forcibly and collect sputum in his mouth and spit carefully into the cup and close the lid tightly".

-At least 4 to 5 ml sputum brought from the lungs after a deep, productive cough was collected.

2. **Laryngeal Swab**

-Using a sterilized absorbent cotton swab Laryngeal swab was collected .

-This is useful in children and patients who cannot bring out sputum or may

-swallow it.

3. **Gastric Lavage**

-The patient was hospitalized for collection of gastric lavage specimen.

-The sample was collected early in the morning before breakfast in a sterilized one oz McCartney bottle.

-The specimen was transported to the laboratory immediately and was processed at the earliest possible to avoid killing of acid-fast bacilli due to the presence of acidic pH in the specimen.

-If the specimen could not be transported immediately, it was neutralized by adding 5 ml of 10% Trisodium Phosphate.

4. **Bronchial Washing, Pleural Fluid and Biopsy**

- **Collection of BAL :**The procedure was carried out by Chest physician. Flexible bronchoscope was passed into the bronchi. About 200 ml of normal saline was injected and allowed to remain there for 2 minutes. The saline was sucked out using a suction apparatus and fluid was collected in a sterile "trap".
- Bronchial Washing was collected in a sterile McCartney bottle taking precaution not to introduce any contamination.
- Pleural fluid was collected in a sterile large saline bottle.
- Biopsy material is collected in a sterilized McCartney bottle with 5 to 10 ml of sterile normal saline.

All the above samples were collected with citrate to prevent coagulation.

B. Transportation of sample :

All the samples were transported to the laboratory and processed as early as possible. If any delay is anticipated in processing, they were stored at 4^{0} c

C) Sample processing:

1. MICROSCOPY :

Smear was prepared from the purulent portion of sputum on a clean slide on an area of 2 x 1 cm. They were air-dried and heat fixed and subjected to Zeil-Neelsen staining as recommended by NTI. The procedure was as follows.[175]

The Smear was flooded with Carbol-fuchsin and heated from below till vapours were seen. Heating was repeated after an interval of 2½mins. The slide was

washed with tap water.

It was decolourised with 20% Sulphuric acid for 2½ mins. and then washed with tap water. The procedure was repeated. The slide was counterstained with 0.1% methylene blue for 10 seconds and washed with tap water once again. The slide was blotted with clean blotting paper and dried in air and observed under oil immersion lens.

The grading of the smear was done as follows :[175]

Number of AFB	**Result**	**Grading**	**No. of fields to be examined**
>10 AFB per oil immersion filed	Positive	3+	20
1-10 AFB per oil immersion filed	Positive	2+	50
10-99 AFB per 100 oil immersion filed	Positive	1+	100
1-9 AFB per 100 oil immersion filed	Scanty	Record exact number	200
No AFB in 100 oil immersion field	Negative	-	100

2. DECONTAMINATION

Modified Petroff Technique was used for decontamination of sputum sample.[176]

4-ml. of sputum was taken in screw capped bottle and an equal volume of 4% NaOH was added to it. Mixture was homogenized by shaking on vortex machine for 15 minutes and then incubated at 37^0C for 20 minutes. The mixture was centrifuged at 3000 rpm, for 15 minutes. The supernatant was poured off in the disinfectant solution and deposit was resuspended in 15 ml of sterile distilled water. The solution was centrifuged once again at 3000 rpm, for 15 minutes. Supernatant fluid was poured off and the deposit was used for further processing.

3. PRIMARY ISOLATION

a. Culture on LJ Medium :[176]

In house prepared LJ medium was used for primary isolation of mycobacteria.

Two loops full of decontaminated deposit was inoculated on the entire surface of two LJ slopes in a pre-sterilized inoculation hood taking necessary asceptic precautions. Date of inoculation was noted. The slopes were incubated at 37^0C for a maximum period of 8 weeks. The slopes were inspected daily for the growth or for the contamination. In case of growth of Mycobacteria, date of appearance of first colony was noted and slopes were further incubated for more growth. In case of contamination, the slopes were removed and processed further as described later.

b. Culture on Middlebrook 7H10 Medium :

Commercially obtained Middlebrook 7H10 agar base as well as OADC supplement were used for study (Hi Media). The medium was prepared as per the manufactures instructions and was dispensed in 10ml aliquots in sterile screw capped bottles.

Two bottles, each of which were inoculated with 500µl of decontaminated deposit aseptically. The date of inoculation was noted. Tubes were incubated at 37^0C for a maximum period of 8 weeks. They were observed daily for appearance of growth.[118].

c) Mb bact:[182]

System Description

Intended Use

The Mycobacterial (MB) configuration of the BacT/ALERT® 3D instrument is designed to grow and detect mycobacteria from patient samples.

Overview

The BacT/ALERT® 3D system uses no radioactive reagents, is non-invasive, and offers walk-away automation for mycobacteria detection. No handling of the bottle inoculated with specimen is required after it is placed in the BacT/ALERT® 3D instrument. Positives are signaled immediately upon detection. After a specified time, which can be determined by the operator, negatives are signaled if no growth has been detected.

The BacT/ALERT® 3D instrument consists of a Controller Module that directs the activity of one or more Incubation Modules. Each Incubation Module may contain one or more MB Drawers for mycobacterial detection. Each drawer can be converted by a service engineer to perform either an MB or non-MB function. One important difference between the drawer configurations is that MB Drawers do not agitate the bottles during incubation. MB Drawers are clearly identified by a red label on the drawer handle. It is important to open and close the door gently to minimize avoidable agitation when adding or removing bottles.

The disposable Mycobacteria bottles contain bioMerieux's patented colorimetric sensor which is continuously monitored for positive sample detection. Unloading or manipulating the bottles when not indicated by the system may interfere with critical bottle readings. In addition, the bottles contain broth and atmosphere which promote the recovery of a wide variety of Mycobacterium species without venting. MB/BacT® Antimicrobial Supplement and Reconstitution Fluid inhibit the growth of contaminating organisms and enhance the recovery of mycobacteria.

The data generated by the BacT/ALERT® 3D are managed by extensive data management software which provides a high level of flexibility. Mycobacterial data can easily be stored, queried, sorted and reported. Predefined reports and macros are available, or they may be customized by the user.

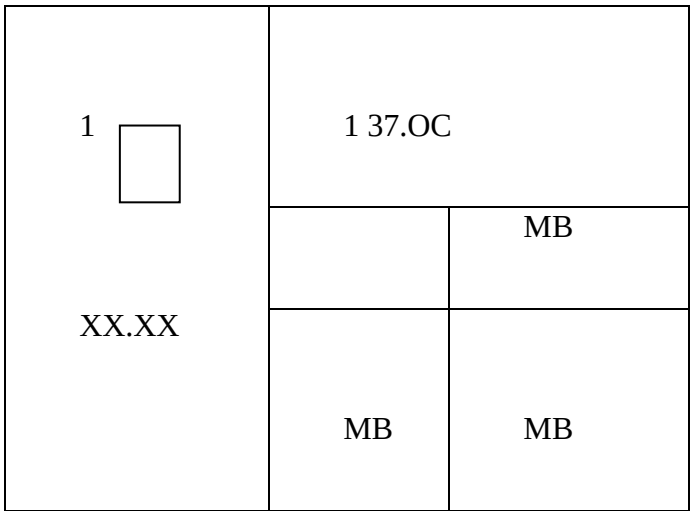
BacT/ALERT® 3D Instrument

The instrument consists of a Controller Module and one or more Incubation Modules. The instrument incubates and continuously monitors the status of each mycobacterial

culture bottle. The Incubation Modules consist of four drawers each with a 60-bottle capacity. MB and non-MB drawers can coexist in the same Incubation Module.

MB Drawers may be provided directly by bioMerieux or may be converted from a non-MB configuration in the field.

Each MB Drawer handle has a red MB label also displaying the Incubation Module number and letter corresponding to that drawer. The MB Drawer faces on the Instrument icon of the Main screen are also labeled .



Instrument Icon for MB-Configured System

Circulation Fan

The circulation fan, normally running to distribute air within the BacT/ALERT® 3D instrument, is turned off whenever a drawer in the Incubation Module is opened.

Rocker Clamp

The BacT/ALERT® 3D drawer configured for MB has the agitation mechanism disengaged so the three racks within each drawer remain immobile.

The BacT/ALERT® 3D has been designed to minimize risks associated with Mycobacterial testing. However, to further reduce the risks of accidental exposure to infectious agents, additional precautions should be taken. It is strongly recommended that the instrument be placed in a laboratory used for the routine culture of M. tuberculosis. For activities involving the propagation and manipulation of M. tuberculosis or Mycobacterium species grown in culture, BioSafety Level 3 Practice, Containment Equipment, and Facilities are required as recommended by CDC and N1H guidelines.

At a minimum, the instrument should be placed in a contained environment with controlled access which has a tuberculosis exposure control plan.

The locations should have surfaces which can be easily decontaminated using an appropriate topical disinfectant. The instrument must not be placed in an open corridor or hallway that is accessible to the general public or the patient population.

Barcodes

MP Process Bottle barcodes are encoded with the bottle type. MP Process Bottle barcodes have an SM (plastic) or !M (glass) prefix When an MP Process Bottle barcode is entered, the BacT/ALERT® 3D system will direct the user to load the bottle into an MB Drawer. All MB Drawers are clearly marked with a red label on the drawer handle.

MB Blood bottle barcodes are encoded with the bottle type. MB Blood bottle barcodes have an !B (glass) prefix When an MB Blood Bottle barcode is entered the BacT/ALERT® 3D system will direct the user to load the MB Blood Bottle into any containing cell available for loading.

Load Bottles

Cells are assigned based on the type of bottle. MP Process Bottles are directed exclusively to MB Drawers.

Note: Anonymously loaded MP Process Bottles must be placed in an MB drawer for proper incubation and detection of positives.

Note: MB Blood bottles must never be loaded anonymously.

Unload Positives and Negatives

If an instrument contains both MB and non-MB bottles, both bottle types will be identified for unloading when any of the unload buttons is pressed. The user then has an option of unloading all indicated bottles, all indicated bottles of a certain type (ex. MB or non-MB), or a portion of the indicated bottles of any type.

Set Maximum Test Time

The system default maximum test time is 42 days for Mycobacteria bottles.

Test times may be changed manually through the Set Maximum Test Time screen.

Positive Detection Algorithm

A special detection algorithm is used to detect growth of Mycobacterium species. This algorithm is designed to detect growth at the earliest possible time.

Bottle Status

The Main Screen on the monitor displays the status of positive or negative Mycobacteria bottles separately from the status of other bottle types.

Located just above the **Instrument** icon on the Main screen are the **Unload** buttons. Above each **Unload** button is a rectangular box containing the number of bottles currently matching the unload criteria for that button.

The Bottle Count Table contains two rows. The top row displays the bottle count for Mycobacteria culture bottles and is labeled MB. The bottom row displays the bottle counts for non-mycobacterial culture bottles and is labeled **BC.**

The leftmost box in each bottle count row contains the culture type BC, MB) and is not associated with an **Unload** button. The number displayed within this box represents the total number of bottles (of any status) currently loaded into the system.

The second leftmost box shows the total number of identified bottles with a positive test status. It appears above the **Positive Bottle** icon.

The box third from the left shows the total number of identified and anonymous bottles with a negative test status. It appears above the **Negative Bottle** icon.

The box second from the right shows the total number of anonymous bottles with a positive test, and is associated with the corresponding icon.

The total number of anonymous bottles with a negative-to-date or negative test status appears rightmost, and is associated with the corresponding icon.

System Startup

General Information

Drawer Configuration

The BacT/ALERT® 3D drawer agitation mechanism can be disengaged at the factory or by a service engineer on site, after which the three racks within the drawer remain immobile. The BacT/ALERT® 3D Controller Module is also configured by bioMerieux personnel to activate the MB status of each drawer.

Theory of Operation

The BacT/ALERT® 3D Mycobacteria! Detection System utilizes a colorimetric sensor and reflectance detector to determine the level of carbon dioxide within the bottle. If microorganisms grow in the bottle, carbon dioxide is produced which will change the color of the sensor on the bottom of the bottle. The instrument monitors this color while incubating the sample and determines if growth has occurred.

The MP Process Bottles contain a media which will, in combination with the MB/BacT® Reconstitution Fluid, promote the growth of mycobacteria. If a non-sterile sample is tested, the Reconstitution Fluid should be combined with the MB/BacT® Antibiotic Supplement, which is then added to the Process Bottle in accordance with the

instructions in the package insert. The addition of these antibiotics inhibits the growth of contaminating, non-mycobacterial microorganisms while permitting the growth, detection, and recovery of mycobacteria.

Principle of Detection

Mycobacteria behave like most other aerobic bacteria with respect to carbohydrate metabolism, energy production, and the biosynthesis of low weight metabolites. They are able to assimilate a large range of carbohydrates, lipids, and proteins.

Glycerol was selected as the primary metabolic source in the MP Process Bottle because of its unique metabolic pathway and its ability to maximize the amount of CO_2 generated by mycobacteria. Once ingested, glycerol is converted to Acetyl-CoA and oxidized through the Krebs or Tricarboxylic Acid Cycle (TCA). CO_2 and reduced electron carriers are the major metabolic byproducts of this oxidation.

A solid-state sensor at the base of each MP Process Bottle detects CO_2 as an indicator of microbial growth. As the concentration of CO_2 increases, the sensor undergoes a color change from green to yellow.

Safety Features

The design of the BacT/ALERT® 3D provides several safety measures to help mitigate hazards associated with mycobacteria and other organisms in the interest of operator and laboratory safety. They are summarized as follows.

- All glass bioMerieux BacT/ALERT media bottles are manufactured with a polymeric safety sleeve to help limit the effects of damage to a bottle-Plastic bottles are made from a break-resistant material.
- Each bottle cell within the BacT/ALERT® 3D incubation Module is sealed to help contain and minimize effects liquid spillage.
- A drip tray is incorporated at the bottom of each drawer beneath the opening end to minimize the effect of any spillage or liquid.
- The circulating fans with in the incubation model turn off whenever any incubator Drawer is open to minimize airflow and the potential for aerosols.
- To reduce the chance of binding a heavily over labeled bottle in a cell, incubation module cells are gentle tapered and fabricated from a compliant polymer.

General Precautions

The following precautions should be observed during Maintainance and Repair, even in situations where a spill is neither observed nor suspected. At minimum, disposable gloves, eye protection, and a laboratory coat should be worn. Any parts removed or tools used should be decontaminated using a 10% bleach solution or other EPA registered tuberculocidal disinfectant before removal from the laboratory. Anything which cannot be disinfected should be sealed in a plastic bag, labeled as biohazardous, and handled accordingly. In addition, the institution's safety precautions should always be observed.(As told in Biosafety in Microbiological and Biomedical Laboratories. HHS Publication No. CDC 93-8395. US Dept of Health and Human Services, Public Health Service, Centers for Disease Control and Prevention, National Institutes of Health, ed 3, 1993, pp 93-96).

Culture in Mb bact; All Mb bact bottles were brought to room temperature along with the Mb bact antibiotic supplement supplied. 0.5 ml of antibiotic supplement provided was injected into the Mb bact bottle using a 2 ml syringe. 0.5 ml of the decontaminated deposit of the sample was aspirated with the help of syringe and injected into the antibiotic supplement containing Mb bact bottle. The bottles were loaded into the Mb bact machine and waited for the machine to signal for the appearance of growth by the beeping sound.

4. CONFIRMATION OF GROWTH :

Growth of tuberculosis bacilli on LJ medium, middlebrook 7H10 agar and Mb bact was confirmed by observing the presence of acid fast bacilli in the ZN stained smears made from colonies.

5. TESTING FOR CONTAMINATION :

Bacterial contamination was detected by performing Gram stain from the suspected colonies. Fungal contamination was detected by observing typical fungal colonies and/or observing yeast cells or hyphal elements in Lacto phenol Cotton Blue (LCB) preparations. The methods of Gram staining and LCB preparation were as follows:

a. Grams Stain :[177]

A thin smear was prepared from colonies or broth, which was air-dried and heat fixed. Gention Violet was overlaid on slide and allowed to act for 1 minute. The slide was washed thoroughly with tap water. Grams iodine was overlayed and allowed to act for 1 minute. The slide was washed with distilled water and decolourised by using 95% ethanol as decolouriser for 10-15 seconds. Safranine was overlayed and allowed to act for about 1 minute. The slide was washed, blot dried and observed under oil immersion lens.

b. LCB Preparation :[178]

A drop of LCB was placed on clean side. A fragment of colony was teased in it using two pointed needles. A cover slip was applied taking care to avoid air bubbles. Excess stain, if any, was removed using blotting paper. Preparation was observed under 10x or 20x magnification.

No attempt was made to speciate bacterial and fungal contaminants.

6. SPECIATION OF MYCOBACTERIAL ISOLATES :

Each strain was subjected to following of tests for species identification :

- Rate of growth.
- Growth at different temperatures.
- Pigmentation.
- Niacin test.
- Nitrate reduction test.
- Catalase test.

a. Rate of Growth :[179]

A LJ slope was inoculated with culture suspension and incubated at 37^0C. It was read daily till the visible growth appeared. The date of appearance of visible growth was noted. The number of days taken to give a visible growth was worked out from date of inoculation and date of appearance of visible growth.

The results were interpreted as follows : Visible growth on third day reading : Rapid growers. Visible growth on 7^{th} day or later : Slow growers.

b. Growth at Different Temperature :[179]

Two LJ slopes were inoculated with culture suspension. One of them was incubated at room temperature and other at 42^0C in an incubator. The growth was observed on 28^{th} day of incubation. If the growth had appeared on any of these slopes it was noted.

c. Pigmentation Test :[179]

Two LJ slopes were inoculated with one loop full mycobacterial suspension. One of the slope was incubated in closed box at 37^0C while another was incubated at

37^0C. In usual manner until visible colonies appeared on slope kept at 37^0C. It was compared with the slope kept in box. If the slope was un-pigmented it was exposed to artificial light for three days with lid loose. Both the slopes were compared for pigmentation after another three days of incubation.

The results were interpreted as follows :

- No pigmentation on either : Non chromogenic mycobacteria.
- Yellow pigmentation on both : Scotochromogenic mycobacteria slopes.
- Yellow or orange pigmentation after exposure to light but not in dark : Photochromogenic mycobacteria.

d. Niacin Test :[179]

About 1 to 2ml of sterile distilled water was added to 3 to 4 weeks old culture on LJ slope. The slope was autoclaved for 30mins at 15 lbs pressure in slanting position. After cooling down few drops of extract of culture was transferred to a test tube to which few drops of 3% benzidine in alcohol and few drops of 10% Cyanogen bromide were added. Tube was shaken intermittently and observed for colour change at the end of 10mins.

Positive control was set by performing all above mentioned steps for a standard strain of Mycobacterium tuberculosis. Negative control was set by putting few drops of Cyanogen bromide and benzidine reagents in few drops of distilled water.

Results were interpreted as follows :

Positive Reaction : Appearance pink to red colour.

Negative Reaction : Appearance of no colour or white precipitate.

e. Nitrate Reduction Test :[180]

2ml of nitrate reduction test substrate was taken in a test tube to which two loops full colony from LJ slope was inoculated. The test tube was shaken and incubated at 37^0C for 2 hours. 1 to 2 drops of reagent A (Sulphanilic acid) and reagent B (naphthalamine) were added. A drop of Hcl was added. Test tubes were observed for colour change.

Positive control was set by performing the test by using a known strain of Mycobacterium tuberculosis. Negative control was set by adding reagent A and B to nitrate substrate only.

Results were interpreted as follows :

Positive Test : Development of red colour.

Negative Test : Absence of development of red colour.

All negative tests were confirmed by adding small amount of zinc powder. If red colour developed the negative test result was confirmed.

f. Catalase Test :[181]

The Semiquantitative method of catalase test was followed.

1 to 2 loop full colonies were emulsified in Middlebrook 7H9 broth. The broth was incubated for 7 days at 37^0C after which was mixed on vortex for 5 to 10 seconds. A small quantity of broth was transferred to LJ deep the deep was incubated at 37^0C for 14 days. 1ml of freshly prepared Tween-80-H_2O_2 reagent was added to deep and it was allowed to stand at room temperature for 5 minutes. Tubes were observed for appearance of column of oxygen bubbles. The height of column was

measured in millimeters.

Interpretation of results was as follows: High catalase activity : >45 mm of column. Low catalase activity : <45 mm of column.

Reactions of Mycobacterium Tuberculosis :

An isolate was designated to be that of Mycobacterium tuberculosis, if it showed following characters :

a. Grew slowly, taking more than 7 days.

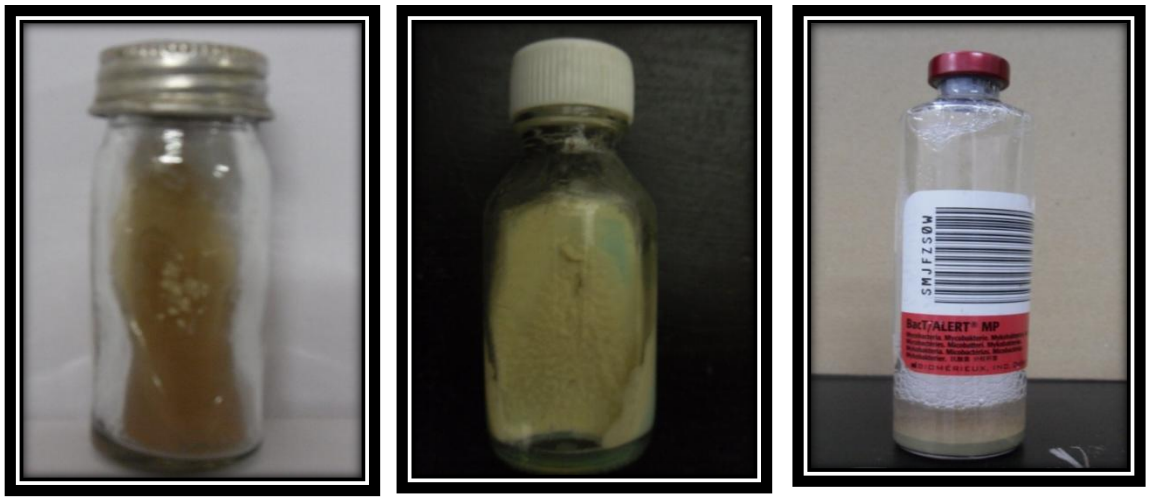
b. Had buff coloured, dry, rough colonies which were difficult to emulsify. c. Non-pigmented colonies.

d. Grew only at 37^0C and not at room temperature or at 42^0C.

e. Positive niacin test.

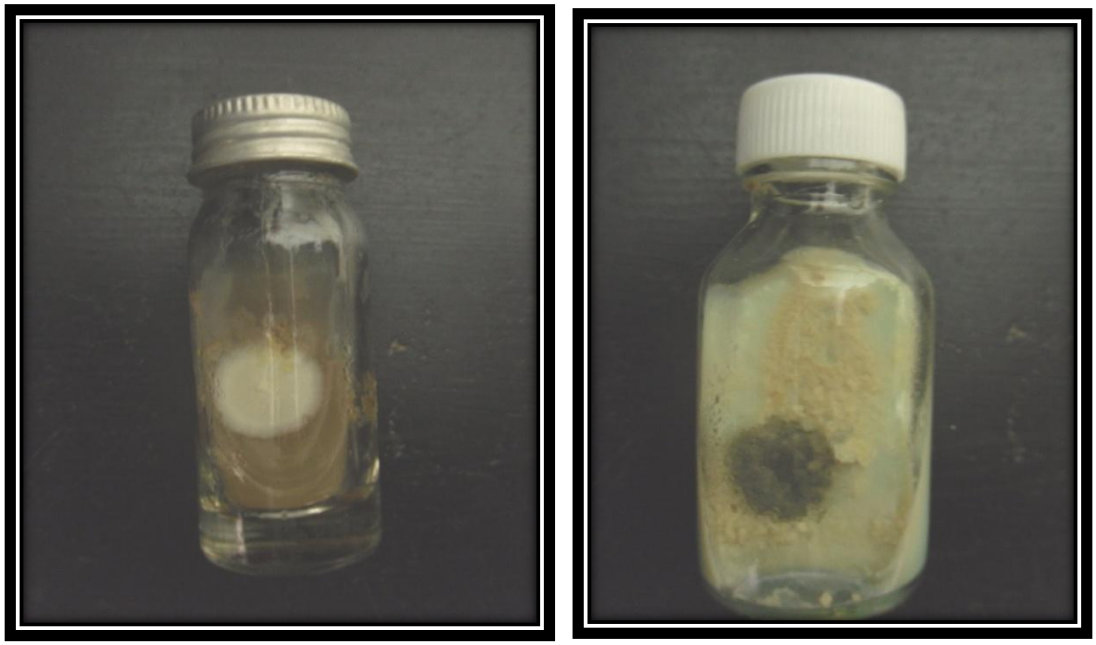
f. Positive nitrate reduction test.

g. Low catalase activity.



(1) **(2)** **(3)**

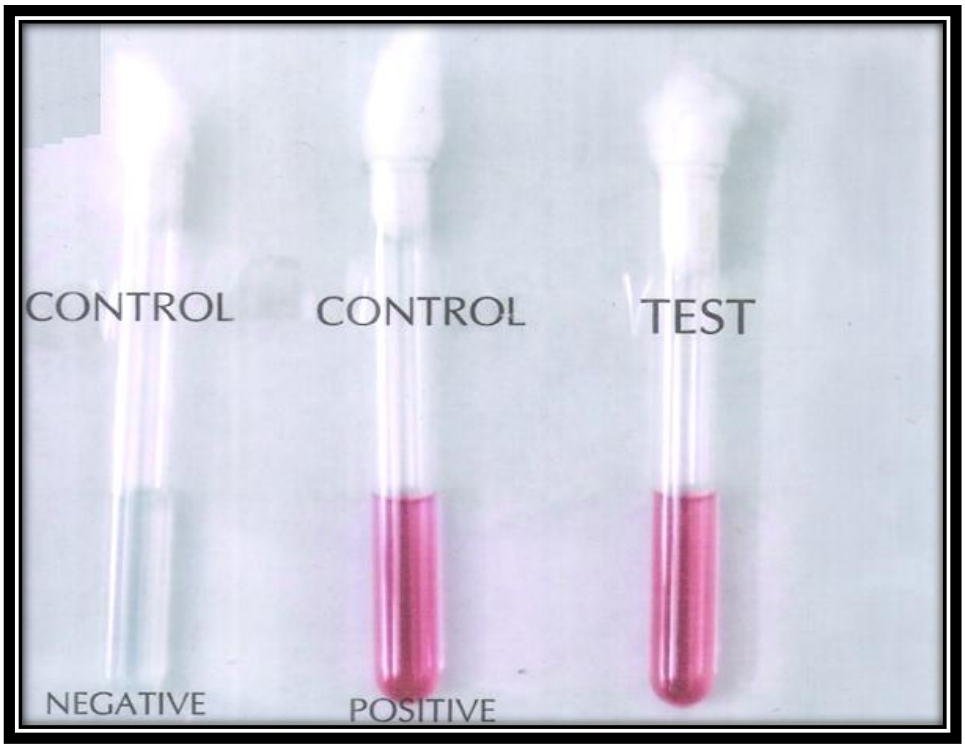
Growth on 1. Middle brook 7H-10 medium 2. Lowenstein Jensen medium 3. Bact alert 3D system bottle



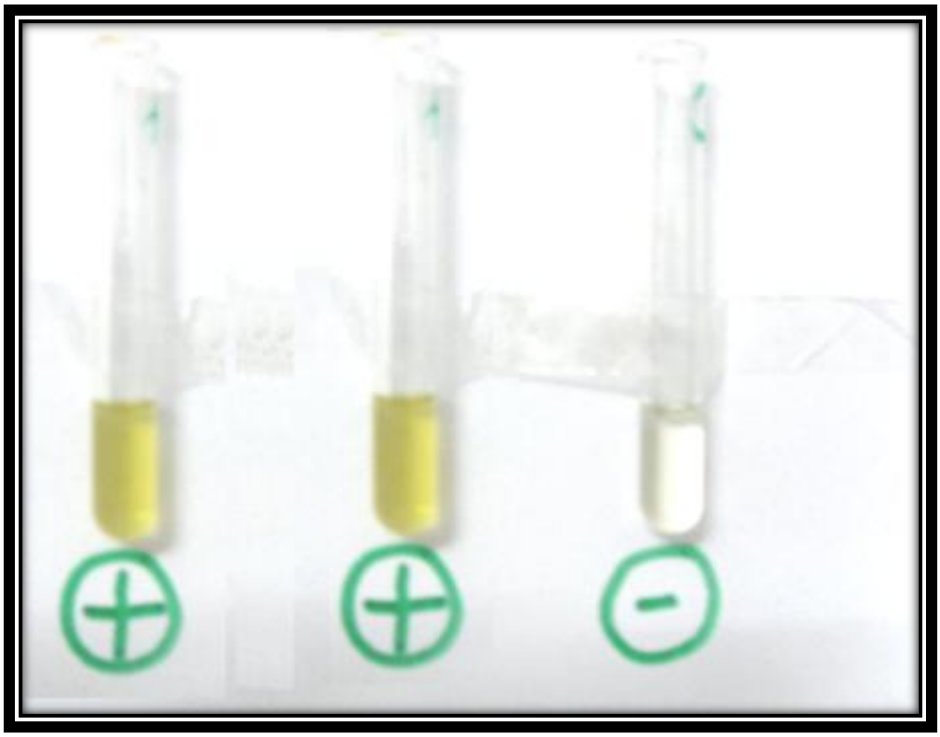
Contamination on Middle Brook 7H – 10 medium and Lowenstein Jensen medium

Bact Alert 3D system for Mycobacteria

Growth in MB BACT Bottles 1) Positive and 2) Negative

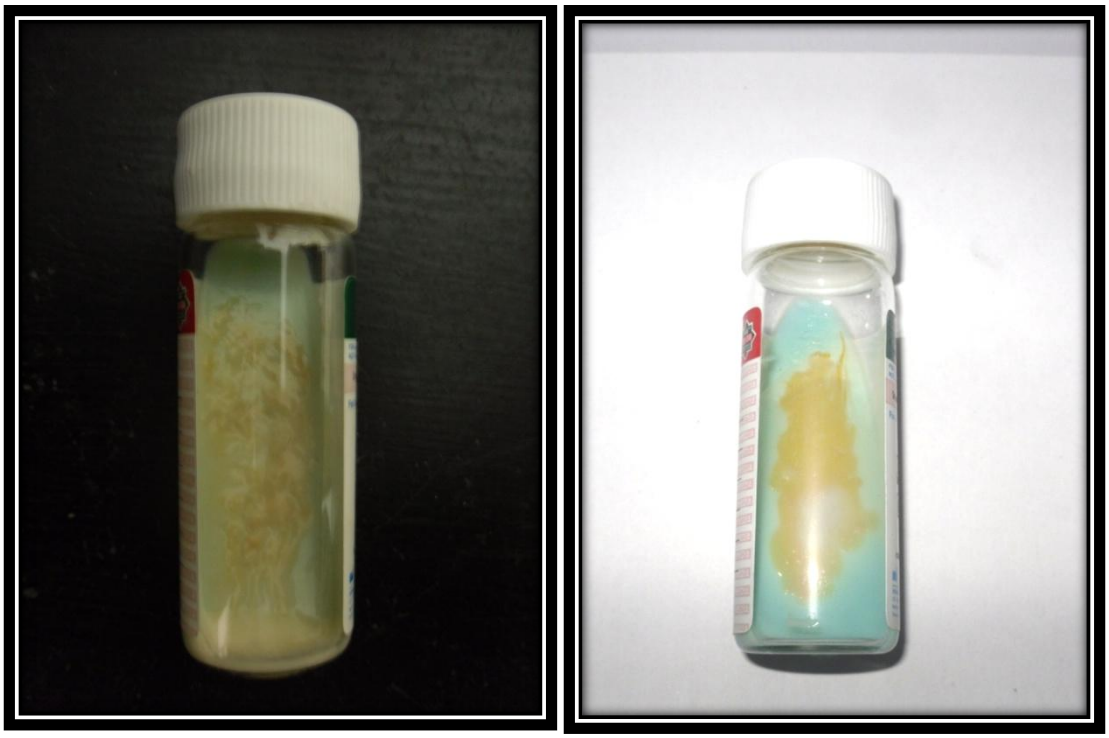


Nitrate Reduction Test

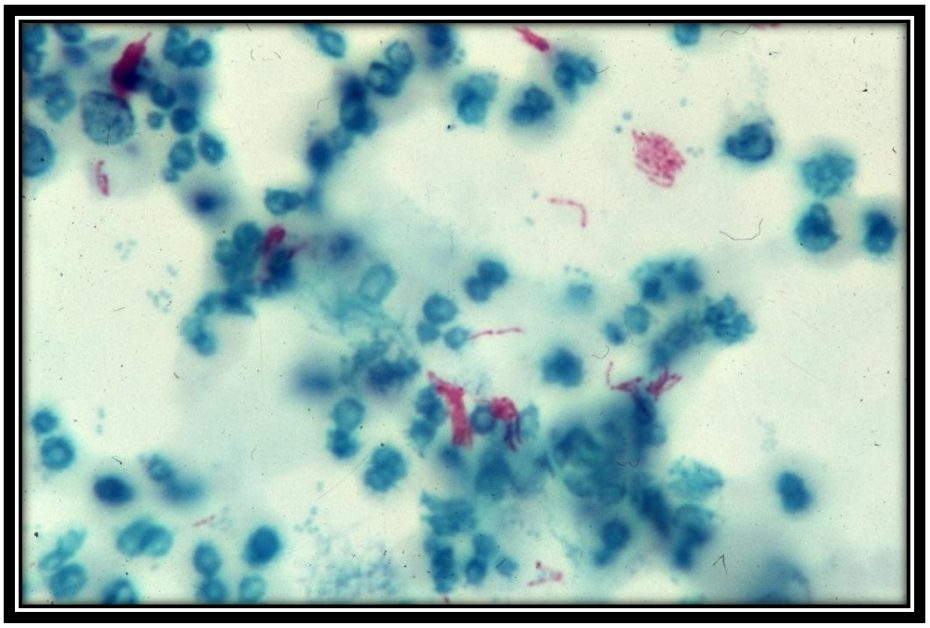


1) Positive 2) Control 3)Negative

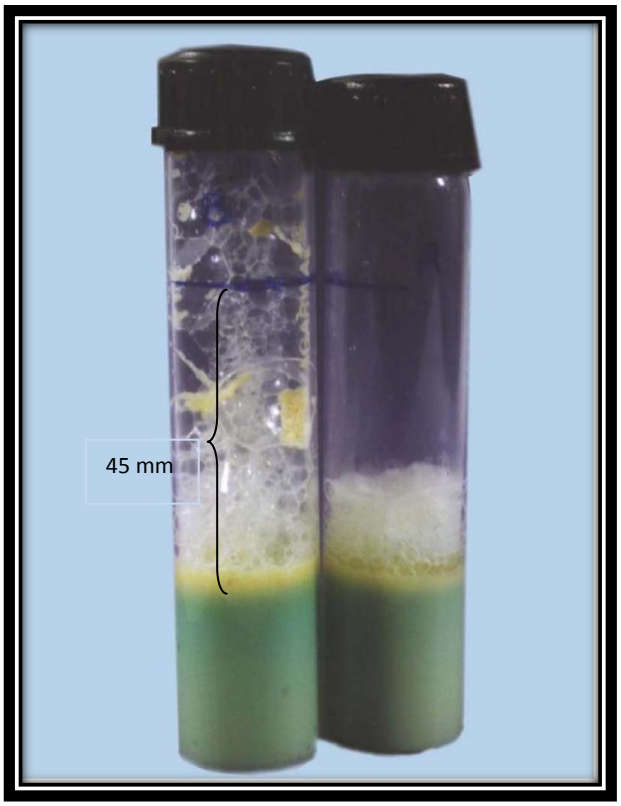
Niacin Test



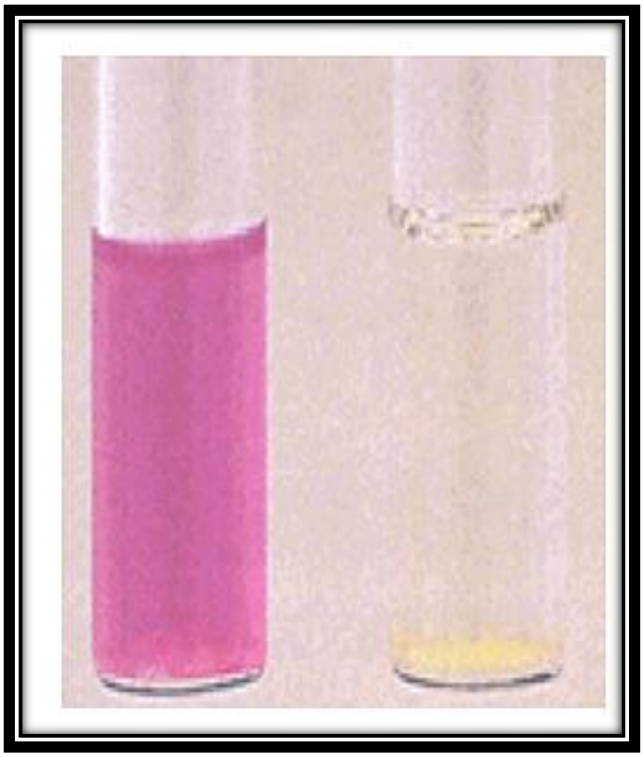
Growth of non tuberculous Mycobacterium on L J Slant



Mycobacterium Seen On ZN Staining



Catalase Test 1. Strongly Positive 2. Weakly Positive



Aryl sulphatase test 1. Positive 2. Negative

RESULTS

The present study was attempted to assess the feasibility of using MB bact and middlebrook7H10 as primary isolation medium for mycobacteria. It has been compared with LJ medium, the gold standard.

For the purpose of study we have included 230, suspected cases of tuberculosis attending the OPDs of Medicine, Paediatrics, Respiratory Medicine, surgery and admitted to the wards of these departments.

The three media were compared with respect to the number of isolates, rate of isolation,type of isolates and contamination rates.

The comparative study is as follows. . .

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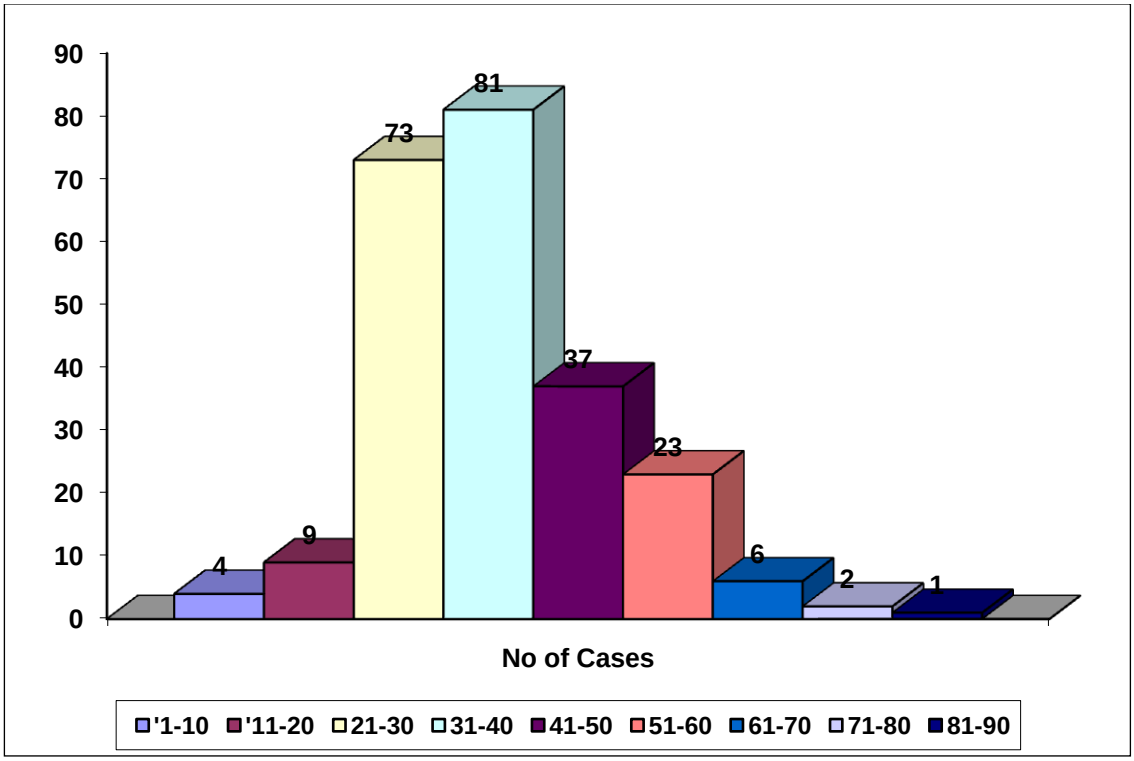
Table-1

Age Distribution of patients

Age Intervals	No. of Cases	Percentage
1-10	04	1.69
11-20	09	3.81
21-30	73	30.93
31-40	81	34.32
41-50	37	15.67
51-60	23	9.74
61-70	6	2.54
71-80	2	0.84
81-90	1	0.42
Total	236	100

The youngest patient among the study group was of 2 years old while oldest patient was of 85 years. The average age of patients was 36.87 years.

The majority of patients i.e. 81 (34.32%) were found to be in the range of 31- 40 years, followed by those in the range of 21-30 years that included 73 (30.93%) patients.



AGE DISTRIBUTION OF PATIENTS

Table-2

Distribution of cases by Sex

Sex	No of cases	Percentage
Male	186	78.8
Female	50	21.18

Out of 236 patients included in the study, 186 (78.8%) were male while 50 (21.18%) were female. Male to female ratio was 3.72:1.

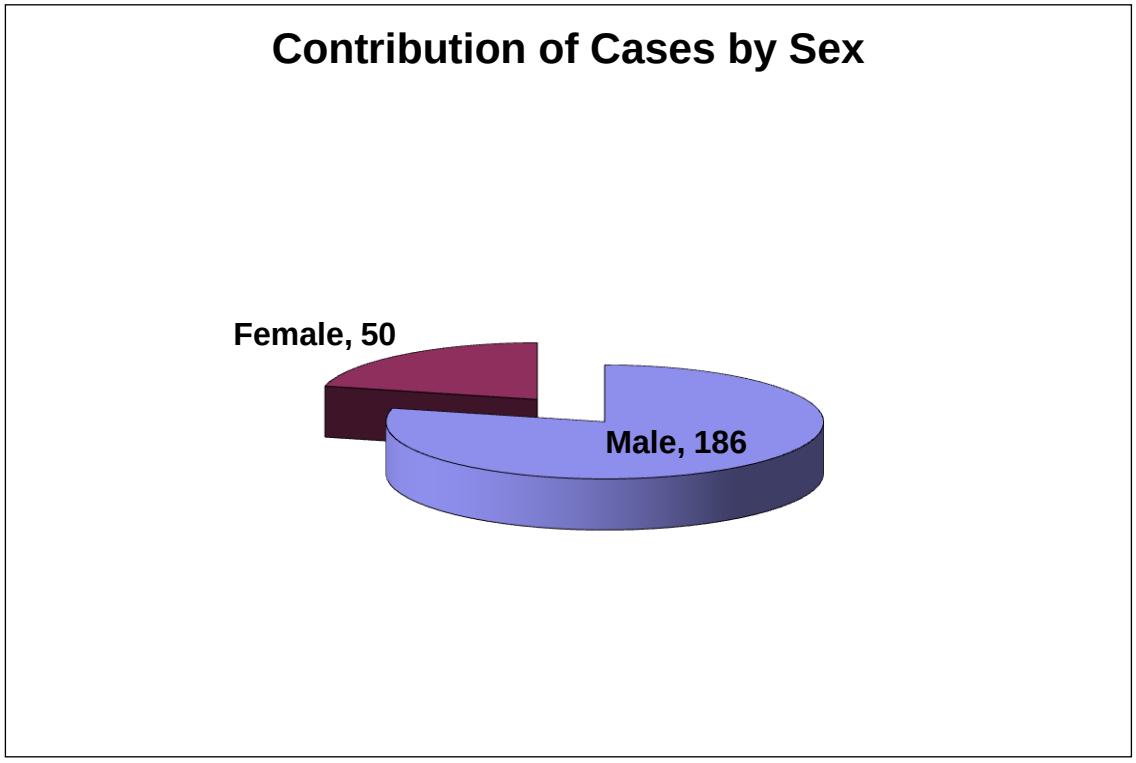


Table-3

Distribution of cases according to Grade of Sputum Smear Microscopy

Grade	No of cases	Percentage
+	51	30.35
+ +	79	47.02
+ + +	38	22.61

Out of 168 sputum smear positive patients, 51(30.35%) patients had 1(+) grading on sputum microscopy while 79(47.02%) had 2(+) grading. Remaining 38(22.61%) had 3(+) grading.

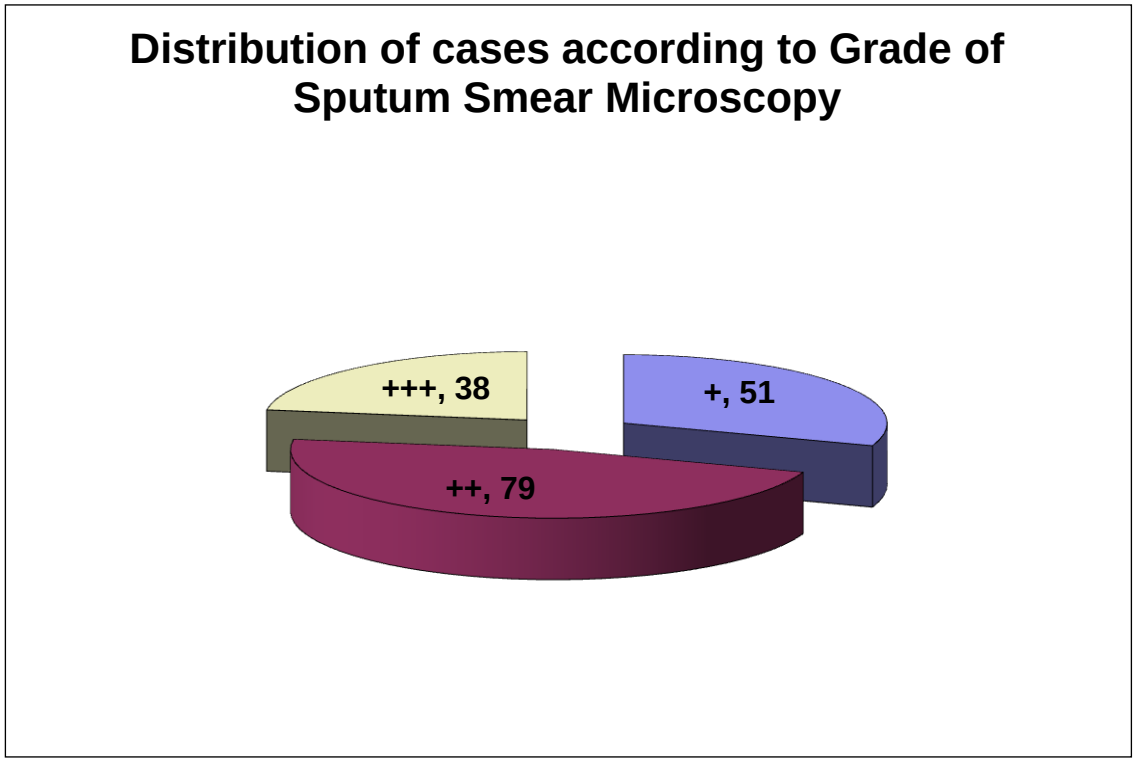


Table-4
Distribution of cases according to Culture status

	LJ		MB7H10		Mbbact	
Culture status	No of cases	Percentage	No of cases	Percentage	No of cases	Percentage
Culture Positive	82	34.74	62	26.27	116	49.15
Culture Negative	154	65.26	174	73.73	120	50.85
Total	236	100	236	100	236	100

Out of 236 cases screened, Mycobacteria were isolated in 82 cases(34.74%) by L J medium, 62 cases(26.27%) by middlebrook 7H10 medium and 116 cases(49.15%) by MB BACT automated system.

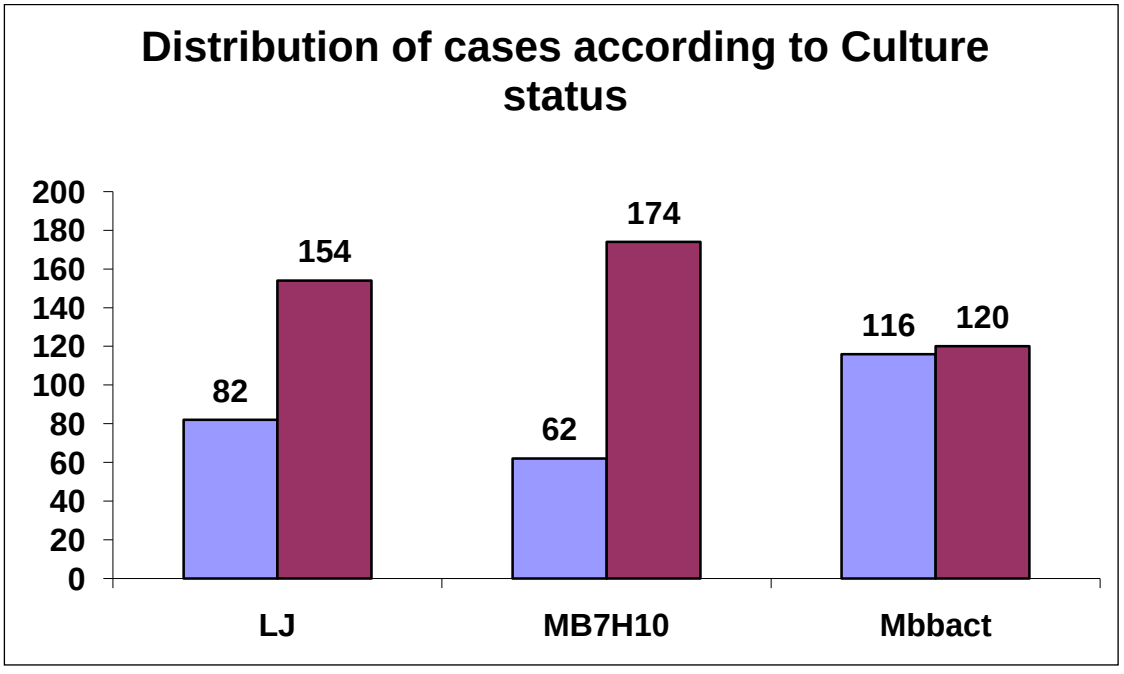


TABLE-5 :

CULTURE STATUS ACCORDING TO SMEAR POSITIVITY

	L J		MIDDLEBROOK 7H10		MB BACT	
CULTURE STATUS	NO. OF CASES	PERCENTAGE	NO. OF CASES	PERCENTAGE	NO. OF CASES	PERCEN TAGE
Culture positive and smear positive	82	48.80	62	36.90	112	66.66
Culture negative and smear positive	86	51.20	106	63.10	52	30.95
Smear negative and culture positive	-	-	-	-	4	2.38
Total smear positive	168	100	168	100	168	100

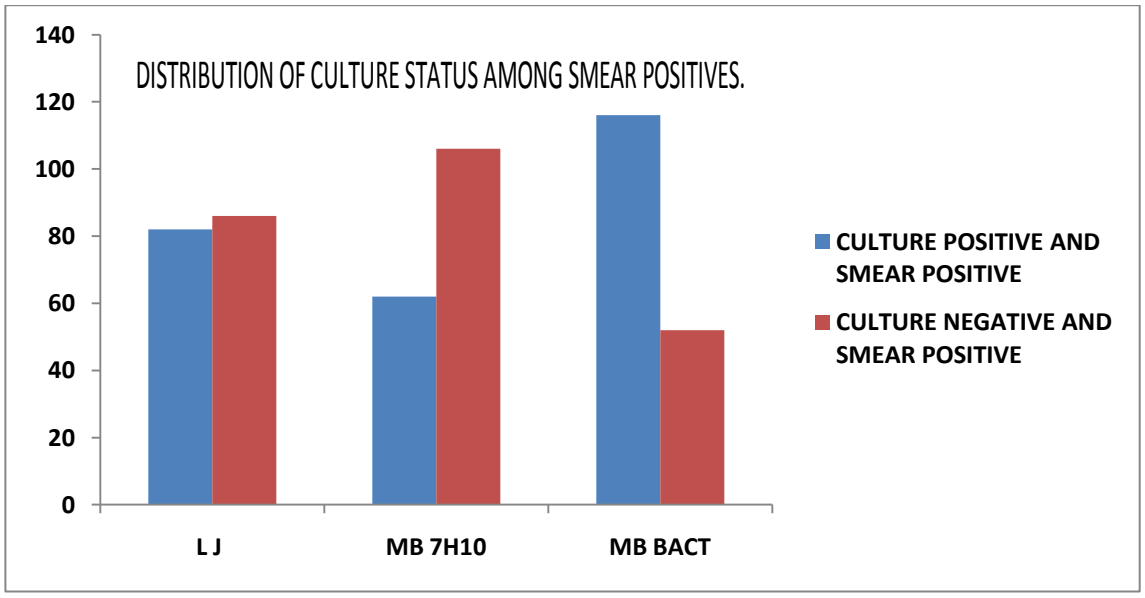


TABLE-6:

ISOLATION OF MTB AND NTB.

TYPE OF ISOLATE	NO. OF CASES	PERCENTAGE
MTB	116	96.66
NTB	04	03.34
TOTAL	120	100

Out of 120 isolates of Mycobacteria, 116 (96.66%) were identified as Mycobacterium tuberculosis (MTB) while remaining 4 (3.34%) were found to be Non- tuberculous Mycobacteria (NTM). Further speciation of NTM was not attempted.

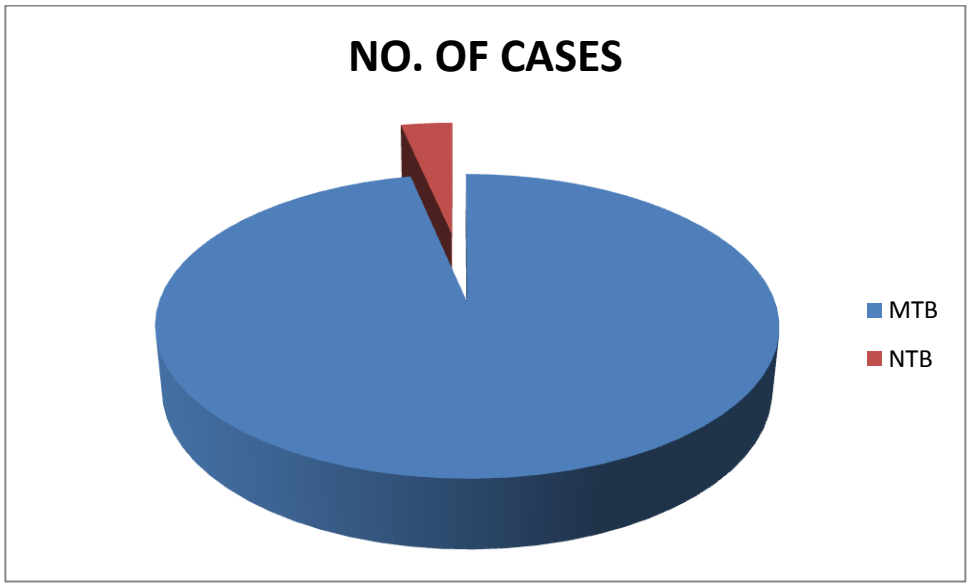


Table-7

Comparison of LJ. MB7H10 & Mbbact isolation of MTB

Medium	No of isolates	Percentage
LJ	82	34.74
MB7H10	62	26.27
Mbbact	116	49.15

Out of 236 cases screened, Mycobacteria were isolated in 82 cases(34.74%) by L J medium, 62 cases(26.27%) by middlebrook 7H10 medium and 116 cases(49.15%) by MB BACT automated system.

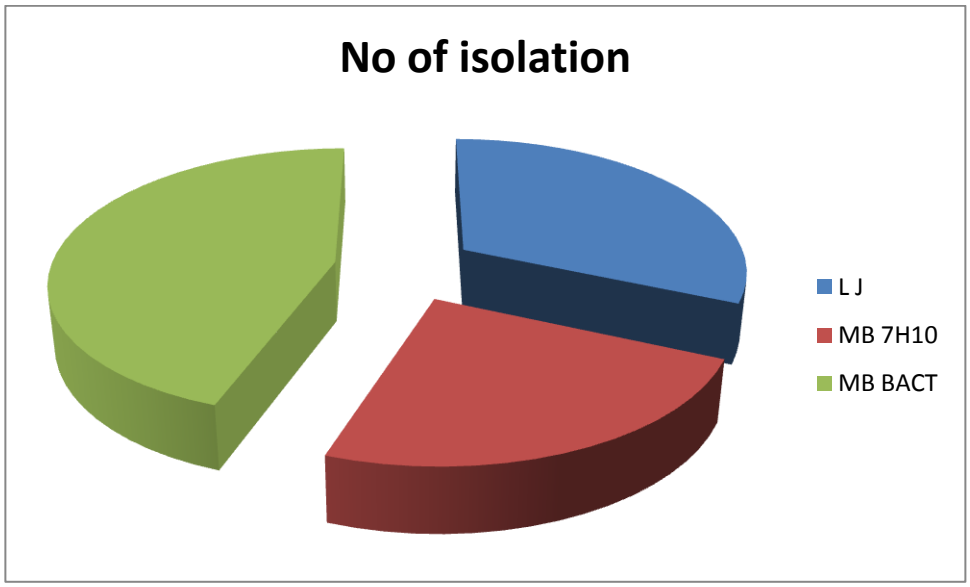


Table 8:

Comparison of LJ. MB7H10 & Mbbact for duration of isolation of MTB

Duration	LJ	MB7H10	Mbaact
1-7	0	0	0
8-14	0	0	23
15-21	1	0	62
22-28	26	16	34
29-35	43	37	0
36-42	12	9	0
Total	82	62	119

Although maximum incubation period for growth was 56 days, maximum time taken by any strain to grow was 42 days.

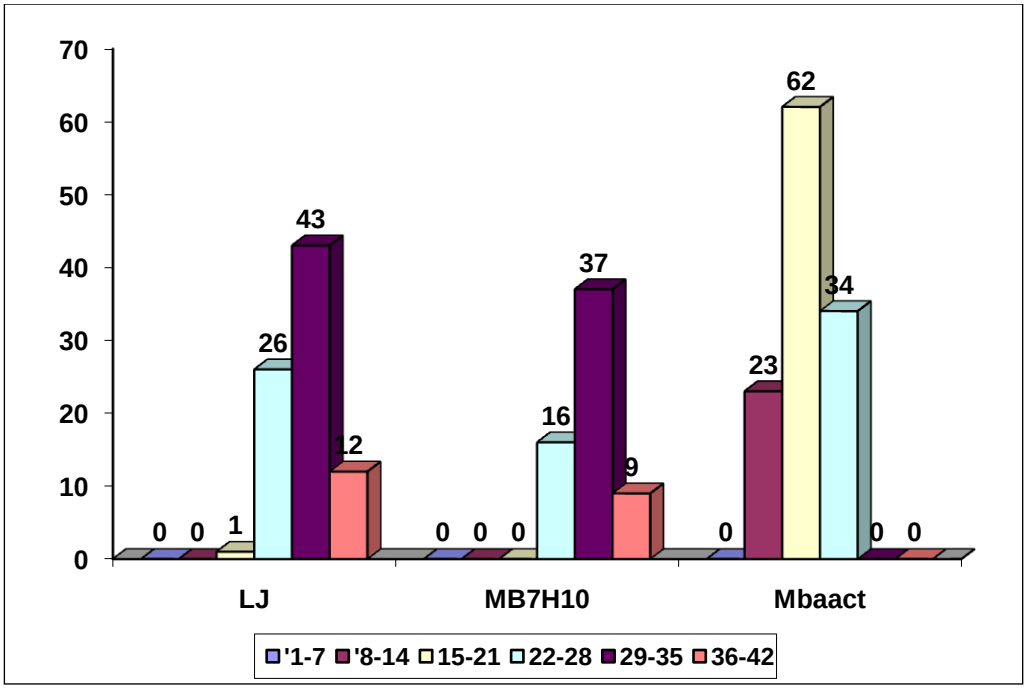
Mean duration of isolation on LJ, MB7H10 medium and MB BACT method was 30.81 days, 31.06 days and 18.70 days respectively. The difference between L J medium and middlebrook 7H10 medium was not significant.

None of the strain was isolated on either medium in first 7 days. In between 8 and 14 days (2^{nd} week) , 23 strains were isolated by MB BACT method and no strains were isolated by L J and middlebrook 7H10 medium. In between 15 and 21 days (3^{rd} week), 62 strains were isolated by MB BACT method and 1 strain was isolated on L J medium. In between 22 and 28 days (4^{th}week), 26 strains were isolated on L J medium, 16 strains on middlebrook 7H10 medium and 34 by MB BACT system. Similarly in between

29 and 35 days(5th week), 43 strains were isolated by L J medium and 37 by middlebrook 7H10 medium. In the 6th week, 12 strains were isolated on L J medium and 9 strains on middlebrook 7H10 medium.

Thus the period of maximum isolation was 3rd week followed by 4th week on MB BACT and it was 5th week followed by 4th week on L J medium and middlebrook 7H10 medium.

Further analysis shows that on L J medium 70(85.36%) of 82 strains were isolated by 34th day of incubation i e.,by the end of 5th week . 53(85.48%) of 62 strains were isolated on middlebrook 7 H10 in this period. 88 (74%) of 119 strains were isolated by 21st day of incubation by MB BACT ie. , by the end of 3rd week and almost 100% by the end of 24th day.



COMPARISION OF L J MEDIUM,MB 7H10 MEDIUM AND MB BACT SYSTEM FOR DURATION OF ISOLATION OF MTB.

Table-9

Comparison of LJ. MB7H10 & Mbbact for duration of isolation of MTB

Duration Days	Median								
	LJ (N=82)			MB7H10 (N=62)			Mbaact (N=119)		
	+	++	+++	+	++	+++	+	++	+++
1-7	0	0	0	0	0	0	0	0	0
8-14	0	0	0	0	0	0	4	2	16
15-21	0	1	0	0	0	0	6	36	20
22-28	4	8	14	1	5	10	6	22	2
29-35	3	23	17	0	20	17	0	0	0
36-42	0	9	3	0	9	0	0	0	0
Total	7	41	34	1	34	27	16	60	38

Only MB BACT could isolate mycobacteria (22 isolates) in the 2nd week,of which 4 of them were 1(+), 2 were 2(+) and 16 were 3(+) by smear microscopy grading initially. In the 3rd week L J medium could isolate 1 mycobacteria which was 1(+) by smear microscopy grading apart from MB BACT , which isolated 62 isolates of which 6 of them were 1(+), 36 were 2(+) and 20 were 3(+) by smear microscopy grading.

In the 4th week L J medium could isolate 26 isolates of which 4 were 1(+), 8 of them were 2(+) and 14 of them were 3(+) by smear microscopy grading. Middlebrook 7H10 could isolate 16 isolates in 4thweek, of which 1 was 1(+), 5 were 2(+) and 10 of them were 3(+) by smear grading . MB BACT could isolate a total of 30 isolates in 4 weeks of which 6 were 1(+), 22 were 2(+) and 2 were 3(+) by sputum microscopy grading.

In the 5th and 6thweek L J medium could isolate a total of 55 isolates of which 3 were 1(+), 32 were 2(+) and 20 were 3(+) by smear microscopy grading. Similarly middlebrook 7H10 in the 5th and 6thweek could isolate a total of 46 isolates , of 29 were 2(+) and 17 were 3(+) by smear microscopy grading.

There were 5 isolates that were negative by sputum smear microscopy but were isolated by MB BACT. Three of which were isolated by 24 days, 1 at 14 days and 1 by 26 days by MB BACT automated method.

Table-10

Comparison of LJ. MB7H10 & Mbbact for nature of contamination

Medium	Contamination			Total
	Fungal	Bacterial		
		GPC/GPB	GNB	
LJ	3	2	2	7
MB7H10	4	0	4	8
Mbaact	2	2	4	8

GPC – Gram-positive Cocci

GNB – Gram-negative bacilli

In 7 cases contaminants grew on LJ medium, in 8 cases contaminants grew on MB 7H10 medium and in 8 cases contaminants grew in MB bact bottles. Thus, **the rate of contamination on LJ , MB 7H10 and MB BACT was 2.9%,3.3% and 3.3% respectively.**

Of the 7 contaminants on LJ 3 (42.8%) were fungi, 2 (28.57%) were GPC and 2 (28.57%) were GNB. Of the 8 contaminants on MB7H10 4 (50%) were fungi and 4(50%) were GNB. Of the 8 contaminants in MB BACT bottles, 2(25%) were fungi, 2 (25%) were GPB and 4(50%) were GNB respectively.

TABLE-11:

HIV and tuberculosis co-infection

NO	AGE (YEARS)	SEX	TYPE OF ISOLATE
1	28	MALE	MTB
2	18	MALE	NTB
3	32	MALE	MTB
4	65	FEMALE	MTB
5	40	MALE	MTB
6	27	MALE	MTB
7	39	FEMALE	MTB
8	24	FEMALE	MTB
9	42	MALE	MTB
10	18	MALE	MTB
11	25	FEMALE	MTB
12	36	MALE	MTB
13	52	FEMALE	MTB
14	20	FEMALE	MTB
15	25	FEMALE	MTB
16	28	MALE	MTB
17	50	MALE	MTB
18	45	FEMALE	MTB

Out of 236 patients, a total of 120 isolates were obtained by culture. Of these 18 cases were positive for HIV antibodies. The mean age of these patients was 34.11 years(25-45). 10 (55.55%) of them were male while 8(44.45%) were female.

Only 1(5.5%) strain was non tuberculous mycobacteria while remaining 17(94.5%) were mycobacterium tuberculosis.

Table-12:

Test of significance between different methods based on duration of isolation

	LJ	MB7H10	Mbaact	Bet LJ & MB7H10		Bet MB7H10 & Mbaact		Bet LJ & Mbaact	
				Z-Value	P-Value	Z-Value	P-Value	Z-Value	P-Value
Mean	30.81	30.18	18.79	0.816	P < 0.420	13.36	P < 0.0001 HS	20.79	P < 0.0001 HS
SD	4.44	5.91	3.45						
N	82	66	116						

Note : HS → Highly Significant Difference

When L J medium was compared with middlebrook 7H 10 medium in terms of number of isolates , the Z value turned out to be 0.816. the P value according to the Z value showed that there was no much significant difference between the two if the maximum allowable error was 42%. When the same 2 media were compared in terms of tunaround time for isolation , most of the isolates were detected earlier by middlebrook 7H 10 medium by the end of 5^{th} week.

When middlebrook 7 H 10 medium and MB BACT were compared with each other , the Z value turned out to be 13.36 with P value <0.0001, showing that there was highly significant difference between the 2 methods. MB BACT method turned out to be more superior than MB 7H 10 medium both in terms of no of isolates and isolation rate.

When L J medium and MB BACT were compared with each other , the Z value turned to be 20.79 giving the P value of <0.0001 . This showed that there is a highly significant difference between the two methods. MB BACT proved to be a superior method over L J medium both in terms of number of isolates and the isolation rate.

DISCUSSION

The present study was attempted to assess the feasibility of using MB bact and middlebrook7H10 as primary isolation medium for mycobacteria. It has been compared with LJ medium, the gold standard.

For the purpose of study we have included smear positive cases only, regardless of their antitubercular treatment status. This helped us in two ways. First, we could isolate more MTB strains from relatively smaller sample size. Second, we could test MB7H10, L J medium and MB Bact method more realistically to assess its use as primary isolation medium for MTB. In practice, a sizable population of patients whose samples are submitted for culture of MTB are already treated with antitubercular drugs for variable period of time.

A. PATIENT CHARACTERISTICS :

1. Age :

The youngest patient included in our study was of 2 years while the oldest patient was of 85 years. Mean age was 36.87 years. Maximum number of patients suffering from TB were in the age group of 31 to 40years (34.32%), followed by 21to 30 years (30.93%). Thus, nearly 2/3 (67.40%) of patients were in the age group 20 to 40 years.

This is economically most productive age group in any society. Therefore resulting in reduction of manpower leading to economic loss.

Our findings are similar to those of S.B. Richards et al.[182] who have reported that 61% of their study population was in this age group. Studies from India show relatively smaller percentage of people belonging to this group. In

the study by Narang P and coworkers, at Wardha 26.30% patients belonged to this age group.[183]

Shivaraman et al. have reported that 40.8% of their study population belonged to this

age group.[184] The difference in our observation and other Indian studies may possibly due to large sample size in those studies and more number of cases of 0 to 14 age group. Number of Paedeatric patients was very less in our study due to difficulty in collection of sample and very few AFB positive cases.

The reasons that make this age group vulnerable to TB are many. They are socially more active and are exposed to an open case of TB more than any others. In some parts of the world HIV pandemic has contributed as people of this age group are sexually most active.

2. Sex :

In our study there were 78.8% males and 21.18 % females. Male to female ratio was 3.72: 1.

Many other investigators have also noted male preponderance in their studies. Peter Eriki et al.[185] and Fandinho FCO et al.[186] have reported male to female ratio of 1.8:1 and 1.6:1 respectively. Narang. P. et al. have reported that 61.03% of their study were male while 38.97% were female.[81] All these findings are comparable to study were males.

Likely reasons for male preponderance are as follows :

a. In a male dominating society, usually he is the earning member. As he goes out for work, he is more likely to come in contact with an active TB case.

b. Men are more likely to acquire habits like smoking, alcoholism etc.

3. Grade of sputum smear microscopy :

In our study grading of ZN stained smears was done as per recommendations of NTI. Percent number of patients with microscopy grade of 1+, 2+ and 3+ was 30.35%, 47.02% and 22.61% respectively.

Our findings are in accordance with the study by Paramashivan C.N. and coworkers. In their study 76.7% patients had 1(+) grade while 0.5% patients had 3 (+) grade.[85]

Grading of smears gives an idea regarding the bacterial load. It depends upon

various factors such as time of collection, number of samples taken, nature of sample, treatment with antituberculous drugs and its duration and method of grading used. In our study large number of patients were on antituberculosis treatment for variable time period. That might have reduced the bacterial load. So majority of patients are of grade 1 (+), which ranks highest among the 3 grades.

B. ISOLATION OF MYCOBACTERIA

In our study **overall rate of isolation was 52.17%** (120/230). It includes both

MTB and non-tuberculous Mycobacteria (NTM) isolated on LJ or MB7H9 or both.

The reported overall culture positivity from the cases of PTB shows wide variations. Following chart shows the details :

	Author, Year	**Rate of Isolation (%)**	**Sample size**	**Reference No.**
	Ghatole M. (2005)	30.46	151	86
	Narang P. (1992)	56.23	1252	81
	Chauhan (1991)	21	1005	87
	Kothadia (1991)	25.47	475	88
	Damale A. S. (1986)	80	208	89
	Vasant Kumari (1987)	32.6	250	90
	Narang P. Mendiratta. (1997)	18.85	960	91
	B. Malhotra (2002)	76.2	164	92
	J. Jena (1997)	85.1	336	26
	Present study	**52.17**	**230**	

Our isolation rate is comparable to that reported by Narang P. et al.[183] and Malhotra B. et al. It is higher than reported by Ghatole M et al.[188] Chauhan et al.

Kothadia et al.[189] Narang P and Mendiratta et al. while lower than that observed by Damle et al.[89] and Jena et al.[26] Important factors responsible for such wide variation are as under :

Case selection criteria are important. Damle et al. (80%) have included smear positive cases in their study while Jena et al.[115] (85.1%) have included clinically and radiologically proven cases of pulmonary tuberculosis. On the contrary, Kothadia et al. (25.47%) and Chauhan et al. (21%) have used clinically "suspected" cases as their subjects. We have included clinically suspected cases of tuberculosis.

Number of sputum samples collected and methodology of collection exerts its influence on rate of isolation. Jena et al. (85.6%)[115] have used three consecutive samples while Narang P. et al. (56.23%)[183] have used 2 samples per patient, one was spot collection and other was overnight collection.

Rate of isolation varies with the method of decontamination used. Damale and Kaundinya have found that Nassau's swab method of decontamination was superior to Pettroff's method and NALC method in giving positive cultures. Studies by Claudio Peirsiomoni et al. have also reported variation in culture yield when different decontamination methods were used.[119] we have included modified petroffs method.

Previous antitubercular treatment and its duration is an important determining factor of isolation of MTB. Jena, Panda B. et al. have conclusively proved that rate of isolation decreases as the duration of antituberculosis treatment increases. In our study 34 out of 48 smear positive and culture negative patients were on antitubercular treatment.

Mycobacteria are difficult to culture on artificial media. A combination of two or more media increases chances of their recovery and thus increases overall rate of isolation. Stager C.E. et al. have found 4 to 6% increase in overall isolation rate when

LJ slants were used along with BACTEC system.[116]

C. ISOLATION OF MYCOBACTERIUM TUBERCULOSIS AND NON TUBERCULOSIS MYCOBACTERIA (NTM) :

In our study, out of 120 isolates 116 (96.66%) were identified as MTB while remaining 4 (3.34%) were identified as NTM. No attempt was made to speciate NTM isolates further.

Varying percentage of isolation of MTB and NTM has been reported in literature. Following chart shows percentage isolation of MTB and NTM in few of the

studies.

Author, year.	**MTB isolation**	**NTM isolation (%)**	**Sample size**	**Ref No.**
Thomas, 1961	100	0	287	94
S.V.R. Shankar, 1989	86.4	14.6	4554	95
Enrico Tortoli, 1998	58.90	41.09	2500	47
Pfyffer G.E., 1997	62.77	37.22	1500	40
Trivedi S.S., 1986	68%	4%	2945	96
Saran, 1973	98%	2%	100	97
Mukhopadhyaya, 1978	99	1	43929	98
Present study	**96.6**	**3.3**	**230**	**-**

Our observations regarding isolation of NTM are comparable to those of Trivedi et al. Saran et al, and Mukhopadhyaya et al. They are considerably lower than that of Pyffer G. E. et al. and Enrico Tortoli et al.[129,136]

Various factors are responsible for the wide variation in the frequency of isolation of NTM. These are as follows :

Geographical region in which study has been carried out is one of the important factor. Most of NTM infections have some environmental source. Prevalence of NTM in the environment varies from one geographic region to another. This is probably reflected in the frequency with which it is isolated in culture. Most Indian studies report isolation of less than 1% to 13% while workers from developed countries have isolated it in much higher frequency.

Number of immuno-compromised patients in a study group is also a determining factor. Pulmonary diseases caused by NTM are believed to be opportunistic and many immuno-compromised disease like HIV, Diabetes, Cystic fibrosis are the predisposing conditions. In our study, both the NTM isolations were from immuno-compromised patients.

It appears from the studies of Claudio Piersimmoni et al. that method of decontamination and medium of culture influence NTM isolation. They could isolate 148 MTB and 31 NTM when N-acetyl-L-cysteine (NALC) and 3% NaoH was used for decontamination. Number of MTB and NTM isolated was 46 and 79 respectively when only NALC was used. Further, they observed that recovery of NTM was considerably higher by BACTEC 460 (67.7%) than by MB/BaCT system (51.6%). They have attributed this difference to variation in temperature of incubation. In MB/BaCT instrument we can incubate bottles only at one temperature i.e. 37°C.[119]

D) COMPARISION OF L J MEDIUM, MB7H10 MEDIUM AND MB BACT METHOD.

1. Rate of Isolation :

In our study, out of 236 cases screened, mycobacteria were isolated in 82 cases(34.74%) by L J medium, 62 cases(26.27%) by middlebrook 7H10 medium and 116 cases(49.15%) by MB BACT automated system. The sensitivity, specificity, positive predictive value and negative predictive value of MB7H10 medium in comparison to LJ was 69.5%, 94.3%, 95% and 66.7% respectively.

Our results are comparable to that of Claudio et al[107], Paul I Lin et a[11], Adler et al[8], A.Carricajo[9] et al and Angeby[10] K.A et al. Sensitivity and negative predictive value of MB7H10 in comparison to LJ was 69% and 63% respectively.[118] Lack of sensitivity as observed in these two studies makes MB7H10 a less preferred medium for primary isolation of MTB on its own.

In our study, no isolates were detected exclusively on MB7H10 medium. Recommendation of Martin T. to use larger size of inoculum did not work out in our study and that of Bhargava A et al. The reason for having a low yield on middlebrook 7H10 medium could be due to not incubating the bottles under co_2 atmosphere.

For the maximal recovery of MTB laboratories worldwide use combination of one or two solid media and one liquid medium. BACTEC system is the most popular liquid medium used but the high cost and use of radioactive material prohibits its routine use in developing world. Other commercially available non-radiometric method like MGIT, ESP-II can also be used. In such settings a simple, non-radiometric medium like MB7H9 as used in MB BACT can be a boon. It can be used in combination with LJ to maximize recovery and characterization of MTB , hence is highly

recommended.

2. **Duration of Isolation :**

In our study, **mean duration of isolation on LJ medium ,MB7H10 medium and MB BACT method were 30.81 days , 31.06 days and 18.70 days respectively.**

The difference was not significant between L J medium and middlebrook 7H10 medium. But there was highly significant difference between MB BACT and the other two media. The period of maximum isolation was 3rd week followed by 4th week for MB BACT. The period of maximum isolation was 5th week followed by 4thweek for L J medium and middlebrook7H10 medium.

Our findings co relate substantially from those of Bhargava A et al.[118] who found average time taken by LJ and MB7H9 for detection of growth to be 5 weeks and 6 weeks respectively. These findings may be due to similarity in selection criteria. Bhargava A. et al. have used smear positive and negative cases as well as pulmonary and extra pulmonary cases.

Duration of isolation of mycobacteria by middlebrook 7H10 medium in our study is comparable with concepcion F, RMT, Myrna T. Mendoza et al who found middlebrook 7H 10 to have an equal isolation time when compared with L J medium.

MB BACT could isolate mycobacteria with an average duration of 18.70 days which was similar to the study carried out by A.Carricajo et al and Claudio piersimoni et al. MB BACT proved to be superior to L J medium and Middlebrook 7H10 medium in isolation rate as it could isolate 7-10 days earlier when compared with the other two media. This finding was similar to the study done by concepcion F, RMT, Myrna T. Mendoza et al.

3. Rate of contamination :

In present study, **rate of contamination on LJ medium, middlebrook 7H10 medium and MB BACT were 2.9%,3.3% and 3.3% respectively.**

Fungi were the predominant contaminants on LJ slopes while Gram-negative bacteria were the commonest contaminants in MB7H10 broth. Higher fungal contamination on LJ medium can be due to two reasons.

a. Presence of Malachite green in LJ medium which suppresses bacterial growth but not of fungi.

b. Slants were incubated in humidified incubators with their caps loose. Fungi grow profusely in such environment and their spores enter the bottles through loosened caps. Absence of any antibacterial drug in this medium allows bacteria to grow faster than fungi and overgrow them.

SUMMARY

The present study was attempted to assess the feasibility of using MB bact and middlebrook7H10 as primary isolation medium for mycobacteria. It has been compared with LJ medium, the gold standard for parameters like rate of isolation , duration of isolation and rate of contamination.

The study was cross sectional one and was carried out over a period of one and a half years from October 2009 to may 2011 at the Department of Microbiology of BLDEU'S Shri B M Patil medical College, Bijapur. Two hundred and thirty suspected cases of pulmonary tuberculosis attending OPDs and admitted to various wards of BLDEU'S Shri B M Patil medical College, Hospital and Medical Research Center Bijapur regardless of their age, sex and antitubercular treatment status were selected. Isolation and identification of MTB were carried out as per standard methods.

Following important observations were made in our study :

- Average age of the study subjects was 36.87 years. Maximum number of patients (34.32%) were in the age group of 31 to 40 years.
- Male preponderance was observed as male to female ratio was 3.72:1.
- Overall rate of isolation of Mycobacteria was 34.74% by L J medium, 26.27% by middlebrook 7 H10 medium and 49.15% by MB BACT automated system.
- Rate of isolation of MTB was 96.66%(116/120) while rate of isolation of NTM was 3.34%(4/120).
- Of the 116 strains of MTB, 82 were cultured on L J medium,62 were cultured on middlebrook 7H10 medium and 116 were cultured by MB BACT system. The sensitivity, specificity, positive predictive value and negative predictive value

of MB7H10 medium in comparison to LJ was 69.5%, 94.3%, 95% and 66.7% respectively.

- Neither L J medium nor middlebrook 7H 10 medium could exclusively isolate any mycobacteria species.

  The rate of isolation of mycobacteria by middlebrook 7H10 medium and L J medium was almost similar(30 days).

  The number of isolates obtained were not significantly different between L J medium and middlebrook 7H 10 medium with the allowable error of 42%.

- The maximum time required for isolation of MTB was 42 days. The average time required for the growth of MTB on LJ medium middlebrook 7 H10 medium and MB BACT medium was 30.81days,31.06days and18.70 days respectively.
- Rate of contamination of LJ medium MB7H10 medium and MB BACT system was 2.9%,3.3% and 3.3% respectively. Fungi were the predominant contaminants on LJ medium while gram-negative bacilli were predominating in MB7H10 medium and MB BACT system.

CONCLUSION

Present study was attempted to assess the feasibility of using MB bact and middlebrook7H10 as primary isolation medium for mycobacteria. It has been compared with LJ medium, the gold standard.

MB BACT is a better medium compared to L J medium and middlebrook 7H10 medium both in terms of number of isolates obtained and isolation rate. MB BACT proved to be a very speedy method and could isolate mycobacteria 7-10 days earlier compared to L J medium and middlebrook 7H 10 medium.

Middlebrook 7H10 medium in our study could isolate mycobacteria at the same speed compared to L J medium and even there was not much significant difference in the number of isolates statistically with the allowable error of 42%. Middlebrook 7H10 medium could be thought of as an alternative to L J medium , as it is cheaper and even easy to prepare.

MB BACT is a safe and quicker method as it is a automated method which involves liquid media and does not involve any radioactive material.

Thus combination of MB BACT with a solid media like L J media or middlebrook 7H10 media helps in increased isolation and better identification.

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ANNUXERE 1

PROFORMA

1)HISTORY

PATIENT DETAILS

Full Name of the patient :

Age / Sex : IPD/OPD No :

Complete Address :

I. Clinical history :

1. Cough with expectoration
2. Fever
3. Night sweats
4. Shortness of breath
5. Chest pain
6. Tiredness
7. Weight loss
8. Loss of appetite
9. Coughing up blood
10. Others

II. Personal history :

History of smoking :

III. Treatment History :

IV. Clinical examination :

A) General physical examination

- ✦ Nutritional Status :
- ✦ Pallor :
- ✦ Icterus :

- ✦ Pulse :
- ✦ Temperature :
- ✦ BP :

B) Systemic Examination :

- ✦ RS :
- ✦ PA :
- ✦ CVS :
- ✦ CNS :

C) Investigations done :

- ✦ Blood :
  - ▪ Hb % :
  - ▪ TC :
  - ▪ DC : N L M E B
    ( )( )( )( )( )
  - ▪ ESR :
- ✦ Urine Examination :
  - ▪ Albumin :
  - ▪ Sugar :
  - ▪ Microscopy :
- ✦ Chest X-ray
- ✦ Weight of the patient

D) Microbiological Study :

- ✦ Sputum smear examination : Grading

  Sample No 1 – AFB No - Positive / Negative

  Sample No 2 – AFB No - Positive / Negative

Culture Study :

	Sample No. 1		**Sample No 2**	
	L.J. Medium	**Middle Brook 7H10 Medium**	**L.J. Medium**	**Middle Brook 7H10 Medium**
Inoculation date				
Growth appeared on				
Culture Morphology				

Growth Characteristics :

Rate of growth	Colony Morphology

Biochemical Reactions :

Niacin	Nitrate reduction test	Catalase	Peroxidase	Arylsulphatase

Bac T / Alert 3D device :

	Sample 1	Sample 2
Inoculation Date		
Indication for appearance of growth by Bac T/ Alert machine		

ANNEXURE 2

PROCEDURE

1. Ziehl-Neelsen (2N) Stain :

Reagents :

A. Concentrated Carbol Fuchsin :

a. Basic Fuchsin 10 gms.

b. Phenol Crystals 50 gms.

c. 95% Alcohol 100 ml.

d. Distilled water 1000 ml.

Basic Fuchsin was dissolved in alcohol to which phenol and distilled water added. The mixture was mixed well and filtered.

B. 25% Sulphuric Acid :

a. Concentrated Sulphuric Acid 250 ml.

b. Distilled water 750 ml.

Sulphuric acid was added to distilled water with constant mixing in a large boiling flask. It should be stored in a stoppered glass bottle.

C. 0.1 % Methylene Blue :

a. Methylene Blue 1 gm.

b. Distilled water 1000 ml.

Stock solution of 1% methylene blue was prepared by dissolving 1gm of powder in 100 ml distilled water. It was further diluted by adding 900 ml of distilled water.

2. Gram Staining

Reagents :

A. Primary Stain :

a. Crystal Violet 10 gms.

b. Absolute alcohol 100 ml.

c. Distilled water 1000 ml.

The dye was dissolved in alcohol and filtered through the filter paper. Distilled water was added to filtered solution.

B. Iodine Solution (Lugol's) :

a. Iodine 10 gm.

b. Potassium iodide 20 gm.

c. Distilled water 1000 ml.

Potassium iodide was dissolved in 250 ml of distilled water to which iodine was added and dissolved. Remaining 750 ml of distilled water was added.

C. Decolourizer :

a. Absolute alcohol (100% ethanol)

D. Counterstain :

a. Safranine 5 gms.

b. Distilled water 1000 ml.

Safranine was dissolved in distilled water.

3. Lactophenol Cotton Blue stain (LCB) :

Reagents :

a. Phenol Crystals 20 gm.

b. Lactic acid 20 ml.

c. Glycerol 40 ml.

d. Cotton blue 0.075 gm.

e. Distilled water 20 ml.

Lactic acid, glycerol were mixed with distilled water in which phenol crystals were dissolved by gentle warming. Finally cotton blue was added.

4. **Lowenstein Jensen Medium :**

Reagents :

a. Mineral Salt Powder (Hi Media) 37.3 gms.

b. Glycerol 12 ml.

c. Malachite Green (2%) 20 ml.

d. Egg Homogenite 1000 ml.

e. Distilled water 600 ml.

Mineral salt powder was dissolved in distilled water by constant shaking and heating. Glycerol was added. The mixture was sterilized by autoclaving at $121^{O}C$ for 15 minutes. It was added to egg homogenite taking asceptic precautions and mixed well. 20 ml of sterile malachite green solution was added. Mixture was dispensed in 10 ml quantities in McCartney bottles. The bottles were inspissated at $85^{O}C$ for 45 minutes for 3 successive days.

5. **Middlebrook 7H10 Medium with OADC Supplement :**

Reagents :

a. Middlebrook 7H10 medium Base (Hi Media)	2.35 gm
b. Glycerol	2ml
c. Oleic Acid, Albumen, Dextrose, Catalase (OADC)	1 vial
Supplement (Hi Media)	2ml
d. Distilled water	450 ml.

A total at 500 ml of broth was prepared at a time.

Weighed quantity of base powder was added to 450 ml of distilled water to which 2 ml of glycerol was added and sterilized by autoclaving at 121^{O}C for 10 minutes. The medium was cooled to 45^{O}C and 1 vial of OADC supplement was added taking asceptic precautions.

The medium was dispensed in 5 ml aliqoutes after proper mixing.

6. NaOH 4 % :

Reagents :

a. Sodium Hydroxide Pellets	4gms.
b. Distilled water	100 ml.

Preparation :

Pellets of sodium hydroxide were added to distilled water and dissolved them by steering and gentle heating. The solution was autoclaved at 12^{O}C for 15 minutes.

7. Niacin Test :

Reagents :

A. Benzidine Solution (3%) :

a. Benzidine 3 gms.

b. Ethanol (95%) 100 ml.

Benzidine was dissolved in Ethanol by shaking.

B. Cyanogen Bromide (10%) :

a. Cyanogen Bromide 10 gms.

b. Distilled water 100 ml.

Cyanogen bromide was dissolved completely in distilled water by constant shaking.

8. Nitrate – Reduction test

Reagents :

A. M/100 Sodium in M/45 Phosphate Buffer :

a. Sodium Nitrate 0.085 gm.

b. Potassium dihydrogen phosphate 0.117 gm.

c. Disodium hydrogen phosphate 0.485 gm.

d. Distilled water 100 ml.

All the ingredients were dissolved in Distilled water.

B. Solution A :

a. Sulphanilic acid 0.8 gm.

b. Acetic acid (IN) 100 ml.

Sulphanilic acid was dissolved in acetic acid completely and stored in stoppered glass bottle.

C. Solution B :

a. Naphthalamine 0.5 gm. b. Acetic acid (5N) 100ml.

Naphthalamine was dissolved in acetic acid and stored in stoppered glass

bottle.

9. Catalase Test :

Reagents :

- Hydrogen peroxide (30%)
- Tween 80 (10%)

Tween 80 was sterilized by autoclave at 121^{O}C for 10 minutes and stored at

4^{O}C. Just before use both were mixed in equal quantities.

KEY TO MASTER CHART

FU	-	fungi
GNB	-	gram negative bacilli
GPC	-	gram positive cocci
LCB	-	lactophenol cotton blue stain
GRAM	-	gram stain
L J medium	-	Lowenstein Jensen medium
MB 7H10	-	middlebrook 7H10 medium
MDR-TB	-	multidrug resistant tuberculosis
MTB	-	mycobacterium tuberculosis
NTB	-	non tuberculous mycobacteria
DM	-	diabetes mellitus
HT	-	hypertension
HIV +	-	positive for retrovirus infection
BAL	-	bronchio-alveolar lavage
ED	-	ear discharge

MASTER CHART

sl. No	name	age(yrs)	sample	sex	lab. no	hospital no	AFB grade	duration of isolation			MTB/NTB	contamination						remarks
								L J	MB7H10	Mbbact		L J		MB7H10		MB bact		
												LCB	gram	LCB	gram	LCB	gram	
1		32	sputum	female	E-102	454	-	-	-	-	_	_	_	_	_	_	_	_
2		30	sputum	male	AFB-14	946	-	-	-	-	_	_	_	_	_	_	_	_
3		7	**biopsy**	**female**	**AL-1**	44141	-	-	-	-	_	_	_	_	_	_	_	_
4		2	**BAL**	**male**	**AL-2**	9052	-	-	-	-	_	_	_	_	_	_	_	_
5		30	**sputum**	**male**	**BLDE-7**	17418	+	-	-	**14**	**NTB**	_	_	_	_	_	_	_
6		6	**ED**	**male**	**AFB-3**	14755	++	28	28	14	MTB	_	_	_	_	_	_	_
7		18	**sputum**	**male**	**D-1**	10001	+	34	28	14	MTB	_	_	_	_	_	_	_
8		24	**sputum**	**male**	**D-2**	10002	+	-	-	-	_	_	_	_	_	_	_	_
9		30	**sputum**	**male**	**D-3**	10003	++	30	28	16	**MTB**	_	_	_	_	_	_	_
10		28	**sputum**	**female**	**D-4**	10004	+++	28	28	16	**MTB**	_	_	_	_	_	_	_
11		28	**sputum**	**male**	**D-5**	10005	+++	30	30	16	**MTB**	_	_	_	_	_	_	HIV+
12		40	**sputum**	**male**	**D-6**	10006	+++	32	32	19	**MTB**	_	_	_	_	_	_	_
13		32	**sputum**	**female**	**D-7**	10007	+	-	-	22	**MTB**	_	_	_	_	_	_	_
14		45	**sputum**	**male**	**D-8**	10008	++	34	34	19	**MTB**	_	_	_	_	_	_	_
15		44	**sputum**	**male**	**D-9**	10009	+	-	-	20	**NTB**	_	_	_	_	_	_	_
16		18	**sputum**	**male**	**D-10**	10010	++	32	29	16	**MTB**	_	_	_	_	_	_	_
17		18	**sputum**	**male**	**D-11**	10011	+++	**30**	**32**	17	**MTB**	_	_	_	_	_	_	_
18		60	**sputum**	**male**	**D-12**	10012	++	-	-	19	**MTB**	_	_	_	_	_	_	_
19		18	**sputum**	**male**	**D-13**	10013	+++	**28**	**26**	11	NTB	_	_	_	_	_	_	HIV+
20		40	**sputum**	**male**	**D-14**	10014	+	-	-	19	**MTB**	_	_	_	_	_	_	_
21		30	**sputum**	**male**	**D-15**	10015	+++	**32**	**29**	17	**MTB**	_	_	_	_	_	_	_
22		34	**sputum**	**male**	**D-16**	10016	++	**34**	**34**	19	**MTB**	_	_	_	_	_	_	_
23		40	**sputum**	**male**	**D-17**	10017	++	**34**	**30**	21	**MTB**		_	_	_	_	_	DM
24		30	**sputum**	**male**	**D-18**	10018	+++	**34**	**34**	22	**MTB**	_	_	_	_	_	_	_
25		32	**sputum**	**male**	**D-19**	10019	+++	**32**	**30**	19	**MTB**	_	_	_	_	_	_	HIV+
26		27	**sputum**	**male**	**D-20**	10020	++	**34**	**30**	19	NTB	_	_	_	_	_	_	_
27		34	**sputum**	**male**	**D-21**	11001	-	-	-	-	_	_	_	_	_	_	_	_
28		30	**sputum**	**male**	**D-22**	11002	+++	**36**	**32**	22	**MTB**	_	_	_	_	_	_	_
29		28	**sputum**	**male**	**D-23**	11003	+	-	-	-	_	_	_	_	_	_	_	_
30		30	**sputum**	**male**	**D-24**	11004	++	-	-	-	_	_	_	_	_	_	_	_
31		40	**sputum**	**male**	**D-25**	11005	+	-	-	-	_	_	_	_	_	_	_	_
32		44	**sputum**	**male**	**D-26**	11006	+	-	-	-	_	_	_	_	_	_	_	_
33		35	**sputum**	**male**	**D-27**	11007	+	-	-	-	_	_	_	_	_	_	_	_

34		35	**sputum**	**male**	**D-28**	11008	**++**	-	-	-	_	_	_	_	_	_	_	_
35		38	**sputum**	**male**	**D-29**	11009	**++**	**40**	**36**	**22**	**MTB**	_	_	_	_	_	_	_
36		45	**sputum**	**male**	**D-30**	11010	**++**	**38**	**38**	**22**	**MTB**	_	_	_	_	_	_	_
37		30	**sputum**	**male**	**D-31**	11011	**+++**	**27**	**29**	**19**	**MTB**	_	_	_	_	_	_	_
38		30	**sputum**	**female**	**D-32**	11012	-	-	-	-	_	_	_	_	_	_	_	_
39		65	**sputum**	**female**	**D-33**	11013	**++**	**28**	**30**	**19**	**MTB**	_	_	_	_	_	_	HIV+
40		30	**sputum**	**female**	**D-34**	11014	**++**	**34**	**30**	**23**	**MTB**	_	_	_	_	_	_	_
41		40	**sputum**	**female**	**D-35**	11015	**++**	**28**	**28**	**18**	**MTB**	_	_	_	_	_	_	_
42		50	**sputum**	**male**	**D-36**	11016	-	-	-	-	_	_	_	_	_	_	_	_
43		35	**sputum**	**male**	**D-37**	11017	-	-	-	-	_	_	_	_	_	_	_	_
44		45	**sputum**	**male**	**D-38**	11018	-	-	-	-	_	_	_	_	_	_	_	_
45		56	**sputum**	**male**	**D-39**	11019	-	-	-	-	_	_	_	_	_	_	_	DM
46		40	**sputum**	**male**	**D-40**	11020	**+++**	28	28	14	MTB	_	_	_	_	_	_	HIV+
47		35	**sputum**	**male**	**D-41**	11021	**+**	-	-	-	_	_	_	_	_	_	_	_
48		50	**sputum**	**male**	**D-42**	11022	**++**	-	-	-	_	_	_	_	_	_	_	_
49		41	**sputum**	**male**	**D-43**	11023	**++**	34	30	20	MTB	_	_	_	_	_	_	_
50		30	**sputum**	**male**	**D-44**	11024	-	-	-	-	_	_	_	_	_	_	_	_
51		35	**sputum**	**male**	**D-45**	11025	-	-	-	-	_	_	_	_	_	_	_	_
52		40	**sputum**	**male**	**D-46**	11026	-	-	-	26	**MTB**	_	_	_	_	_	_	_
53		40	**sputum**	**male**	**D-47**	11027	-	-	-	-	_	_	_	_	_	_	_	_
54		30	**sputum**	**male**	**D-48**	11028	**++**	-	-	-	_	_	_	_	_	_	_	_
55		25	**sputum**	**male**	**D-49**	11029	**+**	-	-	-	_	_	_	_	_	_	_	_
56		34	**sputum**	**male**	**D-50**	11030	**++**	-	-	-	_	_	_	_	_	_	_	_
57		60	**sputum**	**male**	**D-51**	11031	**++**	-	-	-	_	_	_	_	_	_	_	_
58		60	**sputum**	**male**	**D-52**	11032	-	-	-		_	_	_	_	_	_	_	_
59		23	**sputum**	**male**	**D-53**	11033	**+**	-	-	-	_	_	_	_	_	_	_	_
60		31	**sputum**	**male**	**D-54**	11034	**+**	-	-	-	_	_	_	_	_	_	_	_
61		30	**sputum**	**male**	**D-55**	11035	**++**	-	-	-	_	_	_	_	_	_	_	_
62		32	**sputum**	**male**	**D-56**	11036	-	-	-	-	_	_	_	_	_	_	_	_
63		29	**sputum**	**male**	**D-57**	11037	**++**	-	-	-	_	_	_	_	_	_	_	_
64		34	**sputum**	**male**	**D-58**	11038	**++**	-	-	-	_	_	_	_	_	_	_	_
65		31	sputum	female	**D-59**	11039	**+**	-	-	-	_	_	_	_	_	_	_	_
66		30	sputum	female	**D-60**	11040	**++**	-	-	-	_	_	_	_	_	_	_	_
67		32	sputum	**male**	**D-61**	11041	**++**	-	-	-	_	_	_	_	_	_	_	_
68		29	sputum	**male**	**D-62**	11042	-	-	-	24	**MTB**	_	_	_	_	_	_	_
69		34	sputum	female	**D-63**	11043	**+**	-	-	-	_	_	_	_	_	_	_	_
70		31	sputum	female	**D-64**	11044	**++**	-	-	-	_	_	_	_	_	_	_	_

71		36	sputum	male	**D-65**	11045	**++**	-	-	-	_	_	_	_	_	_	_	_
72		38	sputum	female	**D-66**	11046	-	-	-	-	_	_	_	_	_	_	_	_
73		44	sputum	female	**D-67**	11047	**++**	-	-	-	_	_	_	_	_	_	_	_
74		48	sputum	female	**D-68**	11048	**+**	-	-	-	_	_	_	_	_	_	_	_
75		27	sputum	male	**D-69**	11049	-	-	-	-	_	_	_	_	_	_	_	_
76		23	sputum	female	**D-70**	11050	**+**	-	-	-	_	_	_	_	_	_	_	_
77		28	sputum	male	**D-71**	11051	**+**	-	-	-	_	_	_	_	_	_	_	_
78		29	sputum	female	**D-72**	11052	**+**	-	-	-	_	_	_	_	_	_	_	_
79		30	sputum	male	**D-73**	11053	**+**	-	-	-	_	_	_	_	_	_	_	_
80		68	sputum	male	**D-74**	11054	**++**	-	-	-	_	_	_	_	_	_	_	_
81		65	sputum	male	**D-75**	11055	**+**	-	-	-	_	_	_	_	_	_	_	_
82		34	sputum	male	**D-76**	11056	**+**	-	-	-	_	_	_	_	_	_	_	_
83		48	sputum	male	**D-77**	11057	**+**	-	-	-	_	_	_	_	_	_	_	_
84		38	sputum	male	**D-78**	11058	-	-	-	-	_	_	_	_	_	_	_	_
85		72	sputum	male	**D-79**	11059	**+**	-	-	-	_	_	_	_	_	_	_	_
86		50	sputum	male	**D-80**	11060	**+**	-	-	-	_	_	_	_	_	_	_	_
87		60	sputum	male	**D-81**	11061	-	-	-	-	_	_	_	_	_	_	_	_
88		55	sputum	male	**D-82**	11062	-	-	-	-	_	_	_	_	_	_	_	_
89		50	BAL	male	**D-83**	11063	-	-	-	-	_	_	_	_	_	_	_	_
90		35	sputum	male	**D-84**	11064	**+**	-	-	-	_	_	_	_	_	_	_	_
91		45	sputum	male	**D-85**	11065	**+**	-	-	-	_	_	_	_	_	_	_	_
92		45	sputum	male	**D-86**	11066	-	-	-	-	_	_	_	_	_	_	_	_
93		45	sputum	male	**D-87**	11067	**+**	-	-	-	_	_	_	_	_	_	_	_
94		54	sputum	male	**D-88**	11068	**++**	-	-	-	_	_	**GNB**	_	GNB	_	_	_
95		34	sputum	male	**D-89**	11069	-	-	-	-	_	_	_	_	_	_	_	_
96		32	sputum	male	**D-90**	11070	**++**	-	-	-	_	_	_	_	_	_	GNB	_
97		39	sputum	male	**D-91**	11071	**+**	-	-	-	_	_	_	_	_	_	GPB	_
98		29	sputum	male	**D-92**	11072	-	-	-	-	_	_	_	_	_	FU	_	_
99		27	sputum	male	**D-93**	11073	**++**	36	34	22	MTB	_	_	_	_	_	_	HIV+
100		34	sputum	male	**D-94**	11074	**+++**	28	28	15	MTB	_	_	_	_	_	_	_
101		32	sputum	male	**D-95**	11075	**++**	32	29	16	MTB	_	_	_	_	_	_	_
102		39	sputum	female	**D-96**	11076	**+++**	32	29	16	MTB	_	_	_	_	_	_	HIV+
103		29	sputum	female	**D-97**	11077	**++**	30	30	16	MTB	_	_	_	_	_	_	_
104		27	sputum	male	**D-98**	11078	**-**	-	-	14	MTB	_	_	_	_	_	_	_
105		34	sputum	male	**D-99**	11079	**-**	-	-	24	MTB	_	_	_	_	_	_	_
106		38	sputum	male	**D-100**	11080	**++**	29	30	20	MTB	_	_	_	_	_	_	_
107		27	sputum	male	**D-101**	11081	**+**	-	-	-	_	_	_	_	_	_	GNB	_

108		34	sputum	male	**D-102**	11082	**++**	-	-	-	_	_	_	_	_	_	_	_
109		37	sputum	female	**D-103**	11083	**+++**	29	29	13	MTB	_	_	_	_	_	_	_
110		50	sputum	male	**D-104**	11084	**-**	-	-	-	_	_	_	FU	_	_	_	_
111		35	sputum	male	**D-105**	11085	**+**	-	-	-	_	_	_	FU	_	_	_	_
112		30	sputum	female	**D-106**	11086	**+**	-	-	-	_	_	_	_	_	_	_	_
113		33	sputum	male	**D-107**	11087	**-**	-	-	-	_	FU	_	_	_	_	_	_
114		29	sputum	male	**D-108**	11088	**++++**	28	28	13	MTB	_	_	_	_	_	_	_
115		40	sputum	male	**D-109**	11089	**++**	-	-	19	MTB	_	_	_	_	_	_	_
116		44	SPUTUM	male	**D-110**	11090	**++**	-	-	22	MTB	_	_	_	_	_	_	_
117		24	sputum	female	**D-111**	11091	**++++**	28	28	14	MTB	_	_	_	_	_	_	HIV+
118		40	sputum	male	**D-112**	11092	**++**	-	-	-	_	_	_	_	_	_	_	_
119		42	sputum	male	**D-113**	11093	**++**	34	32	16	MTB	_	_	_	_	_	_	_
120		37	sputum	male	**D-114**	11094	**++**	34	36	19	MTB	_	_	_	_	_	_	_
121		33	BAL	male	**D-115**	11095	**+**	-	-	-	_	_	_	_	_	FU	_	_
122		30	sputum	male	**D-116**	11096	**++**	32	32	19	MTB	_	_	_	_	_	_	_
123		32	sputum	male	**D-117**	11097	**+++**	-	-	14	MTB	_	_	_	_	_	_	_
124		35	BAL	male	**D-118**	11098	**+++**	-	-	12	MTB	_	_	_	_	_	_	_
125		35	sputum	male	**D-119**	11099	**+**	-	-	-	_	_	GNB	_	_	_	GNB	_
126		34	sputum	male	**D-120**	11100	**+**	26	-	14	MTB	_	_	_	_	_	_	_
127		35	sputum	male	**D-121**	11101	**-**	-	-	-	_	_	_	_	GNB	_	_	_
128		30	sputum	male	**D-122**	11102	**-**	-	-	-	_	_	GPC	_	_	_	_	_
129		44	sputum	female	**D-123**	11103	**+**	27	-	16	MTB	_	_	_	_	_	_	_
130		38	sputum	male	**D-124**	11104	**-**	-	-	-	_	_	_	_	_	_	GPB	_
131		34	sputum	male	**D-125**	11105	**-**	-	-	-	_	FU	_	_	_	_	_	_
132		42	sputum	male	**D-126**	11106	**+**	29	-	14	MTB	_	_	_	_	_	_	HIV+
133		54	sputum	male	**D-127**	11107	**+++**	-	-	12	MTB	_	_	_	_	_	_	_
134		53	sputum	male	**D-128**	11108	**+**	31	-	23	MTB	_	_	_	_	_	_	_
135		55	sputum	female	**D-129**	11109	**+++**	-	-	13	MTB	_	_	_	_	_	_	_
136		22	sputum	female	**D-130**	11110	**+**	24	-	20	MTB	_	_	_	_	_	_	_
137		24	sputum	female	**D-131**	11111	**++**	22	-	20	MTB	_	_	_	_	_	_	_
138		28	sputum	male	**D-132**	11112	**+**	26	-	21	MTB	_	_	_	_	_	_	_
139		37	sputum	male	**D-133**	11113	**++**	-	-	20	MTB	_	_	_	_	_	_	_
140		42	sputum	male	**D-134**	11114	**++**	29	-	19	MTB	_	_	_	_	_	_	DM with HT
141		47	BAL	male	**D-135**	11115	**++**	-	-	24	MTB	_	_	_	_	_	_	_
142		44	sputum	male	**D-136**	11116	**++**	-	-	26	MTB	_	_	_	_	_	_	_
143		35	sputum	male	**D-137**	11117	**++**	29	-	16	MTB	_	_	_	_	_	_	_
144		18	sputum	male	**D-138**	11118	**+++**	26	_	18	MTB	_	_	_	_	_	_	HIV+

145		40	sputum	male	**D-139**	11119	**++**	29	_	19	MTB	_	_	_	_	_	_	_
146		28	sputum	male	**D-140**	11120	**++**	-	_	19	MTB	_	_	_	_	_	_	_
147		35	sputum	male	**D-141**	11121	**++**	_	_	22	MTB	_	_	_	_	_	_	_
148		45	BAL	male	**D-142**	11122	**+**	-	_	19	MTB	_	_	_	_	_	_	_
149		39	sputum	male	**D-143**	11123	**-**	_	_	_	_	_	_	_	_	_	_	_
150		25	sputum	female	**D-144**	11124	**+++**	24	_	14	MTB	_	_	_	_	_	_	HIV+
151		36	sputum	male	**D-145**	11125	**+++**	28	_	16	MTB	_	_	_	_	_	_	HIV+
152		52	sputum	female	**D-146**	11126	**+++**	23	_	14	MTB	_	_	_	_	_	_	HIV+
153		35	sputum	male	**D-147**	11127	**++**	_	_	22	MTB	_	_	_	_	_	_	_
154		27	sputum	male	**D-148**	11128	**+**	_	_	23	MTB	_	_	_	_	_	_	_
155		35	sputum	female	**D-149**	11129	**+**	_	_	24	MTB	_	_	_	_	_	_	_
156		62	sputum	female	**D-150**	11130	**++**	_	_	23	MTB	_	_	_	_	_	_	_
157		25	sputum	female	**D-151**	11131	**-**	_	_	_	_	_	_	_	_	_	_	_
158		29	sputum	male	**D-152**	11132	**+++**	29	_	14	MTB	_	_	_	_	_	_	_
159		54	sputum	female	**D-153**	11133	**-**	_	_	_	_	_	_	_	_	_	_	_
160		37	sputum	male	**D-154**	11134	-	-	_	_	_	_	_	_	_	_	GNB	_
161		25	sputum	male	**D-155**	11135	**++**	27		19	MTB	_	_	_	_	_	_	_
162		34	sputum	female	**D-156**	11136	_	_	-	_	_	_	GPC	_	_	_	_	_
163		28	BAL	male	**D-157**	11137	**++**	32	34	17	MTB	_	_	_	_	_	_	_
164		35	sputum	female	**D-158**	11138	**++**	_	_	23	MTB	_	_	_	_	_	_	_
165		32	sputum	male	**D-159**	11139	_	_	_	_	_	_	_	_	_	_	_	_
166		24	SPUTUM	female	**D-160**	11140	**+**	_	_	_	_	_	_	_	GNB	_	_	_
167		54	sputum	male	**D-161**	11141	**+++**	36	_	19	MTB	_	_	_	_	_	_	_
168		28	sputum	male	**D-162**	11142	**++**	34	29	20	MTB	_	_	_	_	_	_	_
169		46	sputum	male	**D-163**	11143	**++**	32	_	18	MTB	_	_	_	_	_	_	DM with HT
170		43	sputum	male	**D-164**	11144	**+++**	30	_	19	MTB	_	_	_	_	_	_	_
171		30	sputum	male	**D-165**	11145	_	_	_	_	_	_	_	_	GNB	_	_	_
172		22	sputum	male	**D-166**	11146	**++**	_	_	24	MTB	_	_	_	_	_	_	_
173		40	sputum	male	**D-167**	11147	_	_	_	_	_	_	_	_	_	_	_	_
174		85	sputum	male	**D-168**	11148	_	_	_	_	_	FU	_	_	_	_	_	_
175		26	sputum	male	**D-169**	11149	**++**	_	_	24	MTB	_	_	_	_	_	_	_
176		60	sputum	male	**D-170**	11150	**++**	29	_	13	MTB	_	_	_	_	_	_	_
177		45	sputum	female	**D-171**	11151	_	__	_	_	_	_	_	_	_	_	_	_
178		22	sputum	female	**D-172**	11152	_	_	_	_	_	_	_	_	_	_	_	_
179		20	sputum	female	**D-173**	11153	**++**	29	32	19	MTB	_	_	_	_	_	_	HIV+
180		25	sputum	female	**D-174**	11154	**+++**	32	29	14	MTB	_	_	_	_	_	_	_
181		10	sputum	female	**D-175**	11155	_	_	_	_	_	_	_	_	_	_	_	_

182		74	sputum	female	**D-176**	11156	_	_	_	_	_	_	_	_	_	_	_	_
183		25	BAL	female	**D-177**	11157	**+++**	32	30	19	MTB	_	_	_	_	_	_	HIV+
184		54	sputum	male	**D-178**	11158	**++**	_	_	22	MTB	_	_	_	_	_	_	_
185		55	sputum	male	**D-179**	11159	_	_	_	_	_	_	_	_	_	_	_	_
186		28	sputum	male	**D-180**	11160	**++**	40	40	21	MTB	_	_	_	_	_	_	_
187		30	sputum	male	**D-181**	11161	**+**	_	_	_	_	_	_	_	_	_	_	_
188		34	sputum	male	**D-182**	11162	**+**	_	_	_	_	_	_	_	_	_	_	_
189		34	sputum	male	**D-183**	11163	_	_	_	_	_	_	_	_	_	_	_	_
190		43	sputum	male	**D-184**	11164	**+**	_	_	23	MTB	_	_	_	_	_	_	_
191		30	sputum	female	**D-185**	11165	_	_	_	_	_	_	_	_	_	_	_	_
192		32	sputum	female	**D-186**	11166	**++**	42	40	22	MTB	_	_	_	_	_	_	_
193		29	sputum	male	**D-187**	11167	**+**	_	_	_	_	_	_	_	_	_	_	_
194		18	sputum	female	**D-188**	11168	_	_	_	_	_	_	_	_	_	_	_	_
195		19	sputum	male	**D-189**	11169	_	_	_	_	_	_	_	_	_	_	_	_
196		28	sputum	male	**D-190**	11170	**++**	27	30	18	MTB	_	_	_	_	_	_	HIV+
197		50	sputum	male	**D-191**	11171	**+++**	30	28	17	MTB	_	_	_	_	_	_	HIV+
198		40	sputum	male	**D-192**	11172	**+++**	32	29	19	MTB	_	_	_	_	_	_	_
199		58	sputum	male	**D-193**	11173	**+**	_	_	23	MTB	_	_	_	_	_	_	_
200		40	sputum	male	**D-194**	11174	**++**	_	_	22	MTB	_	_	_	_	_	_	_
201		52	sputum	male	**D-195**	11175	**++**	36	42	19	MTB	_	_	_	_	_	_	_
202		28	sputum	male	**D-196**	11176	_	_	_	_	_	_	_	_	_	_	_	_
203		35	sputum	female	**D-197**	11177	**++**	_	_	23	MTB	_	_	_	_	_	_	_
204		40	sputum	male	**D-198**	11178	**++**	21	23	16	MTB	_	_	_	_	_	_	_
205		50	sputum	male	**D-199**	11179	**++**	_	_	23	MTB	_	_	_	_	_	_	_
206		40	sputum	male	**D-200**	11180	**++**	_	_	24	MTB	_	_	_	_	_	_	_
207		50	sputum	male	**D-201**	11181	**++**	22	26	15	MTB	_	_	_	_	_	_	_
208		56	sputum	male	**D-202**	11182	**+**	_	_	_	_	_	_	_	_	_	_	_
209		62	sputum	male	**D-203**	11183	**+++**	27	23	13	MTB	_	_	_	_	_	_	_
210		27	sputum	male	**D-204**	11184	_	_	_	_	_	_	_	_	_	_	_	_
211		36	sputum	male	**D-205**	11185	_	_	_	_	_	_	_	_	_	_	_	_
212		28	sputum	male	**D-206**	11186	**++**	40	37	22	MTB	_	_	_	_	_	_	_
213		23	sputum	male	**D-207**	11187	_	_	_	_	_	_	_	_	_	_	_	_
214		38	sputum	male	**D-208**	11188	_	_	_	_	_	_	_	_	_	_	_	_
215		37	sputum	male	**D-209**	11189	**++**	42	40	22	MTB	_	_	_	_	_	_	_
216		55	sputum	male	**D-210**	11190	**+++**	29	34	20	MTB	_	_	_	_	_	_	_
217		30	sputum	female	**D-211**	11191	_	_	_	_	_	_	_	_	_	_	_	_
218		50	sputum	male	**D-212**	11192	**+++**	32	34	20	MTB	_	_	_	_	_	_	_

219		57	sputum	male	**D-213**	11193	_	_	_	_	_	_	_	_	_	_	_	_
220		26	sputum	female	**D-214**	11194	_	_	_	_	_	_	_	FU	_	_	_	_
221		53	BAL	male	**D-215**	11195	_	_	_	_	_	_	_	_	_	_	_	_
222		33	sputum	male	**D-216**	11196	**++++**	28	30	13	MTB	_	_	_	_	_	_	_
223		45	sputum	female	**D-217**	11197	**++**	40	42	22	MTB	_	_	_	_	_	_	HIV+
224		29	sputum	male	**D-218**	11198	**++**	_	_	21	MTB	_	_	_	_	_	_	_
225		20	sputum	male	**D-219**	11199	**+++**	36	34	20	MTB	_	_	_	_	_	_	_
226		26	sputum	male	**D-220**	11200	_	_	_	_	_	_	_	_	_	_	_	_
227		27	sputum	male	**D-221**	11201	**+++**	30	26	19	MTB	_	_	_	_	_	_	_
228		68	sputum	male	**D-222**	11202	_	_	_	_	_	_	_	_	_	_	_	_
229		30	sputum	male	**D-223**	11203	**+++**	28	28	14	MTB	_	_	_	_	_	_	_
230		42	sputum	male	**D-224**	11204	**++**	32	29	16	MTB	_	_	_	_	_	_	_
231		25	sputum	male	**D-225**	11205	_	_	_	_	_	_	_	_	_	_	_	_
232		58	sputum	male	**D-226**	11206	_	_	_	_	_	_	_	FU	_	_	_	_
233		42	sputum	male	**D-227**	11207	**++**	27	29	18	MTB	_	_	_	_	_	_	_
234		50	sputum	male	**D-228**	11208	_	_	_	24	MTB	_	_	_	_	_	_	_
235		30	sputum	female	**D-229**	11209	_	_	_	_	_	_	_	_	_	_	_	_
236		32	sputum	male	**D-230**	11210	_	_	_	_	_	_	_	_	_	_	_	_