ISOLATION AND IDENTIFICATION OF *MYCOBACTERIUM TUBERCULOSIS* FROM SPUTUM SAMPLE

Training Report

Internship Training Report

Submitted to

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in partial fulfillment of the requirements

For the degree of

Master of Science in Clinical Microbiology

Submitted by:
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RECOMMENDATION

This is to certify that **Mr. ARBIND KUMAR** has completed this dissertation work entitled "**ISOLATION AND IDENTIFICATION OF *MYCOBACTERIUM TUBERCULOSIS* FROM SPUTUM SAMPLE"** as a partial fulfilment of Master of science in Clinical Microbiology under our supervision. To our knowledge, this work has not been submitted to any other degree.

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CERFICATE OF APPROVAL

On the recommendation of **Dr. Mamta** (chief of Microbiologist, SRL, Gurgaon) and Assistant **(lec.) Rupinder kaur** (Lecturer, LFAMS) this dissertation work **by Mr. Arbind Kumar** entitled "**ISOLATION AND IDENTIFICATIONOF MYCOBACTERIUM TUBERCULOSIS FROM SPUTUM SAMPLE**" has been approved for the examination and is submitted to the Lovely Professional University in partial fulfilment of the requirements for Master degree of science in clinical Microbiology.

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…….

ARBIND KUMAR

ABSTRACT

Tuberculosis is a common and in some cases deadly infectious disease caused by various strains of *Mycobacteria*, usually *Mycobacterium tuberculosis* in humans. Tuberculosis usually attacks the lungs but can also affect other parts of the body. It is spread through the air when people who have an active MTB infection cough, sneeze, or otherwise transmit their saliva through the air. Most infections in humans result in an asymptomatic, latent infection, and about one in ten latent infections eventually progresses to active disease, if it is left untreated, kills more than 50% of its victims.

The Present study was carried for a period of 4 month (1nd Jan 2016 to 31st March 2016) in Microbiology Laboratory of SRL Diagnostic Gurgaon, A total of 598 Sputum samples were received 390, (65.21%) were collected from male patients and 208 (34.78%) were collected from female patients, in which 140 (23.41%) samples were positive followed by male 106, (75.71%) and female 34 (24.28%). 6 samples were found to be as MOTT positive.

Keywords- *Mycobacteria tuberculosis*, MOTT ,infection, latent, asymptomatic, MTB

LIST OF ABBREVIATION

TB	Tuberculosis
PNB	P-nitrobenzoic acid
ZN	Ziehl - Neelsen
AST	Antimicrobial Susceptibility Test
MDR	Multi Drug Resistant
XDR	Extended drugs resistent
WHO	World Health Organization
TEMP	Temperature
AFB	Acid fast bacilli
LJ	Lowenstain –jensen
GC	Growth control
D/W	Distilled water
MTC	Mycobacterium tuberculosis complex
MOTT	Other than mycobacterium tuberculosis

ANTIBIOTICS

STR	Streptomycin
RIF	Rifampin
INH	Ionized
EMB	Ethambutol

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CHAPTER-1

1. INTRODUCTION

Tuberculosis (T.B) is an infectious disease caused by bacteria whose scientific name is *MYCOBACTERIUM TUBERCULOSIS*. It was first isolated in 1882 by a German physician named Robert Koch who received the Nobel Price for this discovery. TB most commonly affects the lungs but also can involve almost any organ of the body. Many years ago, this disease was referred to as consumption because without effective treatment, these patients often would waste away. Today of course tuberculosis usually can be treated successfully with antibiotics. (1).

There is also a group of organisms referred to as atypical tuberculosis. These involve others types of bacteria that are in the Mycobacterium family. Often, these organisms does not cause disease and are referred to as colonizers because they simply live alongside other bacteria in our bodies without causing damage. At times these bacteria can cause an infection that is sometimes clinically like typical tuberculosis. When these atypical mycobacterium causes infection, they are often very difficult to cure. Often, drug therapy for these organisms must be administered for one and a half to two years and requires multiple medications.(1).

The re-emergence of tuberculosis in many countries in different regions of the world is a common public health concern. Rapid and precise diagnosis of each case is necessary for appropriate control of the disease. Isolation identification and susceptibility testing are essential procedures that should be performed as quickly as possible so that adequate treatment can be prescribed. The use of liquid media has been suggested as the most efficient and quickest procedure for the isolation of mycobacterium and susceptibility testing. However as well as being isolated these microorganisms should be promptly identified.

Although Mycobacterium tuberculosis infection is most common infection due to mycobacterium other than M. tuberculosis or non-tuberculosis mycobacterium (NTM) is on the increase in many countries. It is important to establish M. tuberculosis infection at an early stage for the establishment of adequate of tuberculosis patients who follow treatments regimens different from patients infected with other mycobacterium (2).

TB is spread though the air from one person to another. The TB bacteria are put into the air when a person with active TB disease of the lungs or throat coughs, sneezes, speaks or sings. People nearby may breathe in these bacteria and become infected.

1.1 DISEASE TUBERCULOSIS

Tuberculosis MTB or TB (short for tubercles bacillus) is a common and in some cases deadly infectious disease caused by various strains of mycobacterium usually Mycobacterium tuberculosis in humans. Tuberculosis usually attacks the lungs but can also affect other parts of the body. Most infections in humans results in an asymptomatic latent infection and about one in ten latent infections eventually progresses to active disease which if untreated kills more than 50% of its victims (1).

1.2 GENERAL CHARACTERISTICS

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

Mycobacterium tuberculosis is an **obligate aerobe**. For this reason, in the classic case of tuberculosis, MTB complexes are always found in the well-aerated upper lobes of the lungs usually of macrophages, and has a **slow generation time**, 15-20 hours, a physiological characteristic that may contribute to its virulence (2).

CHAPTER-2

TYPES OF INFECTION AND THEIR SIGN AND SYMPTOM

The uncontrolled growth of *M. tuberculosis* inside the human host leads to infection. Human tuberculosis is divided in to **pulmonary** and **extra pulmonary tuberculosis** based on the site of infection. A number of radiopharmacuticals have been used in the evaluation of pulmonary tuberculosis. Their main contribution to clinical investigation is the ability to differentiate active from inactive disease, thus enabling evaluation of therapeutic response and revelation of reactivation. In patient with AIDS,in particular, nuclear medicine procedures are more sensitive than morphologic imaging modalities in localizing site of pulmonary tuberculous infection. **Extra pulmonary tuberculosis** is a disseminated infection occurs after the primary infection due to the immune status and nutritional deficiency of the individual. The granuloma caceates, ruptures and bacilli infects different parts of the body For example, TB of the spine may cause back pain; TB of the kidney may cause blood in the urine; TB meningitis may cause headache or confusion. Extrapulmonary TB disease should be considered in the differential diagnosis of ill persons who have systemic symptoms and who are at high risk for TB disease (2).

2.1 CAUSES OF TUBERCULOSIS

Tuberculosis (TB) is caused by *Mycobacterium tuberculosis* . The condition is spread when a person with an active TB infection in their lungs coughs or sneezes and someone else inhales the expelled droplets, which contain TB bacteria.

Although it is spread in a similar way to a cold or the flu, TB is not as contagious. You would usually have to spend prolonged periods in close contact with an infected person to catch the infection yourself.

For example, TB infections usually spread between family members who live in the same house. It would be highly unlikely to become infected by sitting next to an infected person on a bus or train.(3).

Not everyone with TB is infectious. Generally, children with TB or people with TB that occurs outside the lungs (extra pulmonary TB) do not spread the infection.

2.2 Latent and active TB

In most healthy people the immune system is able to destroy the bacteria that cause TB. However, in some cases the bacteria infect the body but don't cause any symptoms (latent TB), or the infection begins to cause symptoms within weeks or months (active TB).(10).

Up to 10% of people with latent TB eventually develop active TB years after the initial infection. This usually happens when the immune system is weakened – for example, during chemotherapy.

2.3 Risk Factors

People with active TB disease who are coughing, which release bacteria into the air. The risk of infection increase for intravenous drugs users, healthcare workers, and people who live or work in a homeless shelter, migrant farm camp, prison or jail or nursing home.

Most people who are infected with *mycobacterium tuberculosis* don't develop active disease. The following factor increase the risk that latent disease will develop into active disease.

Infection with HIV the virus that cause AIDS and weakness the immune system.

Diabetes mellitus.

Head or neck cancer leukemia or Hodgkin's disease

Some medical treatments, including used corticosteroids or certain medications used for autoimmune or vasculitis disease such as rheumatoid arthritis or lupus, which suppress the immune system .(10).

Table 1: Symptoms of Pulmonary and Extrapulmonary TB Disease

Symptoms of Pulmonary TB Disease (TB disease usually causes one or more of the symptoms)	**Symptoms of Possible Extrapulmonary TB Disease** (Depends on the part of the body that is affected by the disease)
➢ Cough (especially if lasting for 3 weeks or longer) with or without sputum production	➢ TB of the kidney may cause blood in the urine
➢ Coughing up blood (hemoptysis)	➢ TB meningitis may cause headache or confusion
➢ Chest pain	➢ TB of the spine may cause back pain
➢ Loss of appetite	➢ TB of the larynx can cause hoarseness
➢ Unexplained weight loss	➢ Loss of appetite
➢ Night sweats ➢ Fever	➢ Unexplained weight loss ➢ Night sweats
➢ Fatigue	➢ Fever, Fatigue

CHAPTER-3

1. Pathogenicity of mycobacterium tuberculosis.

3.1 Mode of infection

Apart from congenital affection, the infection is commonly by way of ingestion and inoculation into skin .

3.2Virulence

Tuberculosis bacilli resist destruction by lysosomal enzyme when inside macrophages and multiply with them. The virulence is attributable to cell wall mycosides. Cord factor is also important in the pathogenesis. The tissue response is influenced by virulence of bacteria, number of bacilli in inoculum and the host response. The lesions produced by tubercle bacilli are primarily of two types.

3.3 Exudative type

There is an acute inflammatory reaction characterized by out- pouring of fibrinoid inflammatory exudative fluid and polymorph nuclear leucocytes.

Later, monocytes appear around the bacilli. Within 10 days of infection, clones of antigen – specific T lymphocytes are produced, which activate macrophage. The activated macrophage form a compact cluster or granuloma, around the foci of infection. These activated macrophage look like epithelial cells and are called epitheloid cells. At this stage the tuberculin test become positive. Exudative types of lesion is found in lungs and serous cavitis (meningitis, pleurisy). The lesion may heal by resolution and fibrosis, lead to massive necrosis of tissue or may pass on to productive type of lesion.(10).

3.4 Productive type

This phagocytic macrophage engulf the bacilli. Then the bacilli multiply inside the macrophage and the lesion become progressive. Activated macrophage or epitheloid cells fuse together to form giant cells. A productive type of lesion is a chronic specific granuloma (tubercle) and consists of 3 zones- central area of large multinucleated giant cells and a peripheral zone of fibroblast and lymphocytes. A smallest tubercle is of only and microscopic size (1-2 mm in diameter) and their fusion produced visible lesions.

Caseous necrosis develops in about 10-14 days after infection in a previously uninfected animal due to hypersensitivity to tuberculoproteins. Due to formation of endarteritis obliterans the bacilli cannot be recovered from the peripheral zone of tubercle. However, most bacilli get disintegrated by epitheloid cells and only a few remain active.(10).

3.5 TRANSMISSION OF TB

M. tuberculosis is carried in airborne particles, called droplet nuclei of 1– 5 microns in diameter. Infectious droplet nuclei are generated when persons who have pulmonary or laryngeal TB disease cough, sneeze, shout, or sing. Depending on the environment, these tiny particles can remain suspended in the air for several hours. *M. tuberculosis* is transmitted through the air, not by surface contact. Transmission occurs when a person inhales droplet nuclei containing *M. tuberculosis*, and the droplet nuclei traverse the mouth or nasal passages, upper respiratory tract, and bronchi to reach the alveoli of the lungs.(10).

TB is spread from person to person through the air. The dots in the air represent droplet nuclei containing tubercle bacilli.

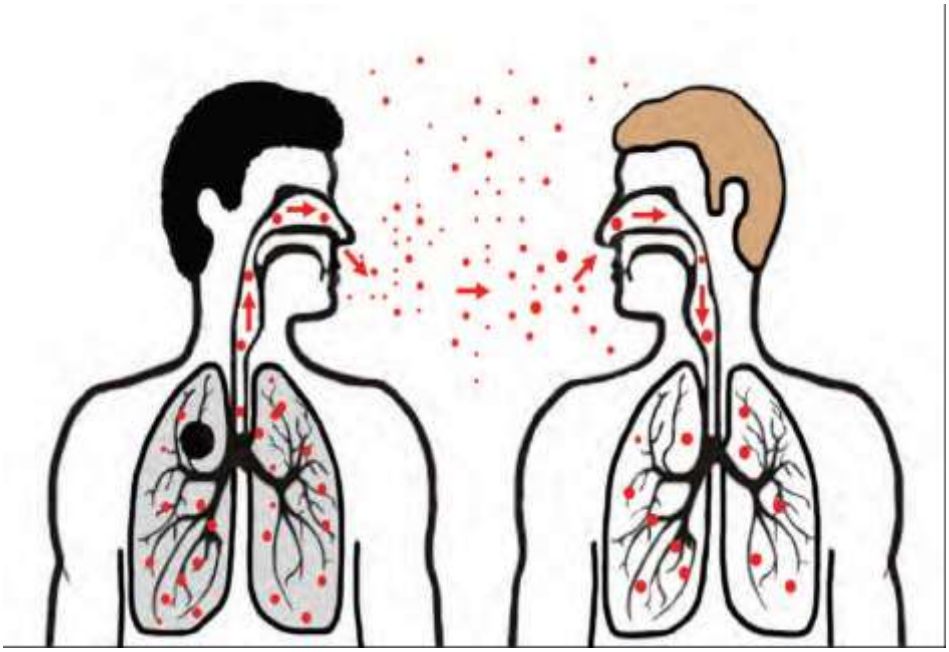


Fig. 1 TB is spread from one person to another person

Table 2: Factors that Determine the Probability of *M. tuberculosis* Transmission

There are four factors that determine the probability of transmission of *M. tuberculosis*

FACTOR	DESCRIPTION
Susceptibility	Susceptibility (immune status) of the exposed individual
Infectiousness	Infectiousness of the person with TB disease is directly related to the number of tubercle bacilli that he or she expels into the air. Persons who expel many tubercle bacilli are more infectious than patients who expel few or no bacilli
Environment	Environmental factors that affect the concentration of *M. tuberculosis* organisms
Exposure	Proximity, frequency, and duration of exposure

Table 4: Environmental Factors that Enhance the Probability that *M. tuberculosis* will Be Transmitted

FACTOR	DESCRIPTION
Concentration of infectious droplet nuclei	The more droplet nuclei in the air, the more probable that *M. tuberculosis* will be transmitted
Space	Exposure in small, enclosed spaces
Ventilation	Inadequate local or general ventilation that results in insufficient dilution or removal of infectious droplet nuclei
Air circulation	Recirculation of air containing infectious droplet nuclei
Specimen handling	Improper specimen handling procedures that generate infectious droplet nuclei
Air Pressure	Positive air pressure in infectious patient's room that causes *M. tuberculosis* organisms to flow to other areas

CHAPTER- 4

4. Material and method

4.1 Laboratory layout and equipment

Persons who work with tubercle bacilli are at risk of laboratory acquired infection, mainly by the airborne route, and it is well-known that sensible laboratory design may contribute much to the prevention of such infections. The detailed arrangement of a tuberculosis laboratory will vary according to the size and shape of the available room, the type of laboratory activity and whether other work is also done in the same room. Nevertheless, the most important aspect in tuberculosis laboratory design is to ensure a logical flow of specimens and activities, from clean to less clean areas.(14).

Mycobacterial cultures should always be performed in containments laboratories, physically separated from other laboratory areas. The objective is reduce the infection risk, not only to tuberculosis technologist, but also to other individuals in the same building.(14)

Contrary to common belief containment laboratories need not be overly sophisticated and expensive. Sophisticated and expensive air conditioning is not an essential requirement in tuberculosis culture laboratories. Rather, the principle should be that, during working hours, air is continuously extracted to the outside of the laboratory either through a biological safety cabinet or through simple extraction fans in walls or windows. Ventilation standards for air changes and pressure gradients should be considered in relation to the number of specimens processed per year and the prevalence of tuberculosis among these specimens. If bacteriological methods are performed with strict adherence to safety standards and high risk procedures are limited to the bio-safety cabinet, air-bone contamination will be minimized. Six to twelve room air changes per hour are sufficient to remove up 99% of airborne particles

within 30 to 45 minutes. Supply and exhaust air devices should be located on opposite side walls, with supply air provided from clean areas and exhaust air taken from less clean areas. An excess of air supply or a similar negative pressure created by extracting air is sufficient to obtain the necessary pressure gradient. Air should be exhausted directly to the outside. Potentially contaminated air should be discharged at least 3m above ground level.(15).

4.1.1Negative pressure room:-

Negative room pressure is an isolation technique used in hospitals and medical centers to prevent cross-contaminations from room to room. It includes a ventilation system that generates negative pressure to allow air to flow into the isolation room but not escape from the room, as air will naturally flow from areas with higher pressure to areas with lower pressure, thereby preventing contaminated air from escaping the room. This technique to used for isolation of patients with airborne contagious diseases such as tuberculosis**.** Negative pressure is generated and maintained by a ventilation system that removes more exhaust air from the room than air is allowed into the room. Air is allowed into the room through a gap under the door (typically about one half-inch high). Except for this gap, the room should be as airtight as possible, allowing no air in through cracks and gaps, such as those around windows, light fixtures, and electrical outlets. Leakage from these sources can compromise or eliminate room negative pressure.(15).

4.1.2 Bio-safety Cabinet Class II (BSC II)

Class II cabinets provide both kinds of protection (of the samples and of the environment) since makeup air is also HEPA-filtered. There are four types: Type A1 (formerly A), Type A2 (formerly A/B3), Type B1, and Type B2. Each type's requirements are defined by NSF

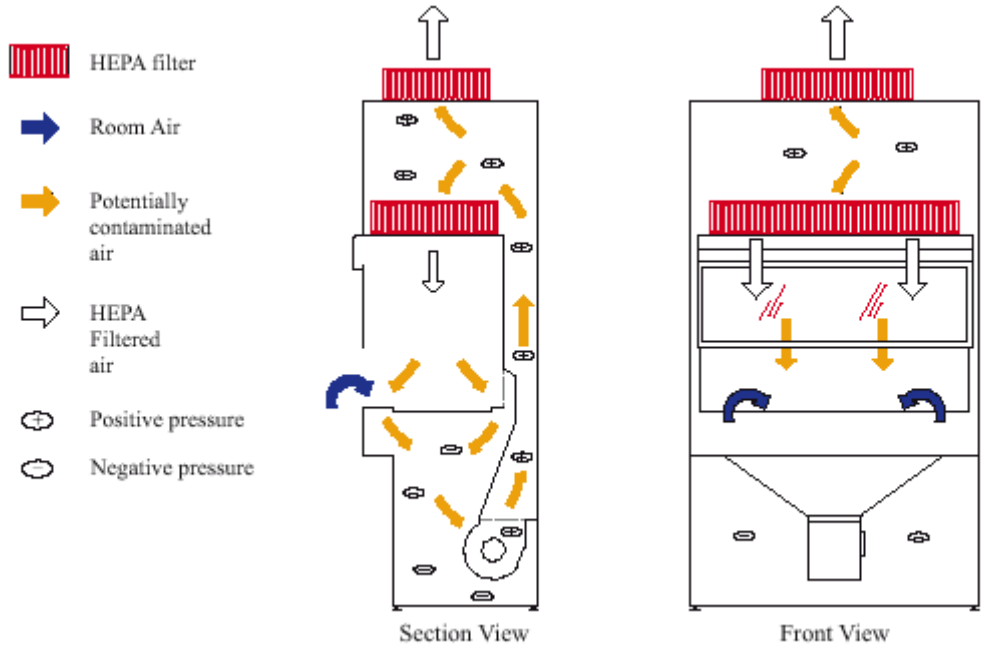
International Standard 49, which in 2002 reclassified A/B3 cabinets (classified under the latter type if connected to an exhaust duct) as Type A2.About 95% of all bio-safety cabinets installed are Type A2 cabinets Cabinet air may be recirculated back into the laboratory or ducted out of the building by means of a "thimble" connection (i.e., a small opening around the cabinet exhaust filter housing) whereby the balance of the cabinet is not disturbed by fluctuations in the building exhaust system. The thimble must be designed to allow for proper certification of the cabinet (i.e., provide access to permit scan testing of the HEPA filter). Maintain a minimum average face velocity of 0.5 m/s (100 ft/min). Have ducts and plenums under negative pressure. Is suitable for work with minute quantities of volatile toxic chemicals and trace amounts of radio-nuclides. The principle of operation involves using a fan mounted in the top of the cabinet to draw a curtain of sterile air over the products that are being handled. The air is then drawn underneath the work surface and back up to the top of the cabinet where it passes through the HEPA filters. The air that is exhausted is made up by air being drawn into the front of the cabinet underneath the work surface. The air being drawn in acts as a barrier to potentially contaminated air coming back out to the operator. The Type A2 cabinet, formerly designated A/B3 has a minimum inflow velocity of 100 ft/min. A negative air pressure plenum surrounds all contaminated plenums that are under positive pressure. In other respects, the specifications are identical to those of a Type A1 cabinet(15).

FEATURES:-

- Negative pressure plenum surrounds contaminated positive pressure plenum; no fabric bags are used.
- Dual, long-life ULPA filters for supply and exhaust (per IEST-RP-CC001.3) with 99.999% efficiency for particle size between 0.1 to 0.3 microns.

- Angled supply filter matches cabinet profile to achieve best down flow uniformity.
- Esco Sentinel™ Gold microprocessor.
- Quick start mode, to turn the blower and lights on/off, by moving the sash window to correct position.
- RS 232 data output port enables remote monitoring of cabinet operating parameters.
- Frameless sash that is easier to clean.
- Ergonomically angled front improves reach and comfort.

- Actual work access height is 45 mm (1.8") higher than tested sash opening.
- Removable one-piece work surface simplifies cleaning.
- Raised airflow grille maintains safety by preventing blockage.
- Esco ISOCIDE antimicrobial coating on all painted surfaces minimizes contamination.
- Enhanced side-capture zones optimize containment.
- Work area on Esco 0.9 meter (3') cabinet is equivalent to work area offered on larger 1.2 meter (4') conventional cabinet.(15).



Class II type A2 Biological safety cabinet

4.1.3 Centrifuge (cold)

Centrifuge are essential in laboratories where tubercle bacilli are cultured. Methods involving the use of a centrifuge are more efficient than simple decontamination and culture of sputum directly onto medium. Centrifuges should preferably be fitted with an electrically operated safety catch which prevents the lid from being opened while the rotor is spinning.

If the RCF is not high enough, many Mycobacteria will remain in suspension following centrifugation and will be proud off with the discarded supernatant. A95% sedimenting efficiency should be attained for optimal isolation of Mycobacteria. This requires a RCF of 3 000 x g. Glass tubes may break under the stress of centrifugation. If a centrifuge tube breaks, the liquid will splash or be blown out and aerosolized. Screw top centrifuge tubes should therefore be used for potentially infectious material.(24).

4.1.4 Incubator

Cultures are incubated at 35E-37EC for six weeks. Incubators are available in various sizes. In general, it is best to obtain the largest possible model that can be accommodated and afforded. Ensure a proper circulation of air by avoiding overloading and by using perforated trays. Maintain a constant temperature by not opening the incubator door unnecessary.(24)

4.1.5 Autoclave

Tubercle bacilli are more readily killed by moist heat (saturated steam) than by dry heat. Steam kills tubercle bacilli by denaturing their protein. Air has an important influence on the efficiency of stem sterilization because its presence changes the pressure-temperature

relationship. For example, the temperature of saturated steam at 15 lb/in^2 is 121EC, provided that all of the air is first removed from the vessel. With only half of the air removed the temperature of air in mixed loads will prevent penetration by steam.(18)

4.1.6 Water bath

Modern water baths are equipped with electrical stirrers and in some the heater, thermometer and stirrer are in one unit, easily detached from the batch for servicing. Water bath must also be lagged so as to prevent heat loss through the walls. A bath that has not been lagged by the manufacturers can be insulated with slabs of expanded polystyrene. Water baths should be fitted with lids in order to prevent heat loss and evaporation.(18)

4.1.7 Bunsen burners

For material that may spatter or that is highly infection a hooded Bunsen burner should be used. Electric burners are available. These are tubular micro-incinerators in which the loop or wire is inserted, and is recommended for use in BSCs (18).

4.1.8 Glassware and plastics

Soda-glass or pyrex are satisfactory for tuberculosis culture and the use of more expensive resistance glass is not justified. New unwashed soda-glass should be soaked in hydrochloric acid overnight to partially neutralize the alkali content of the glass (7)

4.1. 9 Culture bottles

Several sizes of culture bottles are useful for tuberculosis bacteriology. The most useful sizes are the small McCartney (14ml), the standard McCartney (28ml) and the Universal container (28ml), which has a larger neck than the others and is also used as a specimen container. These bottles usually have aluminium screw caps with rubber liners. The liners should be made of black rubber some red rubbers are thought to give off bactericidal substances.(7).

4.1.10 Test tubes

Rimless test tubes of heavy quality are most suitable for tuberculosis bacteriology. Thin glass, lipped chemical tubes should not be used. The most frequently used sizes of test tubes are 152x16mm, holding 5-10 ml and 152x19mm, holding 10-15ml.(8)

4.1.11 Pasteur pipettes

Pasteur pipettes are probably the most dangerous pieces of laboratory equipment in unskilled hands. Safer Pasteur with integral test and made of low density polypropylene (rather than glass) are available and are supplied pre-sterilized. Pasteur pipettes are used once only.(8).

4.1.12 Graduated pipettes

Straight side blow out pipettes, 1-10ml capacity are often used. They must be plugged with non-absorbent cotton wool at the suction end to prevent bacteria from entering from the teat and contaminating the material in the pipette. These plugs must be tight enough to stay in place during pipetting but not so tight that they cannot be removed during cleaning.(7)

4.1.13 Inoculating loops and wires

These are usually made of 25 SWG Nicrome wire. They should be short (not more than 15cm long) in order to minimize vibration and therefore involuntary discharge of contents. Loops should be small (not more than 5mm in diameter). Large loops are inclined to empty spontaneously and scatter infected airborne particles. Loops should be completely closed. This can be achieved by twisting the end of the wire round the shank, or by taking a piece of wire 15cm long. Bending the centre round a nail or rod of appropriate diameter and twisting the ends together in a drill chuck.(7).

4.1.14 Racks and basket

Test tube culture bottle racks should preferably be made of polypropylene or nylon so that they can be autoclaved. This also minimizes breakage, which is not uncommon when metal racks are used. Wooden racks are unhygienic(6).

4.2 SPECIMEN TYPE AND COLLECTION:

The presence of acid-fast bacilli in a clinical specimen may be confirmed either by microscopy or by culture. However, since individual mycobacterial species cannot be identified by smear examination, the definitive diagnosis of tuberculosis can only be made if *M. tuberculosis* is isolated from the clinical specimen (10).

In tuberculosis bacteriology attention tends to be focused on the problems of microscopy, culture and identification systems, while an often overlooked problem is that of obtaining adequate specimens. The advantages of subtle decontamination techniques, sensitive culture

media and simple identification schemes will not be fully realized unless specimens are collected with the most care and promptly transported to the laboratory (10).

4.2.1 CONTAINERS:-

An essential prerequisite for the safe collection of a satisfactory specimen is a robust, leak proof and clean container. Containers must be rigid to avoid crushing in transit and must possess a water-tight wide-mouthed screw top to prevent leakage and contamination.(10)

To facilitate the choice of a container the following specifications are recommended:

- Wide-mouthed (at least 35mm in diameter) so that the patient can expectorate easily inside the container without contaminating the outside
- Volume capacity of 50ml
- Made of translucent material in order to observe specimen volume and quality without opening the container
- Made of single-use combustible material to facilitate disposal
- Screw-capped to obtain an airtight seal and to reduce the risk of leakage during transport
- Easily-labeled walls that will allow permanent identification

4.3 COLLECTION PROCEDURE

4.3.1 SPUTUM (EXPECTORATED)

Three early morning specimens obtained on different days should be submitted. A volume of 5 to 10 ml is adequate and there is no advantage in collecting a larger volume. The sample should contain recently discharged material from the bronchial tree with minimal saliva content (14).

4.3.2 SPUTUM (INDUCED)

If the patient has difficulty producing a sputum specimen, then induction should be considered. Sputum production may be induced by the inhalation of a warm aerosol of sterile 5-10% sodium chloride in water produced by a nebulizer (7)

The specimen should be clearly marked "INDUCED" on the request slip since nebulized sputa is watery in consistency and could be mistaken for saliva (7).

4.3.3 GASTRIC LAVAGE

This procedure can be employed where sputum production is unsuccessful. This technique requires professional attention and should only be attempted in the hospital. Gastric lavage is performed early in the morning before eating and at least 8 hours after the patient has eaten or taken oral drugs. 5-10mL specimen is required and must be neutralized with 100 mg of sodium carbonate (5).

4.3.4 URINE

An early morning midstream specimen should be collected. Send entire specimen. Multiple specimens over several days may be required to obtain a positive specimen. Due to contamination and deterioration, 24 hour urine specimens are NOT acceptable. Keep specimen refrigerated until transport (5).

4.3.5 FLUIDS

Body fluids (spinal, pleural, pericardial, synovial, ascitic, blood, pus, and bone marrow) must be aseptically collected and submitted in sterile containers. Keep refrigerated until transport (4).

4.3.6 TISSUE

Any tissue to be cultured must be collected aseptically into sterile a container without fixatives or preservatives. If the specimen may dry, add sterile saline to keep moist. Do not place tissue specimen for culture into formalin. Keep refrigerated until transport.

Note:-

Body fluids (spinal, pleural, pericardial, synovial, ascitic, blood, pus, bone-marrow) should be aseptically collected in a sterile container by the physician using aspiration techniques or surgical procedures. For fluids that may clot, sterile potassium oxalate (0.01-0.02ml of 10% neutral oxalate per ml fluid) or heparin (0.2mg per ml) should be added. Specimens should be transported to the laboratory as quickly as possible (7).

4.4 BACTERIOLOGICAL EXAMINATION:-

4.4.1 Sample Preparation: Decontamination / Digestion:-

NaOH-NALC procedure is the standard recommended procedure to be used with MGIT, which is also recommended by CDC. In this procedure, the initial concentration of NaOH is 4%. This 4% NaOH solution is mixed with an equal quantity of sodium citrate solution (2.9%) to make a working solution (NaOH concentration in this solution is 2%). When an equal quantity of NaOH-NALC-citrate and sputum are mixed, the final concentration of NaOH in the specimen is 1%.

4.4.2 Materials and Methods

1. Disposable 50 ml plastic tubes (Falcon tubes)

2. Sterile NaOH-NALC-sodium citrate solution, preferably, MycoPrepared and sterilized.

3. Phosphate buffer pH 6.8 prepared and sterilized.

4. Centrifuge with a minimum 3000-3500x g force and safety shield (refrigerated centrifuge is preferred).

5. Timer,

6. Vortex mixer,

7. Shaker,

8. Pipettes/transfer pipettes or a pipettor with cotton plugged pipette tips

9. Add NaOH-NALC-sodium citrate solution in a volume equal to the quantity of specimen. Tighten the cap.

10. Vortex lightly or hand mix for about 15-30 seconds. Invert the tube so the whole tube is exposed to the NaOH-NALC solution.

11. Wait 15-20 minutes (up to 25 minutes maximum) after adding the NaOH-NALC solution. Vortex lightly or hand mix/invert every 5-10 minutes or put the tubes on a shaker and shake lightly during the whole time.

12. Make sure the specimen is completely liquefied. If still mucoid, add a small quantity of NALC powder (3030-35 grams) directly to the specimen tube. Mix well.

13. At the end of 15-20 minutes, add phosphate buffer (pH 6.8) up to the top ring on the centrifuge tube (plastic tube has a ring for 50 ml mark). Mix well (lightly vortex or invert several times).

14. Centrifuge the specimen at a speed of 3000 g or more for 15-20 minutes. Use of refrigerated centrifugation at a higher speed is known to increase recovery of mycobacteria.

15. After centrifugation, allow tubes to sit for 5 minutes to allow aerosols to settle .Then carefully decant the supernatant into a suitable container containing a mycobacterial disinfectant. Make sure the sediment is not lost during decanting of the supernatant fluid. Add a small quantity (1-2 ml) phosphate buffer (pH 6.8) and resuspend the sediment with the help of a pipette or vortex mixer.

16. Use the resuspended pellet for making smears and for inoculation of MGIT tubes and other media.(15).

4.5 MICROSCOPY

Microscopy is the simplest and most rapid procedure currently available to detect acid fast bacilli in clinical specimen by Ziehl-Neelsen staining method or its modification. There are several methods of determining the acid fast nature of an organism. In the carbol fuchsin procedures (Ziehl-Neelsen, Kinyoun), acid fast organisms appear red, and in the fluorochrome procedures (auramine O, auramine rhodamine), the acid-fast organisms fluoresce yellow to orange (the color may vary with the filter system used).(7).

4.5.1 ZN- STAINING (ACID- FAST BACILLI STAIN)

Ziehl-Neelsen stain, also known as the **acid-fast stain**, was first described by two German doctors; Franz Ziehl (1859 to 1926), a bacteriologist and Friedrich Neelsen (1854 to 1894), a pathologist. It is a special bacteriological stain used to identify acid-fast organisms, mainly Mycobacteria. *Mycobacterium tuberculosis* is the most important of this group, as it is

responsible for the disease called tuberculosis (TB) along with some others of this genus. It is helpful in diagnosing *Mycobacterium tuberculosis* since its lipid rich cell wall makes it resistant to Gram stain. It can also be used to stain few other bacteria like *Nocardia*. The reagents used are Ziehl–Neelsen carbol - fuchsin, acid alcohol and methylene blue. Acid-fast bacilli will be bright red after staining (26).

PRINCIPLE:

Acid fast bacilli are difficult to stain because of the lipid content of the cell wall. The exact nature of this unique staining reaction is not completely understood, but it is believed that the phenol dissolves the lipid sufficiently to allow penetration of the primary stain. The cell wall retains the primary stain even after exposure to the decolorizing agent, acid alcohol. This resistance to decolorizing by acid alcohol is required for an organism to be termed acid fast (25).

(ii) PROCEDURE:-

1. Place the slide on the rack

2. Flood the slide and completely cover with carbol fuschin

3. Using the metal forceps, take a piece of cotton wool soaked in alcohol, pass it through the flame and heat the slide from below until the stain emits a vapor, but do not bring to boiling point.

4. Add fuschin if necessary ; the slide should be covered.

5. Rinse with water, drain.

6. Apply decolorizing solution till stain comes out.

7. Rinse, drain.

8. Apply Methylene blue counter stain, 30 seconds.

9. Rinse, drain.

10. Air dry or blot it dry.

11. Observe under microscope.

(iv) Results

1. Acid fast organisms: Bright red bacilli on blue -black ground.

2. Other organisms: Dark blue

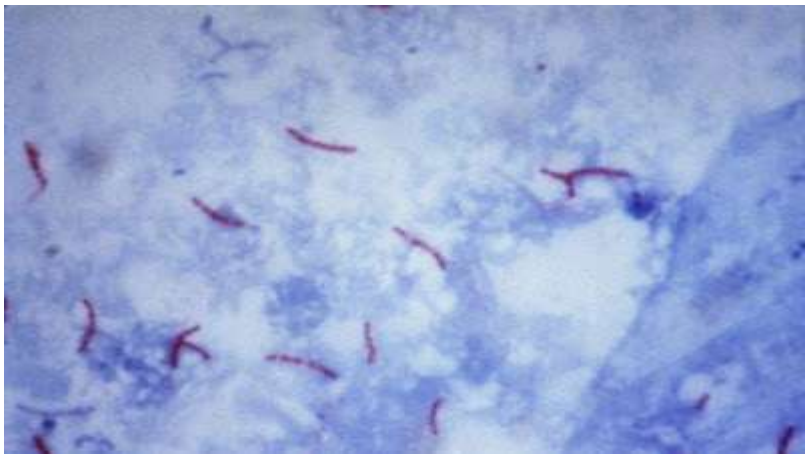


Fig. **An Acid Fast stain (Ziehl-Neelsen) shows numerous bacilli.**

(vi) REPORTING RESULT:

Negative

Report "No acid fast bacilli observed" for all smears in which no acid fast bacilli have been seen.

Positive

Indicate the staining method, and give information on the quantity of acid fast bacilli observed on the smear. For smears examined with a 100x oil immersion objective, the following may be used as a guide.

Examination	**Results**	**Grading**
More than 10 AFB per oil immersion field	Positive	3+
1-10 AFB per oil immersion field	Positive	2+
10-99 AFB per 100 oil immersion fields	Positive	1+
1-9 AFB per 100 oil immersion fields	Scanty	Record exact number seen
No AFB in 100 oil immersion fields	Negative	0

(vii) QUALITY CONTROL

A control slide should be inoculated with each run of strains. This will verify the correct performance of the procedure as well as the staining intensity of the acid fast organisms.

4.6 Culturing

Several types of media are used for the cultivation of mycobacteria, and each facility determines which ones are most appropriate for use. A combination of culture media is often used to optimize recovery of mycobacteria as well as inhibit the growth of contaminants. Mycobacteria require a pH of 6.5-6.8 for growth and grow best at higher humidity. A definitive diagnosis of tuberculosis can only be made by culturing *Mycobacterium tuberculosis* organisms from a specimen taken from the patient (most often sputum, but may also include pus, CSF, biopsied tissue, etc.) automatically and manually.(16)

(a)Manual Method

The manual method for definite diagnosis of tuberculosis demands that ***M.tuberculosis*** be recovered on culture media and identified using differential *in vitro* tests. Many different media have been devised for cultivating tubercle bacilli and three main groups can be identified, viz egg-based media, agar-based media and liquid media.(16)

(i)Egg Based Media

The **Lowenstein-Jensen medium**, more commonly known as **LJ medium**, is a growth medium specially used for culture of **Mycobacterium**, notably *Mycobacterium tuberculosis.*

When grown on LJ medium, *M. tuberculosis* appears as brown, granular colonies (sometimes called "buff, rough and tough"). The media must be incubated for a significant length of time, usually four weeks, due to the slow doubling time of *M. tuberculosis* compared with other **bacteria (15-20 hours).**

Composition

The usual composition as applicable to *Mycobacterium tuberculosis* is:

Ingrredients	**Amount**
Potato Flour(potato starch)	30.0 gm
L- Asparagine	3.6 gm
Monopotassium Phosphate	2.4 gm
Magnesium citrate	0.6 gm
Malachite Green	0.4 gm
Magnesium Sulfate	0.24 gm
Glycerol	12 ml
Egg suspension	1000 ml
Distilled Water	600 ml

This is a close-up of a Mycobacterium tuberculosis culture revealing this organism's colonial morphology. Note the colorless rough surface, which are typical morphologic characteristics seen in Mycobacterium tuberculosis colonial growth. Macroscopic examination of colonial growth patterns is still one of the ways microorganisms are often identified (17).

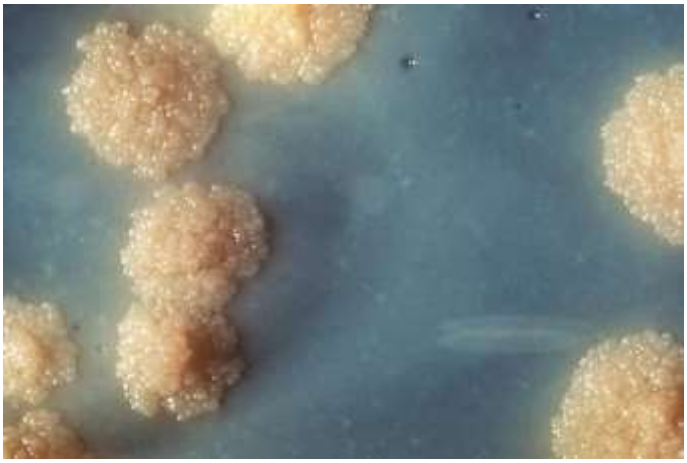


Fig. colony on L-J Medium

(ii)Agar based media

Middle brook 7H10 and Middle brook 7H11 agar are two types g agar media commonly used for detection of Mycobacterium tuberculosis. Middle brook 7H11 is better and more commonly used agar as it is a modification of Middle brook 7H10 agar special as recommended by Cohn, Waggoner and McClately. Cohn et al added an enzyme digest of casein and found organism growth was stimulated for fastidious strains of Mycobacterium.(15).

(iii)Liquid media

Mycobacteria have been cultured in a number of different types of liquid media throughout the years. Currently the most commonly used medium is Middle-brook 7H9 with OADC (oleic acid, albumin, dextrose and a catalase) supplementation, (7H9-OADC). During mycobacterial protein analysis, we have found that cell lysates from washed whole cells grown in this medium contain non-mycobacterial proteins, such as albumin. Furthermore, these contaminants create a background that obscures the protein profile analysis of secreted and cellular mycobacterial products. Unfortunately, the currently available protein deficient media do not allow for rapid growth of mycobacteria. In order to overcome these problems, the OADC carbon and amino acid enrichment was eliminated from the 7H9 medium, and D-glucose and tryptose were added as substitute nutrient sources to the 7H9 salt base. The new formula has been named 7H9-T&G. The growth rates of M. avium were subsequently tested in both 7H9-ADC and in 7H9-T&G and were found to be equivalent. *Mycobacterium spp.* grow more rapidly in liquid media; solid media takes approximately 17 days for the isolation of acid-fast bacilli whereas liquid media takes only about 10 days (7).

(b)Automated System

A culture of the AFB can distinguish the various forms of mycobacteria, although results from this may take four to eight weeks for a conclusive answer but automated liquid culture for *M. tuberculosis* (MTB) is widely used in industrialized countries and increases the sensitivity and reduces the time required for culture based tuberculosis (TB) diagnosis. The use of this highly sensitive technique increases the importance of robust protocols and quality control to prevent and if this occurs detect laboratory cross-contamination. Automated systems that are faster include the BACTEC 960, and the Mycobacterial Growth Indicator Tube (MGIT). (BD AUTO-14)

(i) INTRODUCTION

The new ***BD MGIT™*** 960 System is the new generation in a proven line of mycobacteria testing instruments from BD Microbiology Systems - the undisputed world leader in microbiology. The ***BD BACTEC™*** **BBL MGIT** 960 System builds on the legacy of simplicity, efficiency, performance and safety of the ***BD BACTEC™*** 460TB and 9000MB instruments. The ***BD BACTEC™*** 460TB System, the world's first automated system for mycobacteria testing, continues to serve as the benchmark of quality and reliability. The ***BD MGIT™*** 960 System combines these outstanding features with the technology used in the **BD BBL MGIT** (Mycobacteria Growth Indicator Tube) System. The MGIT Mycobacteria is the liquid media based technique most commonly used for the growth incubator tube contains 7 ml of modified Middle brook 7H9 broth base. The complete medium with OADC enrichment and PANTA antibiotic mixture is liquid media most commonly used for the cultivation of mycobacteria. All types of clinical specimens,

pulmonary as well as extra pulmonary can be processed for primary isolation in the MGIT tube using conventional methods.

(ii) **PRINCIPLE**

A fluorescent compound is embedded in silicone on the bottom of tubes. The fluorescent compound is sensitive to the presence of oxygen dissolved in the broth. Initially, the large Amount of dissolved oxygen quenches emissions from the compound and little fluorescence can be detected. Lately , actively respiring microorganisms consume the oxygen and allow the fluorescence to be detected. Tubes entered into the BACTEC MGIT 960 system are continuously incubated at 37°c and monitored every 60 min for increasing Analysis of the fluorescence is used to determine if the tube is instrument-positive; i.e., the test sample contains viable organisms. Culture tubes which remain negative for a minimum of 42 days (up to 56 days) and which show no visible signs of positivity are removed from the instrument as negatives.(14).

(iii) BBL MGIT Mycobacteria Growth Indicator Tube

Each tube contains 110 L of fluorescent indicator and 7 Ml of broth. The indicator contains Tri 4, 4-diphenyl-1, 10-phenanthroline ruthenium chloride pentahydrate in a silicone rubber base. The tubes are flushed with 10% CO2 and capped with polypropylene caps. The approximate formula, per 1000 ml of purified water, contains:

Modified Middle brook 7H9 broth base	5.9 gm
Casein peptone	1.25g

(iv) BACTEC MGIT960 Supplement Kit : Each kit contains:

MGIT Growth supplement (enrichment)

MGIT growth supplement is provided for the BACTEC MGIT 960, 7 ml tube. For manual MGIT, a different enrichment (BBL MGIT OADC, 15 ml) is used. The enrichment must be added to the MGIT medium prior to inoculation of a specimen. MGIT growth supplement contains 15 ml of the following approximate formula:

Bovine Albumin ----------50.0gm

Dextrose----------20.0gm

Catalase----------0.03gm

Oleic Acid----------0.1 gm

Polyoxyethyline state (POES) ---------- 1.1 gm

(v) MGIT PANTA:-

Each vial contains the following Lyophilized mixture of Antimicrobial Agent:

Polymyxin B……6,000 units

Trimethoprim……. 6,00 µg

Amphotericin…… 6,00 µg

Azlocillin…….. 6,00 µg

Nalidixic Acid…….. 2,400 µg

(vi) PROCEDURES

Reconstituting PANTA

Reconstitute MGIT PANTA with 15.0 ml MGIT growth supplement. Mix until completely dissolved. Add 0.8 ml of this enrichment to each MGIT tube. The enrichment with reconstituted PANTA should be added to the MGIT medium prior to inoculation of specimen in MGIT tube. Do not add PANTA/enrichment after the inoculation of specimen. Do not store MGIT tube after the addition of enrichment/ PANTA.(14).

Inoculation of MGIT medium

Label MGIT tubes with specimen number.

Unscrew the cap and aseptically add 0.8 ml of MGIT growth supplement/PANTA to each MGIT tube. Use of an adjustable pipette is recommended.

Using a sterile pipette or a transfer pipette, add up to 0.5 ml of a well mixed processed/concentrated specimen to the appropriately labeled MGIT tube. Use separate pipette or pipette tip for each specimen.

Immediately recap the tube tightly and mix by inverting the tube several times.

Wipe tubes and caps with a mycobacterial disinfectant and leave inoculated tubes at room temperature for 30 minutes. Work under the biologic safety cabinet for the specimen inoculation.

(vii) **INCUBATION:-**

Incubation Temperature:

All inoculated MGIT (7mL) tubes should be entered in the BACTEC MGIT 960 instrument after scanning each tube. It is important to keep the cap tightly closed and not to shake the tube during the incubation. This helps in maintaining the oxygen gradient in the medium. The instrument maintains 37ºC + 1ºC temperature. Since the optimum temperature for growth of *M. tuberculosis* is 37ºC, make sure the temperature is close to 37ºC. (16).

Length of incubation:

MGIT tubes should be incubated until the instrument flags them positive. After a maximum of six (6) weeks, the instrument flags the tubes negative if there is no growth.

(viii) Detection of positive growth

- The instrument signals a tube positive for growth, and an indicator green light shows the exact location of the positive tube in the drawer of the instrument.
- At this point, the tube Should be removed and scanned outside the instrument.
- The tube should be observed visually.
- Mycobacterial growth appears granular and not very turbid while contaminating Bacterial growth appears very turbid.
- Growth especially of the *M. tuberculosis* complex, settles at the bottom of the tube.
- Information about the time-to-detection of positive growth can be retrieved from the unloaded positives report.(22).

Work-up of Positive Cultures

(ix) AFB smear from a positive MGIT tube

Once a MGIT tube is positive by fluorescence or by visual observation, prepare a smear and stain with carbol fuchsin stain.

Procedure

1. Use a clean slide.

2. Mix the broth by vortexing and then by using a sterile pipette, remove and aliquot. Place 1-2 drops on the slide and spread over a small area (approx. 1½ x 1 cm).

3. Let the smear air dry.

4. Heat-fix the smear by passing it over a flame a few times .

5. Stain the smear with Ziehl-Neelsen. Air dry but do not blot dry.

6. Place a drop of oil on the stained and completely dried smear and screen under a low power objective to locate stained bacteria. Switch to an oil immersion objective lens for detailed observation.

Smear made from a positive MGIT broth also helps in tentative differentiation of *M.tuberculosis* complex from other mycobacteria. Cultures belonging to the TB complex form typical clumps and serpentine cords while other mycobacteria appear as loose, smaller clumps and cording or single cells.

4.16.4 DIFFERENTIATION OF MYCOBACTERIUM TUBERCULOSIS COMPLEX FROM OTHER THAN MYCOBACTERIUM TUBERCULOSIS SPECIES:

After getting positive tubes further diffentiation between MTC and MOTT is necessary for treatment purpose, this can be achieved by two ways:-

(a) Selective inhibition test with p- nitrobenzoic acid (PNB)

(b) SD Bioline Ag MPT 64 rapid test.

(a) Selective inhibition test with p-Nitrobenzoic acid using BECTEC MGIT 960

Mycobacteria growth in media with the addition of inhibitory substances has been used in species identification. Growth of the Mycobacterium tuberculosis complex (MTC) is inhibited by rho-nitrobenzoic acid (PNB), whereas non-tuberculous mycobacteria (NTM) are resistant.

(i) PREPERATION OF PNB

- Dissolve 4.15 g of PNB in 1N NaOH (or more until it dissolves).
- Add 80ml of distilled water.
- Add 1- 2 drops of 0.1% Phenolphthalein solution.
- Add 3 ml of 1N hydrochloric acid, drop by drop shaking continuously until the final color of the PNB solution should becomes pale yellow.
- Make up the total volume of the solution upto 100ml with distilled water.

Autoclave at 120 for 10 min. Store the PNB in aliquot (0.5ml) at 20 degree. This reagent is stable up to 6 months.(15).

(ii) INOCULUM PREPARATION

- From a positive 7 ml MGIT tube
- Day of positivity = Day 0
- Incubate for 1 more day after flagging positive and then treat as day 1
- If the tube is day 1 or day 2, carry out 1:5 dilution of the culture by adding 1 ml of part broth to 4 ml of sterile saline and use for the inoculation procedure.
- If the tube is day 3 to day 5 then adjust the suspension by visually compare to 0.1 Mc Farland turbidity standard. Carry out 1:5 dilution and use.

(iii) INOCULATION PROCEDURE

- Label 2 MGIT 7ml, one as GC(Growth Control) and one as test PNB.
- Add 800 ml of Bactec MGIT OADC supplement to each of the tube.
- Add 100 ml of PNB reagent to MGIT tube labeled as PNB.
- Inoculate both the MGIT tubes with 500ml of inoculums.
- Streak 0.1ml of inoculums to blood agar plate for sterility checking.
- Scan and incubate both the tube in BECTEC MGIT 960 instrument through tube entry operation.

(iv) QUALITY CONTROL

M.tuberculosis – ATCC 27294(H37Rv) as a positive control (sensitive to PNB).

M.fortuitum – ATCC 6841 as a negative control (Resistant to PNB).

(V) Interpretation

MTC: A strain was considered sensitive when the tube containing PNB remained negative 2- days after positive result was observed in growth control tube.

MOTT: A strain was considered resistant when the tube containing PNB remained positive , 2- days after positive result was observed in the growth control tube.

(b) .SD BIOLINE TB Ag MPT64 Rapid

Tuberculosis is a highly infectious disease caused by Mycobacterium tuberculosis and potentially fatal disease of human. Biochemical, immunological and molecular biological characterization Mycobacterium tuberculosis has led to the identification of several antigens which may be useful in the development of improved diagnostics methods in order to discriminate between the M. tuberculosis Complex (MTC) and mycobacteria other than M. tuberculosis(MOTT) bacilli .M .tuberculosis has been found to secrete more than 33 different proteins. One of the predominant proteins, MPT64 was found in the culture fluid of only strains of the MTC . SD BIOLINE TB Ag MPT64 Rapid is a rapid immune-chromatographic identification test for the M.tuberculosis complex that use monoclonal anti- MPT64 (16).

(i) PRINCIPLE

This test cassette consists of a sample pad, a gold conjugate pad, a nitrocellulose membrane, and an absorbent pad . Mouse monoclonal anti- MPT64 were immobilized on the nitro cellulose membrane as the capture material (test line).Another antibodies which recognized another epitope of MPT64, conjugate with colloidal gold particles were used for antigen capture and detection in a sandwich type assay (16).

(ii) SPECIMEN PREPARATION:-

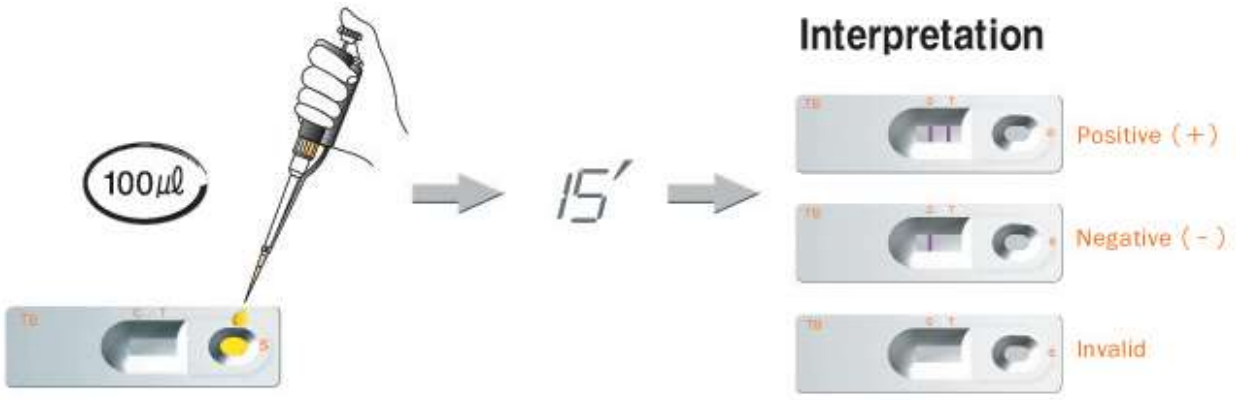
Liquid cultures

- 100microlitre of sample taken from liquid cultures processed by sputum specimen can be applied directly to the sample well without use of the sample preparation procedure.

Solid cultures

- For sample preparation from solid cultures, 3 – 4 colonies should be suspended in 200 microlitre of extraction buffer prior to test.
- If there is condensation fluid of slant agar tubes, 100 microlitre of sample taken from condensation fluid can be applied directly to the sample well.

(iii) INTERPRETATION OF THE RESULT



1. A colour band will appear at left section of the result window to show that the test is working properly. This band is the control band

2. The right section of the result window indicates the test results. If another color band appears at the right section of the result window, this band is the test band.

CHAPTR- 5

Review of literature

Yone *et; al.* (2014) reported that the determinants of sputum culture non-conversion following intensive phase of treatment, and assessed the effects on the outcome among patients treated for a first episode of smear positive tuberculosis (TB). Among patients treated for a first episode of smear positive pulmonary tuberculosis in the Chest service of the Yaounde Jamot Hospital, Cameroon. Logistic regressions models were used to relate baseline characteristics with non-conversion of sputum cultures after the intensive phase of treatment. A total of 953 patients were admitted to the service during the study period, including 97 (10.2%) who had a positive sputum smear at the end of the intensive phase of anti-tuberculosis treatment. Eighty-six patients with persistent of smear positive sputa at the end of intensive phase of TB treatment were included, among whom 46 (53%) had positive sputum culture for *M. tuberculosis.* The absence of haemoptysis were the main determinants of sputum culture non-conversion. The sensitivity and specificity were 78.6% and 55.4% for culture non-conversion after intensive treatment, in predicting anti-TB treatment failure. Failure rate is high among patients with positive sputum culture after intensive treatment, even in the absence of multi-drug resistant bacilli. Treatment should be closely monitored in these patients and susceptibility to anti- tuberculosis drugs tested in the presence of persistent positive smears following the intensive phase of treatment.

Kumar *et al.* (2014) reported that the pulmonary tuberculosis with multi-drug resistant *Mycobacterium tuberculosis* is a major cause of concern in many of the developing countries. The present study was carried out to study the prevalence of multi-drug resistant tuberculosis in clinical isolates at DR.PSIMS & RF general hospital in Gannavaram, which is a tertiary care hospital. Two hundred and fifty seven sputum samples were collected from clinically suspected cases of tuberculosis and subjected to Zeihl-Neelsen stains (ZN) and culture on Lowenstein-Jensen(LJ) medium and 50 cultural isolates were obtained and subjected to economic variant of proportion method for drug susceptibility against Isoniazid (INH) and Rifampicin(RIF). A total of eight (16%) isolates were found to be resistant against INH and one strian (2%) was found to be resistant against both RIF and INH. No strain was found to be resistant against RF alone. The present study revealed the presence of 2-3% of multi-drug resistant M. tuberculosis infection in patients attending DR. PSIMS general hospital. This emphazises the need for strengthening laboratory services for timely diagnosis of MDR TB.

Mycobacterial infections have been shown to be increasing in number worldwide, mainly due a global increase in developing countries, the increased number of patients with HIV infection and AIDS disease worldwide, an increasing number of elderly patients and the emergence of multidrug resistant tuberculosis. Inhalation is the predominant pathway of *M. tuberculosis* infection, making pulmonary tuberculosis the most common form of tuberculosis. Tuberculosis may arise either from a recent infection with M. tuberculosis, or from the reactivation of dormant bacilli, years or decades after initial infection. Extrapulmonary tuberculosis mainly results from

reactivation of a tuberculous focus after hematogenous dissemination or lymphogenous spread from a primary, usually pulmonary focus. Tuberculosis may demonstrate a variety of radiological features depending on the organ site involved and may mimics other pathologies. The final diagnosis of tuberculous disease mainly depends on the detection of the causative organism on histopathological examination, culture and polymerase chain reaction-based assay for mycobacterial DNA on material obtained during bronchoscopic washings, fine needle aspiration cytology (FNAC) or biopsy.

Zumla *et al.* (2013) reported that the availability of a cheap and effective treatment, tuberculosis still accounts for millions of cases of active disease and deaths worldwide. The disease disproportionately affects the poorest persons in both high-income and developing countries. However, recent advances in diagnostics, drugs, and vaccines and enhanced implementation of existing interventions have increased the prospects for improved clinical care and global tuberculosis control.

European Respiratory Journal Feb(2011) Sputum smears and culture conversion are frequently used to evaluate treatment response in pulmonary tuberculosis patients. Limited data are available on the evaluation of the correlation between under-treatment sputum smear results and culture conversion.

This prospective study included sputum culture-proven pulmonary tuberculosis patients at six hospitals in Taiwan. At least two sets of sputum were collected at the completion of 8 weeks of TB treatment. The sensitivities and specificities of 2-month sputum smears were estimated based on culture conversion status.

A total of 371 patients were enrolled for analysis. Factors associated with culture conversion included having a smear positive before treatment, presence of a cavity on radiography, rifampicin resistance and usage of the DOTS (directly observed therapy, short course) strategy. The sensitivities of 2-month sputum smears for culture conversion among all patients, initially smear-positive patients and initially smear-negative patients were 64.3, 71.4 and 38%, respectively, and the specificities were 81.6, 69.9 and 92.8%, respectively. In patients who were 2-month sputum smear-positive, the 2-month culture conversion rate was 80% if the patients were under DOTS and without cavitary lesions in radiograms.

The predictive value of 2-month sputum smears in culture conversion was limited and highly influenced by clinical factors in pulmonary tuberculosis patients.

CHAPTER-6

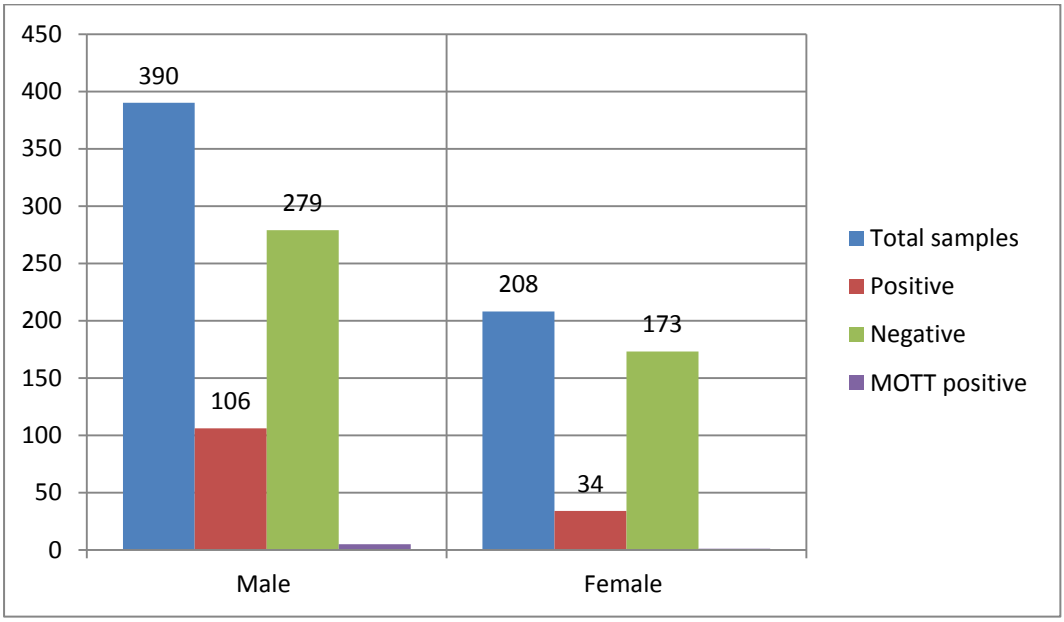
Interpretation of laboratory results

Table 6: Gender wise distribution of laboratory results

Gender	Total samples	Positive	Negative	MOTT positive
Male	390	106	279	05
Female	208	34	173	01

A total of 598 samples were received from 1st January 2016 to 31st march 2016, 390, (65.21%) were collected from male patients and 208 (34.78%) were collected from female patients, in which 140 (23.41%) samples were positive followed by male 106, (75.71%) and female 34 (24.28%). 6 samples were found to be as MOTT positive.

Chart 1: Gender wise distribution of laboratory result1



CHAPTER-7

DISCUSSION

Tuberculosis (TB) is a common and often deadly infectious disease caused by mycobacterium in humans, mainly by *Mycobacterium tuberculosis*. TB is a major air-borne, infectious bacterial disease. It remains a major worldwide health problem with global mortality ranging from 1.6 to 2.2 million lives per year. Direct smear microscopy for acid-fast bacilli (AFB) is rapid, inexpensive, highly specific, and capable of identifying the most infectious cases of TB.

The Gold standard for diagnosing pulmonary TB remains culture. Decontamination of clinical specimens such as sputum is an important and critical step in the isolation of mycobacteria, and the mildest decontamination procedure which provides sufficient control of contaminants is likely to yield best results.

Decontamination and concentration of sputum specimens by modified Petroff's method is one of the most commonly used methods for *M.tuberculosis* culture. *N*-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH) solution is recommended as a gentle but effective digesting and decontaminating agent.

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