IDENTIFICATION OF *MYCOBACTERIUM TUBERCULOSIS* AND CULTURE SENSITIVITY

Submitted in partial fulfilment of the requirements of
Degree of

MASTER OF SCIENCE
(CLINICAL MICROBIOLOGY)

By

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CERTIFICATE

This is to certify that the **Full Term Internship Training** was carried out by **Mr. ASHOK THAKUR** under my direct supervision. This is to further certify that this report embodies the original work carried out by the candidate himself/herself and has not been submitted elsewhere in any form or for any other degree/diploma.

(Dr. Nasib Singh)
Assistant Professor

Date:

Place: Phagwara

DECLARATION

I hereby declare that work embodied in this Full Term Internship Training report was carried out by me under the direct supervision of **Dr. Nasib Singh,** Assistant Professor, Dept. of Paramedical Sciences, Lovely Professional University (Phagwara, Punjab). This work has not been submitted in part or in full in any other university for any degree or diploma.

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ASHOK THAKUR

Contents

List of Abbreviations

MTC	*Mycobacterium tuberculosis*
MOTT	*Mycobacterium* other than tuberculosis
MDR	Multiple drugs resistance
DST	Direct susceptibility test
HIV	Human Immune Deficiency Virus
AIDS	Acquired Immune Deficiency Syndrome
CDC	Centre for Disease Control
WHO	World health organization
INH	Isoniazid
RIF	Rifampin
STR	Streptomycin
EMB	Ethambutol
SLD	Second line drugs
XDR	Extensives drugs resistance
DOT	Direct observed treatment
LJ	Lowenstein Jensen medium
MGIT	Mycobacterium growth indicator tube
PANTA	Polymyxin B, Amphotericin B, Nalidixic acid, Trinetho-Prim, Azlocillin.
NALC	N- acetyl L- cysteine
ZN stain	ZiehlNeelsen
AFB	Acid fast bacilli

Abstract

The following project is an exhibition of the methodology used for analysis of *Mycobacterium tuberculosis* complex. It provides a view to the project analysis – the prime focus was on *Mycobacterium tuberculosis* identification further comprising of drugs pattern.

A random data was collected from January 2015 to April 2015. The research project included 445 patient (Bronchoalveolar lavage fluid) including male and female of different age group. In which 75 patient are following CDC guideline procedure as reported tuberculosis. The report also exhibits anti-tuberculosisdrugs pattern. The project conducted comprised of the respondents of age group between 40-60 years of age affected with *Mycobacterium tuberculosis*. The following research project also demonstrated high incidence of first line anti tuberculin drugs. It's an overview to the case of *Mycobacterium tuberculosis.*

1.1 Introduction

Mycobacterium tuberculosis is slender rod organism that show sometime separate filamentous form similar to fungus mycelium. It is straight or slightly curved rod, about 3×0.3 µm in size. Occurring singly, pair or as small clumps. The size depends on environment condition of growth. In liquid media they form mold-like pellicle, slow growing. Hence the name *Mycobacterium* meaning fungus like bacteria. They are aerobic, non-motile and non-capsulated. It is obligate aerobes, opportunistic pathogen and saprophytes. *M. tuberculosis* has been present in the human population from thousands of years; fragments of the spinal column from Egyptian mummies from 2400 BCE show definite pathological signs of tubercular decay. Called "consumption," tuberculosis was recognized as the leading cause of mortality by 1650. Using a new staining technique, Robert Koch identified the bacterium responsible for causing consumption in 1882. The production of antibiotics in the 1940's allowed physicians to begin effectively treating patients, leading to huge drops in the death rate of the disease. Tuberculosis is still a major cause of mortality in young as well as adults worldwide, but is less of a problem in developed countries.

1.2 General characteristics

Mycobacterium tuberculosis is a non-motile, acid-fast, obligate aerobes. These bacilli are 2-4 µm in length and having very slow generation time of between 15-20 h. Colonies appear after 2 weeks,it's may takes longs as up to 8 weeks also. Optimum temperature is 37℃ and growth does not occur below 25℃ or above 40℃. Optimum pH 6.4-7.0. The cell wall of *Mycobacterium* is unique it is compose of three major components, mycolic acid, cord factor and wax-D.

Mycolic acid: These are unique alpha-branched lipid found in cell wall of *Mycobacterium* and *Corynebacterium.* They make up 50% of dry weight of the *Mycobacterium* cell envelope. Mycolic acid is strong hydrophobic molecule that form lipid shell around the organism and effect permeability properties at the cell surface. Mycolic acid are thought to be a significant determinant of virulence in MTB. Probably, they prevent attack of the *Mycobacterium* by cationic protein, lysozyme and oxygen radical in the phagocytic granules. They also protect extracellular *Mycobacterium* from component deposition in serum.

Cord factor :is toxic for mammalian cell and is inhibitor of neutrophil migration and also abundantly produce in virulent strains of *Mycobacterium tuberculosis*.

Wax-D :In the cell envelop is the major component of freund's complete adjuvant(CFA)

The high concentration of lipid in the cell wall of *Mycobacterium tuberculosis* have been associated with these properties of the bacterium-

a. Impermeabilities of stains and dyes
b. Resistance of many antibiotic

c. Resistance to killing by acidic and alkaline component
d. Resistance to lethal oxidation and survive inside macrophage

Strategies for survival and multiplication inside the host cell.

a. The inhibition of phagosome- lysosome fusion
b. The inhibition of phagosome inhibition
c. The recruitment and of tryptophan asparted containing coat protein phagosome to prevent their delivery to lysosome and
d. The expression of membrane of the host-induce rapititive glycine – rich family of protein.

1.3 Encounter/Entry

Tuberculosis is mainly transmitted through inhalation of aerosols containing tuberculin bacilli. The require inoculum size for infection is usually high but easily occur with the patient who is currently infected. The product of dried aerosols, dried nuclei are particularly infectious because they remain in air for long time and upon inhalation easily move to alveoli. They severe damage related to infection is caused by reaction of host. The tuberculosis infection has two phases, primary and secondary.

1.3.1 Primary infection

Primary infections initials infectious stages of host usually mild and asymptomatic. A healthy person recently infected show flu like symptoms has no reason to suspect tuberculosis. Left untreated, the bacilli infect and multiple within pulmonary alveolar macrophages, migrating to hilar lymph node. This occur about 2-3 week from the time of infection and associated with the development of tuberculin hypersensitivity, a person may show positive tuberculin test with chest x-ray show opacities in lungs. The primary lesion may enlarge and cause military, meningeal or other forms of disseminated tuberculosis.

1.3.2 Secondary infections

The secondary type of infection is due to reactivation of latent infection or those who has weakened immune system rapidly progress to secondary infection. While in healthy and untreated person this disease latent for many year. In some people, may never develop in tuberculosis disease. Its affect mainly upper lobes of lungs, the lesions undergoing necrosis and tissue destruction, leading cavitation.

1.4.Pathogenicity of *Mycobacterium tuberculosis*

Mode of infection

Apart from congenital affection, the infection is commonly by way of ingestion and inoculation into skin (e.g. lupus vulgaris).

1.4.1 Virulence- 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.

1.4.2 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.

1.4.3 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.

1.5 Types of tuberculosis:

They are categorized as **pulmonary tuberculosis** and **extra pulmonary tuberculosis.**
In case of pulmonary tuberculosis mainly affect the lungs. People may cough blood in small amounts, and in very rare cases, the infection may spread into the pulmonary artery, resulting in bleeding.
But in case of extra pulmonary tuberculosis affective sites includes the pleura (in tuberculosis pleurisy), the central nervous system (in tuberculosis meningitis), and the bones and joints.
While it spreads to the bones; it is also known as “osseous tuberculosis” a forms of

osteomyelitis. Sometimes, bursting of a tubercular abscess through skin results in tuberculosis ulcer.

1.6 Symptoms

a) A bad cough that last 3 weeks or longer
b) Pain in chest
c) Cough with blood
d) Weakness or fatigue
e) No appetite
f) Chills
g) Fever
h) Sweating at night.

1.7 Risk factor

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.

a. Infection with HIV the virus that cause AIDS and weakness the immune system.
b. Diabetes mellitus
c. Head and neck leukemia or Hodgkin's disease
d. Some medical treatments, including used corticosteroids or certainmedications used for autoimmune or vasculitis disease such as rheumatoid arthritis or lupus, which suppress the immune system.

1.8 Transmission

Generally, People with active pulmonary tuberculosis while cough, sneeze, speak, sing, or spit, they releasing infectious aerosol droplets 0.5 to 5.0 μm in diameter. Once sneeze can release up to 40,000 droplets. Each one of these droplets transmit the disease. The inhalation of fewer than 10 bacteria may cause an infection. 22% infection rate estimated people those who prolonged,

frequent or close contact with TB are at particularly high risk.

Following are the factor which helps to transmission of tuberculosis.

a) Number of infective droplets expelled by the carrier :-The number of organisms being expelled into the air.
b) The effectiveness of ventilation:- The concentration of organisms in the air determined by the volume of the space and its ventilation.
c) The duration of exposure:- The length of time an exposed person breathes the contaminated air.
d) The virulence strain of *Mycobacterium tuberculosis* strain.
e) The level of immunity in the uninfected person.

Review of literature

Gupta et al (2004) studies on simultaneous resistance of ethambutol and isoniazid in clinical isolation of *Mycobacterium tuberculosis.*

Background and objective: There exist a critical need to understand the nature of drugs resistance patterns and predictors of emergence of drugs resistance regarding *Mycobacterium tuberculosis.* Isoniazid and ethambutol exhibiting drugs resistance acting on cell wall. The conduct study analysis the anti-mycobacterium susceptibility pattern of *Mycobacterium tuberculosis* and to measure the variation of ethambutol and isoniazid resistance.

It seems to be showing a global prevalence of 32% infection of *Mycobacterium tuberculosis* in 22 countries contributed to 80% of all incidence belonging to five south Asian countries like India, Indonesia, Bangladesh, Philippines, and Vietnam. It isestimated that 30% of global tb patients live in India.

To treat opportunistic infection of AIDS patient as well as of tb, ethmbutol is used successfully in multidrug therapy. Resistance to ethambutol reported to be 0-4.2 percent as global prevalence. During ethambutol susceptibility testing micro colonies did not show resistance. Better understanding of clinical response of drugs resistance of *Mycobacterium tuberculosis* isolated requires analysis with focus on achievable plasma concentration.

Methods: A test comprisisng of 380 *Mycobacterium tuberculosis* isolates for the susceptibilities to ethambutol at 2,4,6 µg/ml, isoniazid at 1µg/ml and rifampicin at 64 µg/ml using MIC method, were performed.

Result: resistance to ethambutol at concertration of 2,4,6 µg/ml happened to be 44.21,24.73 and 14.21 percent.

85.18% of Ethambutol resistance showed resistance to isoniazid too. With the same ethambutol concentration, isoniazid resistance isolated happened to be resistant to ethambutol at a fraction of 28.75%.

Interpretation and conclusion: Large percent of isolates demonstrated ethambutol resistance along with isoniazid resistance or the other hand ,ethambutol displayed little multidrugs resistance

Esra et al 2014 study that the resistance to first-line drugs and its pattern:

Isoniazid (H), rifampicin (R), ethambutol (E) and streptomycin (S) among pulmonary tuberculosis patients.

Methods: Strains were obtained from 1584 culture positive pulmonary tuberculosis patients.

All specimens were inoculated into Lowenstein-Jensen media (LJ); drugs susceptibilities test (DST) were performed for first-line drugs.

Results: Multidrug resistance (MDR) were detected in 146 (9.2%) isolates. Three hundred (18.9%) isolates were resistant to H; 220 (13.9%) to R; 168 (10.6%) to S; 137 (8.6%) to E

Any drug resistance was detected in 442 (27.9%) isolates.

MDR rate was higher in male patients than females (P = 0.006). MDR rates were different according to the age groups (P = 0.02). The highest rate was in 35 - 44 years and the lowest rate was in 15 - 24 years. Conclusions: We found an association between middle age and male gender and MDR tuberculosis.

Ikeda *et al* 1968 reported that flexible bronchofibroscope has become very useful tool in patient care and medical research. Proper selection of instrument is necessary to ensure effective and safe procedure. Ability to collect BAL provides a role for flexible bronchoscope in research. The insignificant difference in the clinical presentations, X-ray and CT findings in our study suggest that though the signs and symptoms, and radiographic findings provide important clue for pulmonary tuberculosis, they cannot confirm the diagnosis of pulmonary tuberculosis. Acid fast stain positivity and culture isolation can only provide the definitive diagnosis. Therefore, patients with radiographic and clinical findings compatible with PTB but sputum smear negative are a challenge for the physician - as to start ATT or not. It has been reported that 74% of these patients develop active tuberculosis in five years if not treated. Flexible fiberoptic bronchoscopy is considered as a safe diagnostic and interventional tool, even in young or extremely premature infants. Caminero *et al*, concluded that bronchoscopy should be conducted on all patients without expectoration and negative sputum bacilloscopy and that BAL performance should be a routine procedure as it is simple and usually uncomplicated technique. Among various bronchoscopic specimens, BAL is considered best for diagnosis of TB.

Lopez et al (2015) study that Non-tuberculous mycobacteria (NTM) are a large family of acid-fast bacteria, widespread in the environment. In children, NTM cause lymphadenitis, skin and soft tissue infections, and occasionally also lung disease and disseminated infections. These manifestations can be indistinguishable from tuberculosis on the basis of clinical and radiological findings and tuberculin skin testing. A diagnostic and therapeutic problem for respiratory physicians and other clinicians is therefore evident, particularly in settings where childhood tuberculosis is common, and bacteriological confirmation of any mycobacterial disease is difficult because of low availability of laboratory services in low-resource settings and the inherent paucibacillary nature of mycobacterial disease in childhood. The epidemiology of NTM varies by world region, and attempts to understand the burden of NTM disease and to identify risk factors in the paediatric population are hampered by inadequate mandatory NTM reporting and the overlap of clinical presentation with tuberculosis. The immune response to both NTM and Mycobacterium tuberculosis is based on cellular immunity and relies on the type-1 cytokine pathway. The disruption of this immune response by genetic or acquired mechanisms, such as Mendelian susceptibility to mycobacterial disease or HIV, might result in predisposition to mycobacterial infections. Published diagnostic and management guidelines do not provide specific advice for diagnosis of NTM in children, from whom the quantity and quality of diagnostic samples are often suboptimum. Treatment of NTM infections is very different from the treatment of tuberculosis, depends on the strain and anatomical site of infection, and often involves antibiotic combinations, surgery, or both.

Salim et al (2015) study that Bacteriological diagnosis of tuberculosis has benefited in recent years from many technological advances to improve rapidity and sensitivity of the techniques. Thus, new LED fluorescence microscopes are in the process of replacing the optical microscopes

and the Ziehl-Neelsen technique, making the examination more precise, faster and easier. The manual and automatic liquid culture has improved Lowenstein-Jensen culture and helped shorten antibiotic sensitivity test, allowing appropriate management of patients. The development and standardization of molecular biology methods led to the rapid detection and identification of mycobacterium directly in clinical samples but also of resistance genes for early diagnosis of MDR-TB and dealing with them quickly. However, the performance of these techniques does not sufficiently cover the diagnosis of smear-negative tuberculosis, extrapulmonary forms, children- and immune-compromised tuberculosis where sensitivity is limited. The diagnosis of latent tuberculosis is reinforced by the in vitro release testing of gamma interferon overcoming the lack of specificity of the tuberculin skin test. Despite considerable progress, more amelioration is still needed to improve these techniques in order to extend them to the paucibacillary tuberculosis and to facilitate their access to low-resource countries.

Moraes et al (2015) reported that Tuberculosis (TB) is the second leading cause of human mortality from infectious diseases worldwide. The WHO reported 1.3 million deaths and 8.6 million new cases of TB in 2012. Mycobacterium tuberculosis (M. tuberculosis), the infectious bacteria that causes TB, is encapsulated by a thick and robust cell wall. The innermost segment of the cell wall is comprised of peptidoglycan, a layer that is required for survival and growth of the pathogen. Enzymes that catalyse biosynthesis of the peptidoglycan are essential and are therefore attractive targets for discovery of novel antibiotics as humans lack similar enzymes making it possible to selectively target bacteria only. In this paper, we have reviewed the structures and functions of enzymes GlmS, GlmM, GlmU, MurA, MurB, MurC, MurD, MurE and MurF from M. tuberculosis that are involved in peptidoglycan biosynthesis. In addition, we report homology modelled 3D structures of those key enzymes from M. tuberculosis of which the structures are still unknown. We demonstrated that natural substrates can be successfully docked into the active sites of the GlmS and GlmU respectively. It is therefore expected that the models and the data provided herein will facilitate translational research to develop new drugs to treat TB.

Gladwin et al (1998) the relatively new Mycobacterium tuberculosis direct test (MTDT) enzymatically amplifies M tuberculosis complex 16s ribosomal RNA. The sensitivity of the test ranges from 75 to 100%, with specificity of 95 to 100%, positive predictive value between 78% and 100%, and negative predictive value between 95% and 100%. Similar test characteristics have been documented in non-respiratory specimens and in specimens that ultimately grow nontuberculous mycobacterium (NTM). This test allows for rapid identification of M tuberculosis in the smear-positive patient and may greatly improve sensitivity over acid-fast bacilli smear alone. A negative test result with a positive smear suggests infection with NTM or Mycobacterium avium complex. We present a case that illustrates the value of MTDT for analysis of tissue specimens in immunocompromised patients with suspected mycobacterial disease and review the rapidly developing literature about this test. We propose an algorithm using MTDT, acid-fast smear, and mycobacterial culture for the diagnosis and treatment of the immunocompromised patient with suspected mycobacterial infection.

Marta et al (2014) study that Drug resistance is considered one of the main threats for tuberculosis control. Our aim was to identify risk factors for drug resistance in tuberculosis patients in the Northern Portugal.

Study Design and Methods: Retrospective case-control study. The medical records and drug susceptibility test data from TB patients diagnosed between 31 March 2009 and 1 April 2010 were examined. We enrolled 119 patients with any drug resistance to first line anti-TB drugs and 238 with drug-susceptible TB, matched by age group. Variables analyzed included: gender, country of origin, employment situation, site of disease, previous treatment, presence of diabetes mellitus, HIV infection, alcohol abuse, intravenous drug use, abuse of other drugs and smoking habits. Multivariate conditional logistic regression was used to identify independent predictors for drug-resistant.

TB. Results: Diabetes mellitus [adjusted odds ratio (OR): 3.54; 95% CI: 1.45 - 8.66], intravenous drug use (OR: 4.77; 95% CI: 1.24 - 18.32) and previous TB treatment (OR: 2.48; 95% CI:

1.12 - 5.49) were found to be risk factors for drug-resistant disease development. Conclusions:

Diabetes mellitus, prior tuberculosis treatment, and intravenous drug use were risk factors for

Drug-resistant disease. The association between diabetes and drug-resistant TB should be further explored. Identifying clinical predictors of drug resistance can allow prompt identification of patients at risk for drug-resistant TB.

5. Materials and methods:

This is based on fluorescent technique

a. Biological safety cabinet
b. 1000 µl adjustable pipettes
c. Sterile aerosol pipettes tips
d. Sterile phosphate buffer, sterile distilled water, 2.9% sodium citrate, 4% NaOH and Nalc.

5.1 MGIT

Thiscontain 110microleter of fluorescent indicator and 7ml of broth. The indicator contain **Tris 4, 7-diphenyl-1, 10-phenanthroline ruthenium chloride pentahydrate in a silicon rubber base.** The tubes are flushed with 10% CO2 and capped with polypropylene caps.

BACTEC MGIT 960 supplement (OADC) with the following active following ingredients; **oleic acid, bovine albumin, dextrose, catalase and polyoxyethylene**stearate.

MGIT PANTA antibiotic mixture. Each vial contain of antimicrobial agents. PolymyxinB, Trimethoprim, Amphotericin B, Azlocillin, Nalidixic acid.

5.1.1 Principle:

A florescent compound is embedded in silicon on the bottom of 16x100mm round bottom 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 compound and little florescence can be detected. Later, actively respiring microorganism consume the oxygen and allow the fluorescence to detect.

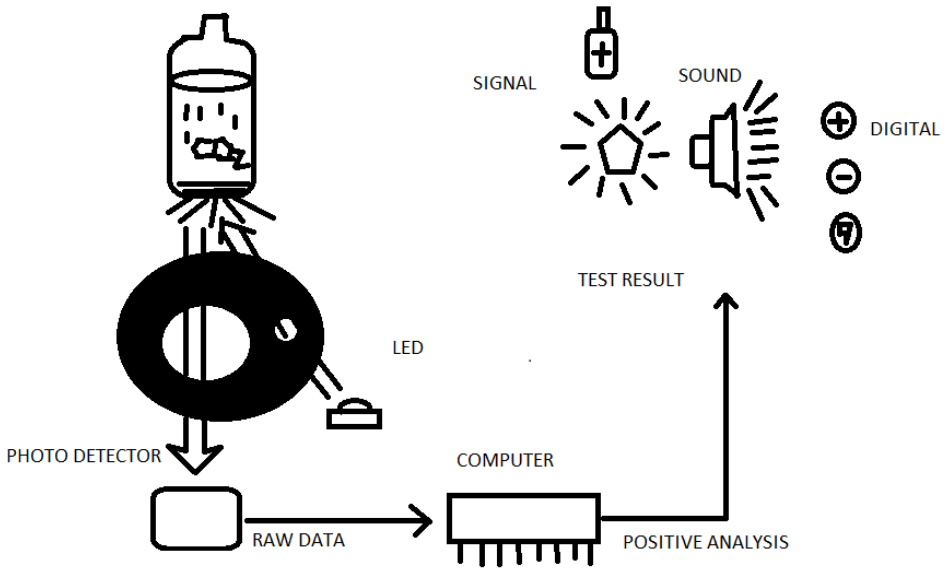

Fig 1.1 show working principle of MGIT 960

Tube entered into the BACTEC MGIT instrument are continuously incubated at 37℃ and monitored every 60 min for increasing fluorescence. Analysis of the fluorescence is used to determine if the tube is instrument positive; i.e. the test sample contains viable organisms. An instrument positive tube contains approximately 1000000 to10000000 colony forming units per mL (CFU/mL). Culture vials which remain negative for a minimum of 42 days (up to 56 days) and which shows no visible signs of positivity are removed from the instrument as negatives and sterilized before discarding.

The BACTEC MGIT Growth Supplement is added to each MGIT tube to make available substances essential for the rapid growth of mycobacteria. Oleic acid is utilized by the tubercle bacteria and plays an important role in the metabolism of *Mycobacteria.* Albumin acts as a protective agent by binding free fatty acids which may be toxic to *Mycobacterium* species, thereby enhancing their recovery. Dextrose is an energy source. Catalase destroys toxic peroxidase that may be present in the medium.

Contamination is reduced when supplementing the BBL MGIT broth base with BACTEC MGIT Growth Supplement/BBL MGIT PANTA antibiotic mixture prior to inoculation with a clinical specimen.

Following are the specimen from the different body site are processed

a. **Sputum**- sputum should be processed using modified petroff method.
b. **Gastric aspirate**- specimen should be decontaminate as sputum.
c. **Tissue**- tissue is processed as recommended by CDC public health microbiology.
d. **Body fluid**- (CSF, pleural fluid, BAL, synovial fluids.

If the volume of fluids is more than 10 ml centrifuge at 3000 rpm and discard supernatant part.

Fig 1.2 patient sample

NALC, NaOH, sodium citrate, buffer mixture is mixed with sample and wait for 15 minute.

For all specimen processing methods, a phosphate buffer solution (PH 6.8) should be used to QS (quality standard) the sample of decontamination mixture to 50 ml prior to centrifugation.

Re-suspension of pellet must be done using a fresh preparation of phosphate buffer solution (PH 6.8).

5.1.2 Procedure:

Material provide MGIT 960 suspension of pellet must be done using a fresh preparation of phosphate buffer solution (PH 6.8).

Decontaminate and digestion

Process specimen using NALC- NaoH method

1. Specimen is transferred to a 50 ml plastic centrifuge tube. Nalc is added in equal volume of sodium citrate and NaoH solution and mixed well.
2. Specimen is allowed to stand in room temperaturefo 15 minute.
3. Tube is filled up to 50 ml mark with sterile phosphate buffer, PH 6.8.
4. It is centrifuged at 4100 rpm in cooling centrifuged machine for 30 minute and supernatant is discarded.
5. Supernatant fluid is discarded carefully from pellet.
6. Pallet is re-suspend with 1-2 ml sterile phosphate buffer. And
7. Acid- fast smear is made.

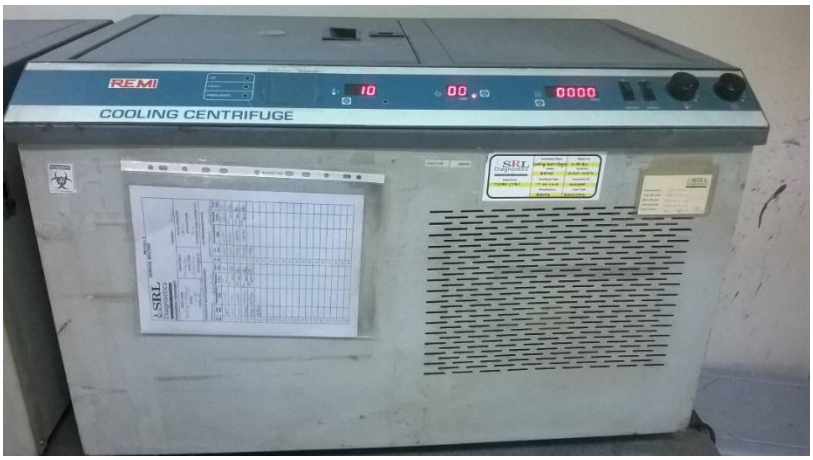

Fig 1.3 cooling centrifuge

5.2 ZiehlNeelsen Stain

5.2.1 Principle

Acid fast bacilli are difficult to stain due to 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 experience to the decolorizing agent, acid alcohol. This resistance to decolorizing by acid alcohol is required for an organism to be termed acid fast. A counterstain is employed to highlight the stained organisms for the microscopes' recognition. There are several methods of determining the acid fast nature of an organism. In the carbolfuchsin procedures (ZiehilNeelsen), acid fast organisms appear red.

5.2.2 Procedure of ZiehilNeelsen method

i. Prepared an air-dried heat-fixed smear.
ii. Slide is covered with concentrated carbol fuchsine.
iii. After the slide is heated till steaming. Should not boiled.
iv. Waiting for 10 minutes.
v. It is washed with tap water.
vi. The slide is covered with acid-alcohol - rock gently and drained.
vii. Then cover the slide with acid-alcohol and stand for 2 minutes.
viii. Washed in tap water, if stain is still evident in the washing, repeat 6 & 7.
ix. Counterstain with methylene blue for 2 minute.
x. Wash in tap water, drained and air dry.
xi. Examine under 100-X oil immersion

5.2.3Smear examination

i. Examine carbolfuchsin stained smears with a 100x oil immersion objective.
ii. Thoroughly examine each slide for the presence of acid fast bacilli, regardless of the time required to complete the task. Mycobacteriologists recommend that a minimum of 100 fields be examined before a smear is reported as negative.

5.2.4Morphological characteristics

i. Acid fast bacilli are approximately 1 to 10 mm long and typically appear as slender, rod shaped bacilli, but they may appear curved or bent.
ii. Individual bacteria may display heavily stained areas referred to as beads and areas of alternating stain producing a banded appearance.
iii. Some Mycobacteria other than *M. tuberculosis* may appear pleomorphic, ranging in appearance from long rods to coccoid forms, with more uniform distribution of staining properties.

5.2.5Result

i. Acid fast organism- Bright red bacilli on blue back ground.
ii. Other organism- Dark blue

5.2.6Reporting pattern

Table 1.1 showing grading according to bacteria seen in microscope.

Examination	Result	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

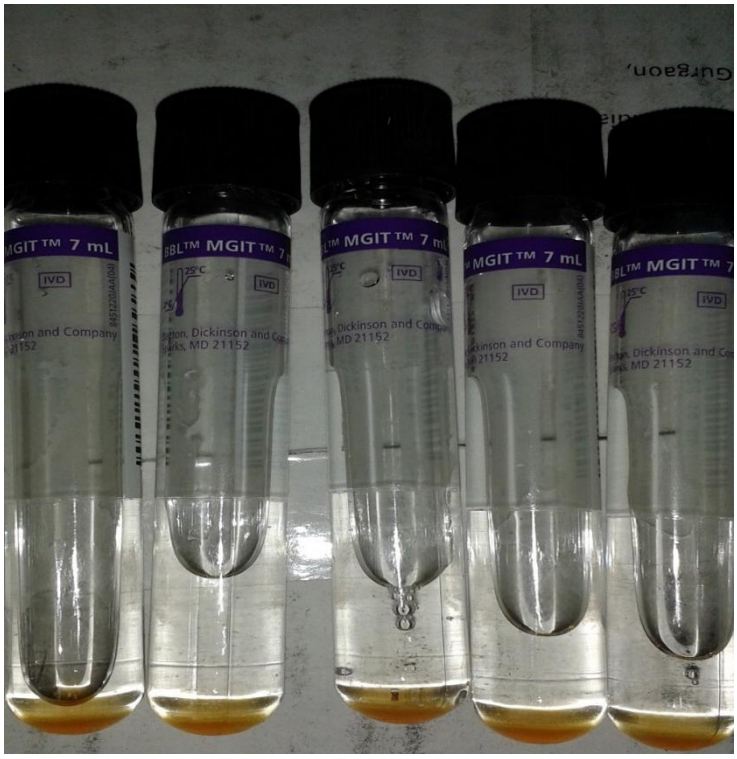

Fig 1.4 MGIT tube

5.3 Inoculation of MGIT tube

BBL MGIT 7ml Tube must be used with a BACTEC MGIT instrument.
MGIT 960 Growth Supplement: Provides mycobacterial growth
Supplements. Oleic Acid: important in mycobacterial metabolism
Albumin (**bovine**): binds free fatty acids, which may be toxic to mycobacteria.
Dextrose: energy source
Catalase: destroys toxic peroxides
Polyoxyethylene stearate (POES): enhances growth of *M. Tuberculosis* and assists in providing a uniform inoculum.

a. A lyophilized vial of BBL MGIT PANTA antibiotic mixture with 15ml of BACTEC MGIT PANTA growth supplement is reconstituted.
b. The MGIT Tube is labelled with the specimen number
c. Unscrew the cap and aseptically 0.8ml of growth supplement/ MGIT PANTA is added antibiotic mixture is made just prior to specimen inoculation
d. Tube entered into the instrument is automatically tested for duration of the recommended 42 days testing protocol.

Positive tube. Identified by the BACTEC MGIT instrument is tosubcultured and an acid- fast smear is prepared.

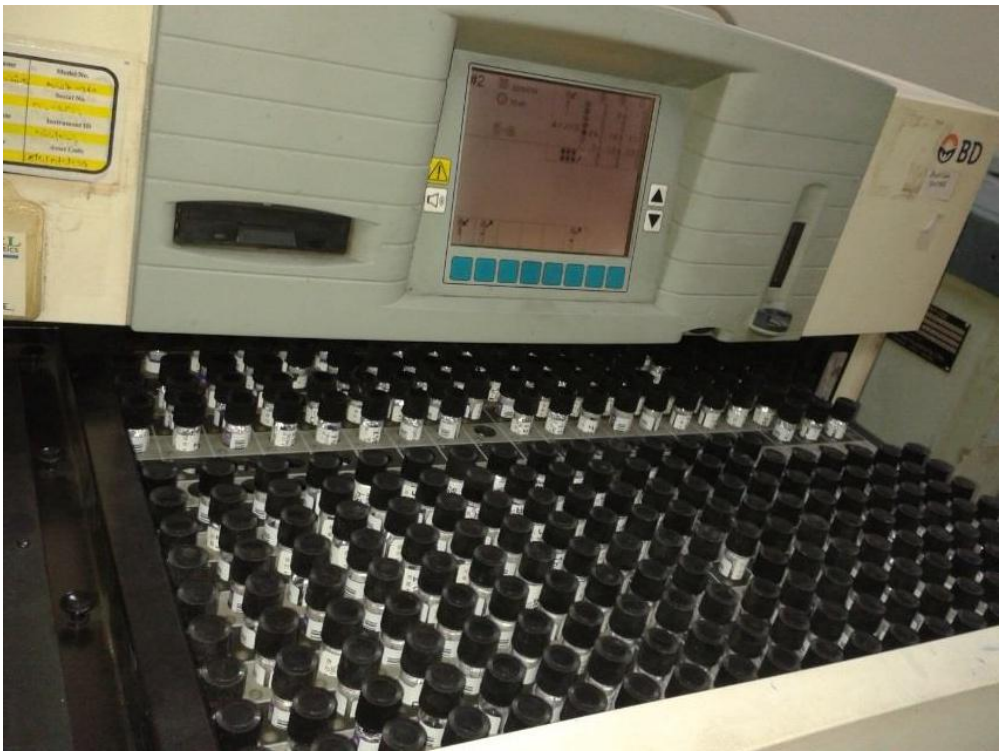

Fig 1.5 BACTEC MGIT device

Add 0.5ml of the concentrated specimen suspension prepared above. Also add a drop of 0.1ml of specimen to L J medium. And incubate it at 37 degree Celsius.

Fig 1.6 showing LJ media in incubator

Tightly recap the tube and mixed well. Being slowing growing colony may come 2-8 weeks looks like Buff, rough.

5.3.1 Processing of the positive tube

1st of all here to check the contamination. So, stick in the 5% sheep blood agar.

If no growth, then there may be chances of mycobacterium species and are conformed by an acid- fast smear prepared by same positive MGIT tube.

AFB then there may be chances of mycobacterium species and are conformed by an acid- fast smear prepared by same positive MGIT tube.

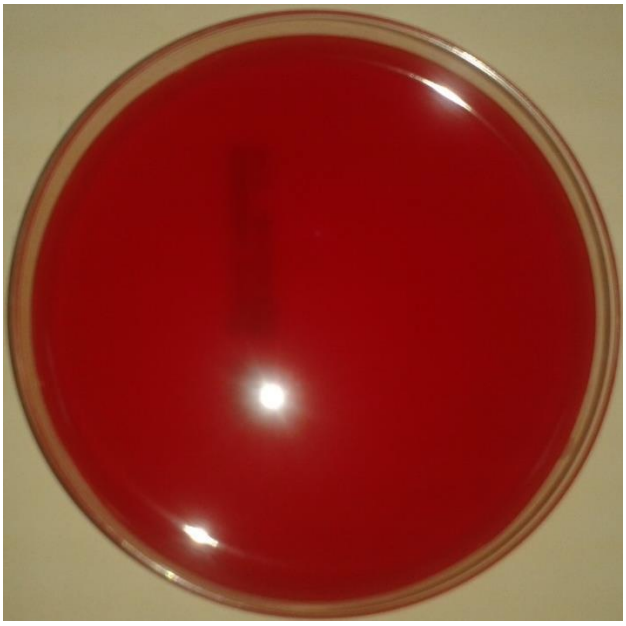

Fig 1.7 showing blood agar plate

Here is neededto be identified MTC (*mycobacterium tuberculosis* complex) and MOTT (*Mycobacterium* other than *tuberculosis). Mycobacterium tuberculosis* looks like serpentile cord.

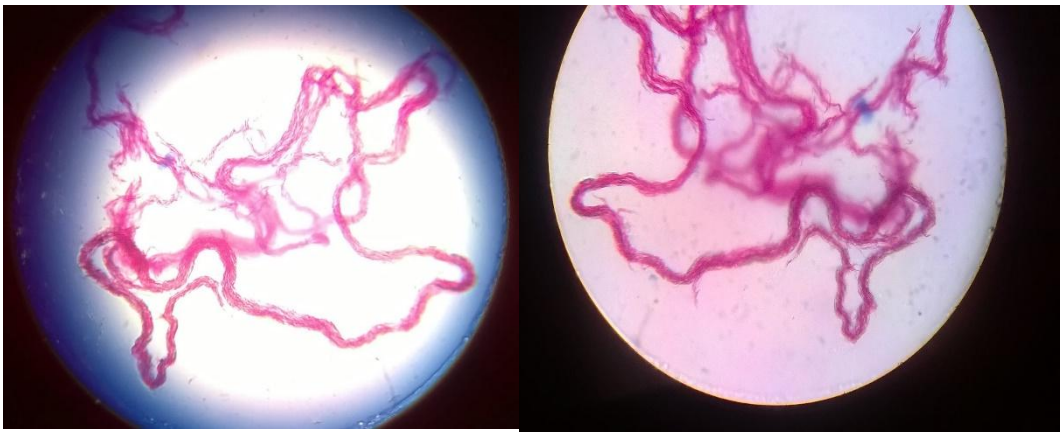

Fig 1.8 MTC in serpentile cord.

5.4SD BIOTINE TB ANTIGEN MPT64 RAPID CARD

5.4.1 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 nitrocellulose 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 is sandwich type assay.

SD BIOTINE TB ANTIGEN Rapid test device has a letter of T and c as “Test line “and “control line” in result window are not visible before applying any sample. The “control line” is use for procedural control. Control line should always appear if the test procedure is performed properly and the test reagent of control line are working. As the test sample applied in sample well flow laterally through the member, the antibody-colloidal gold conjugate bind to the MPT64 antigen in the sample, liquid media. The complex then flows further and bind to the mouse monoclonal anti-MPT64 on the solid phase in the test line, producing red to purple color band. In the absence of MPT64, there is no line in the test band region.

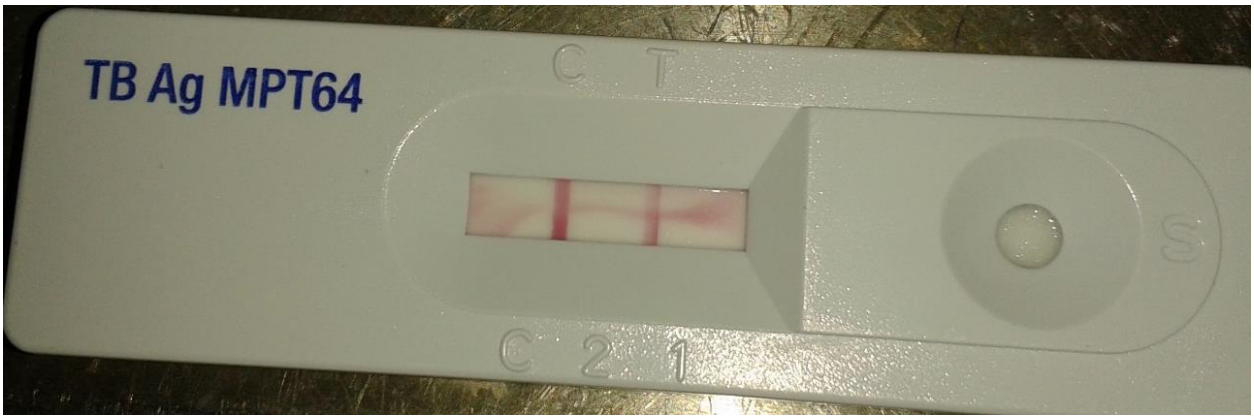

Fig 1.9 SD BIOTINE TB ANTIGEN rapid test device.

5.4.2 Procedure of test

I. The test device is removed from the foil pouch, and placed it on a flat, dry surface.
II. 100 µl of liquid culture is added (or 100 µl of suspended solid culture in buffer) into the sample well.
III. As the test begins to work, purple color is seen more across the result window in the center of the test device.
IV. The result is interpreted in 15 minutes after sample application.

5.4.3 Interpretation of the test

I. A color band will appear at left section of the result windows to show that the test is working properly. This band is the control band.
II. The right section of the windows indicates the test results. If another color band appear at the right section of the result window, this band is the test band.

Negative result- the presence of only control band ("C" band) with the result window indicates a negative result.

Positive result- the presence of two color bands ("T" band and "C" band) within the result window, no matter which band appear first, indicates a positive result.

Note – depending on the MPT64 antigen concentration, the intensity of test line may vary. A positive result will not change once it has established at 15 minutes.

Invalid result- if the control band not the visible within the result window after performing the test, the result is considered invalid. The direction may not have been followed correctly or the test may have deteriorated. It is recommended that the specimen should be re- tested.

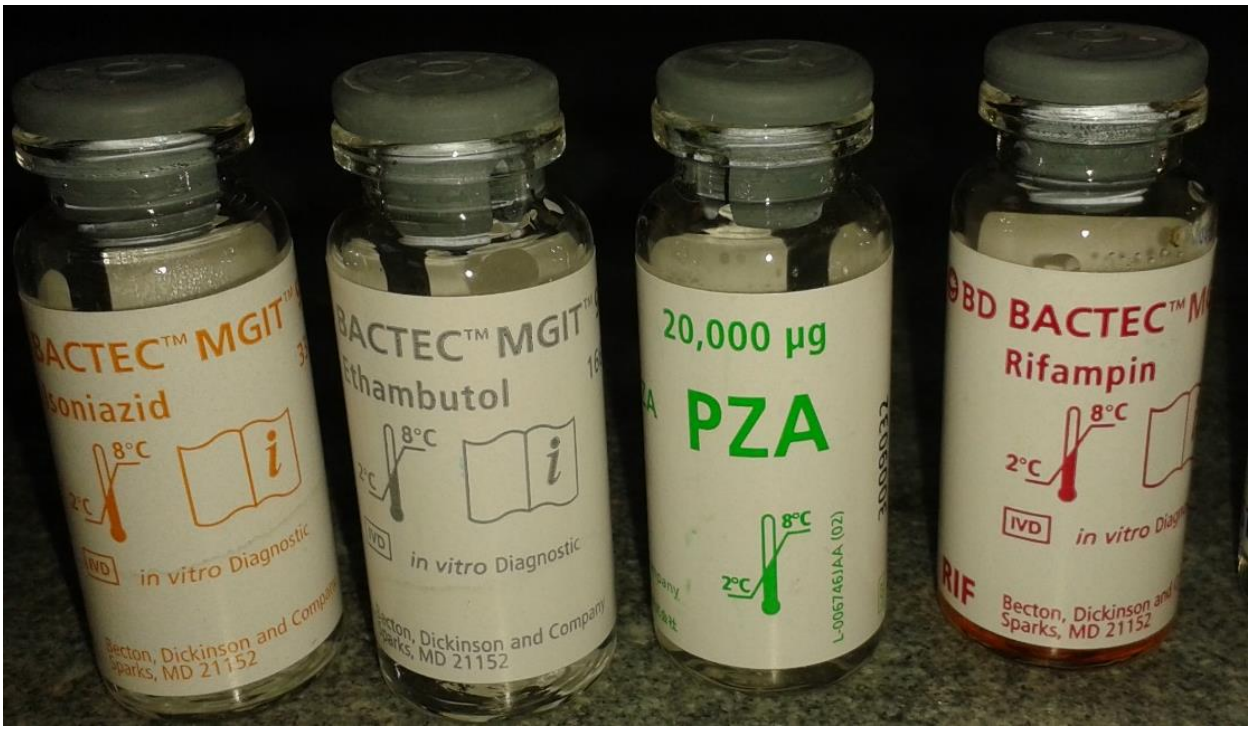

Fig 1.10 Different types of antibiotics

5.5Different generation of anti- tuberculin drugs

1st line dugs- Isoniazid, Rifampin, Pyrazinamide, Ethambutol.

2nd line drugs- Streptomycin, Kanamycin, Amikacin, Capreomycin, Ofloxacin, Levofloxacin, Ciprofloxacin Para amino salicylic acid.

3rd line drugs -Amoxicillin plus clavulanate, clarithromycin, Linezolid.

Results

Total number of sample is 450 among of them 75 patient are reported as positive including *Mycobacterium tuberculosis* and *Mycobacterium* other than *tuberculosis*. Where 18 are male reported as *Mycobacterium tuberculosis* and 11 are *Mycobacterium tuberculosis* in case of female remaining are *Mycobacterium* other than *tuberculosis*. Mostly the high frequent evidence of number of positive case found at the age group of 40-70 in both cases of male and female in which 13 are male and in female it shows 4.

2nd highest case is seen at the age group of 20-40 in which 9 and in case of male and female, the age group 10-20 it shows 3.

Multidrugs resistance (MDR) is well defined as resistance to minimum isoniazid and rifampin. Other case were characterized as follow: drug sensitive-susceptibity to all drugs confirmed mono resistance – resistance to only one drug; polyresistance – resistance to two drugs excluding the Isoniazid and Rifampin combination. The high prevalence for resistance of anti- tuberculosis drugs is 1st line drugs specially isoniazid and rifampin enhance 44% and 76% sensitive, and 56% and 24% resistance respectively. The most effective drugs are Capromycin, kanamycin, Amikacin, Ciprofloxacin, Ethionamide are 96%, 96%, 96%, 96%, 96% sensitive and 4%, 4%, 4%, 4%, 4% resistance respectively.

Table no 6.1 Data of positive and negative sample

Total sample	445
Positive	75
Negative	370

Total number of sample is 445 in which 29are positive and 416 are negative.

Table no 6.2*Mycobacterium tuberculosis* and *Mycobacterium* other than *tuberculosis* including male and female.

	Male		female	
Age	*Mycobacterium tuberculosis*	*Mycobacterium* other Than *Tuberculosis*	*Mycobacterium tuberculosis*	*Mycobacterium* other Than *Tuberculosis*
0-10	1	2	2	0
10-20	2	1	1	0
20-30	9	3	4	5
30-40	5	1	4	1

40-50	10	4	6	3
50-60	7	1	0	2
60-70	1	0	0	0

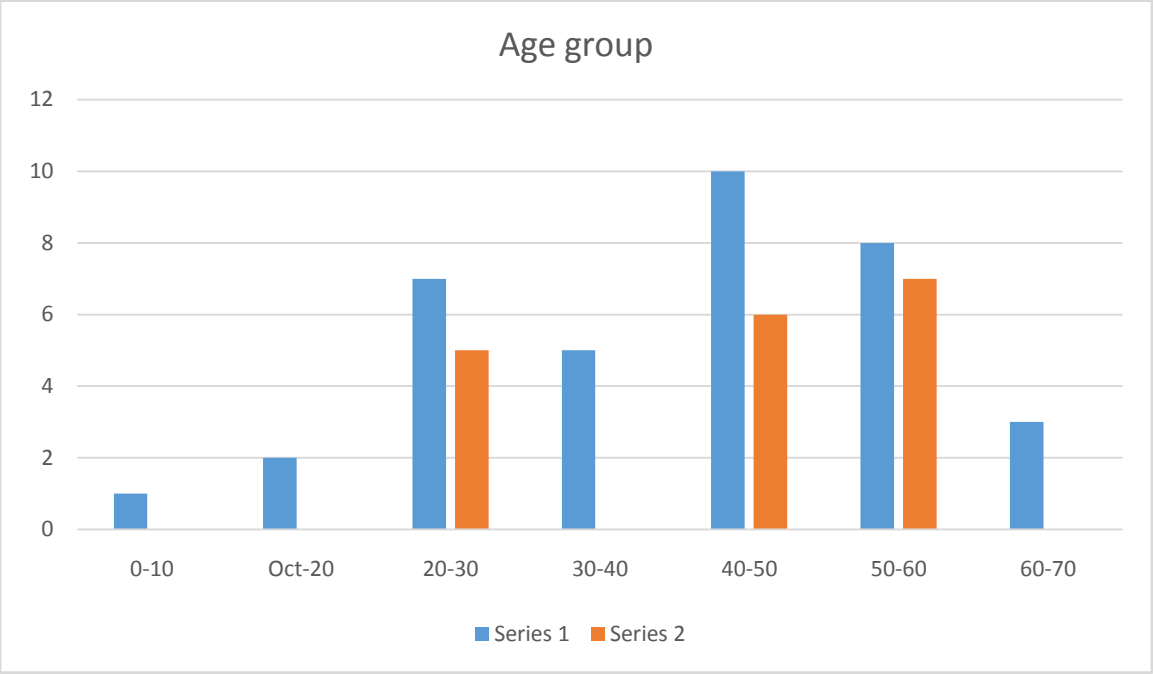

Fig 6.1Show comparison of*Mycobacterium tuberculosis*and*Mycobacterium* other than *tuberculosis* isolates.

The table show that high frequent number of evidences of *mycobacteriumtuberculosis* I at age group 40-50 in both male and female. Least frequent numberof evidence shows at age group 0-10 in both male and female.

Table No. 6.3 the given data show Infection with *Mycobacterium tuberculosis* and other than *Mycobacterium tuberculosis* isolated particularly on the basis of age in Male

	Male	
Age	*Mycobacterium tuberculosis* (MTC)	*Mycobacterium* other than *tuberculosis* (MOTT)

0-10	1	2
10-20	2	1
20-30	9	3
30-40	5	1
40-50	10	4
50-60	7	1
60-70	1	0

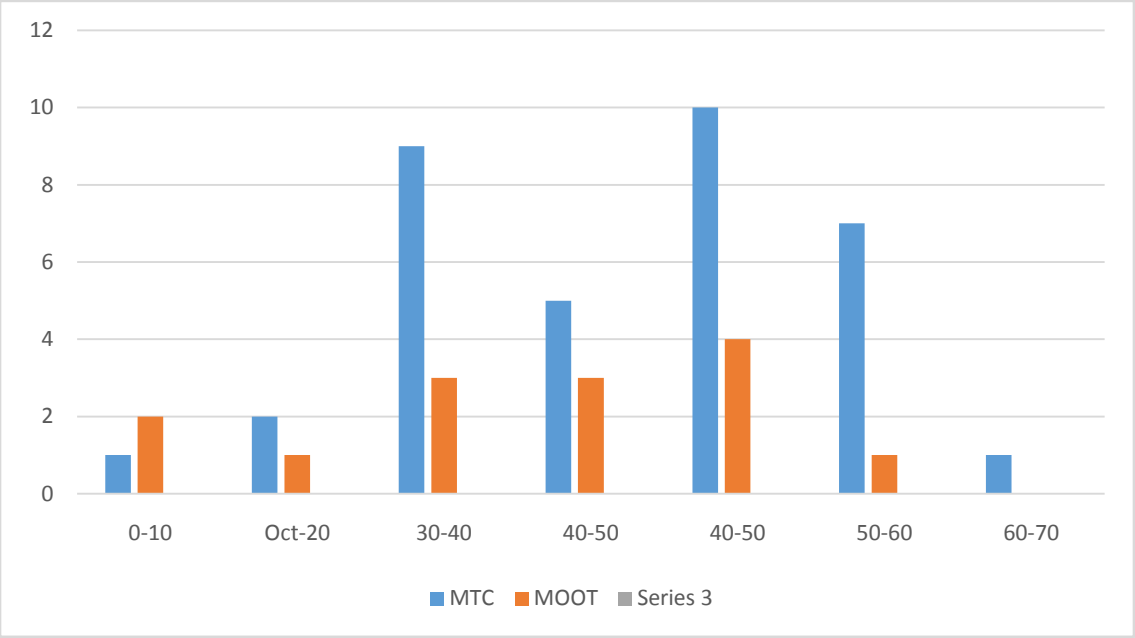

Fig 6.2 Show tuberculosis with*Mycobacterium tuberculosis*and*Mycobacterium*other than *tuberculosis* on the basis of age**.**

Table no. 6.4 the given data show tuberculosis with *Mycobacterium tuberculosis* and *Mycobacterium other than tuberculosis* on the basis of age in Female

	Female	
Age	*Mycobacterium* *Tuberculosis*(MTC)	*Mycobacterium* other than *Tuberculosis*(MOTT)

0-10	2	0
10-20	1	0
20-30	4	5
30-40	4	1
40-50	6	3
50-60	0	2
60-70	0	0

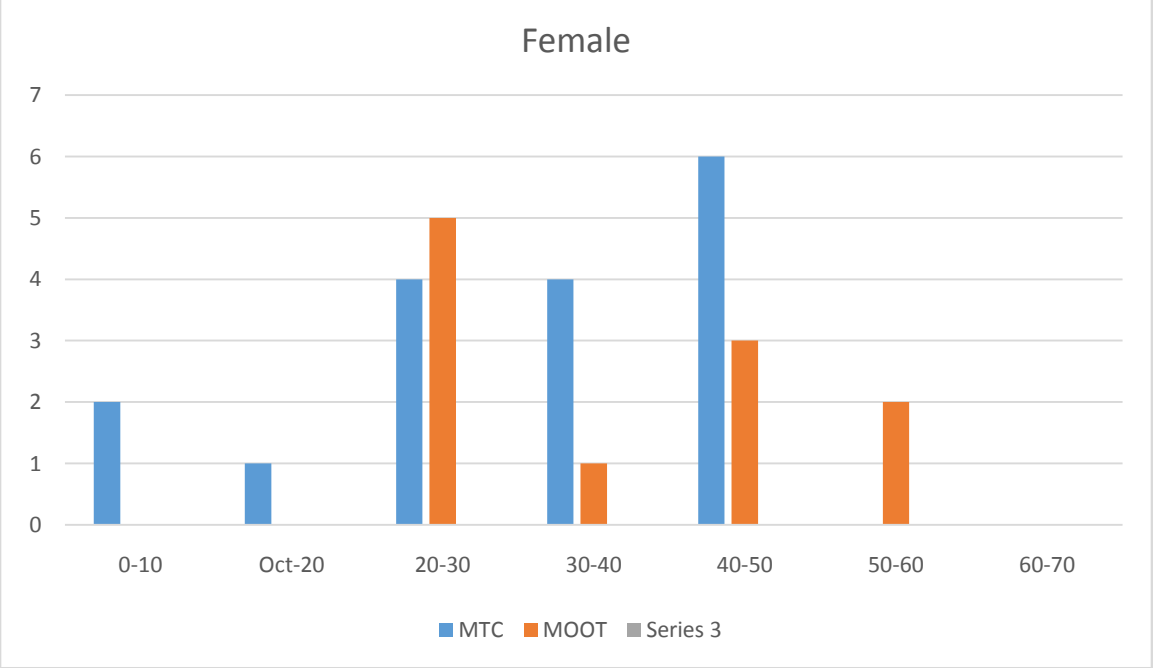

Fig.6.3 Show tuberculosis with *mycobacterium tuberculosis* and *mycobacterium* other than *tuberculosis*particularly in Female on the basis of age.

Table no. 6.5 show anti- tuberculin drugs pattern in *M. tuberculosis* isolated organism.

Number of sample for antibiotic susceptibility	25	
Drugs	sensitivity	Resistant
Streptomycin	19%	6%
Isoniazid	11%	14%
Rifampin	16%	9%
Ethambutol	21%	4%
Pyrazinamide	21%	4%
Capreomycin	24%	1%
Kanamycin	24%	1%
Amikacin	24%	1%
Ciprofloxacin	24%	1%
Ethionamide	24%	1%
Levofloxacin	21%	4%
Para amino salicylic acid	23%	2%
Rifabutin	23%	2%
Ofloxacin	20%	5%

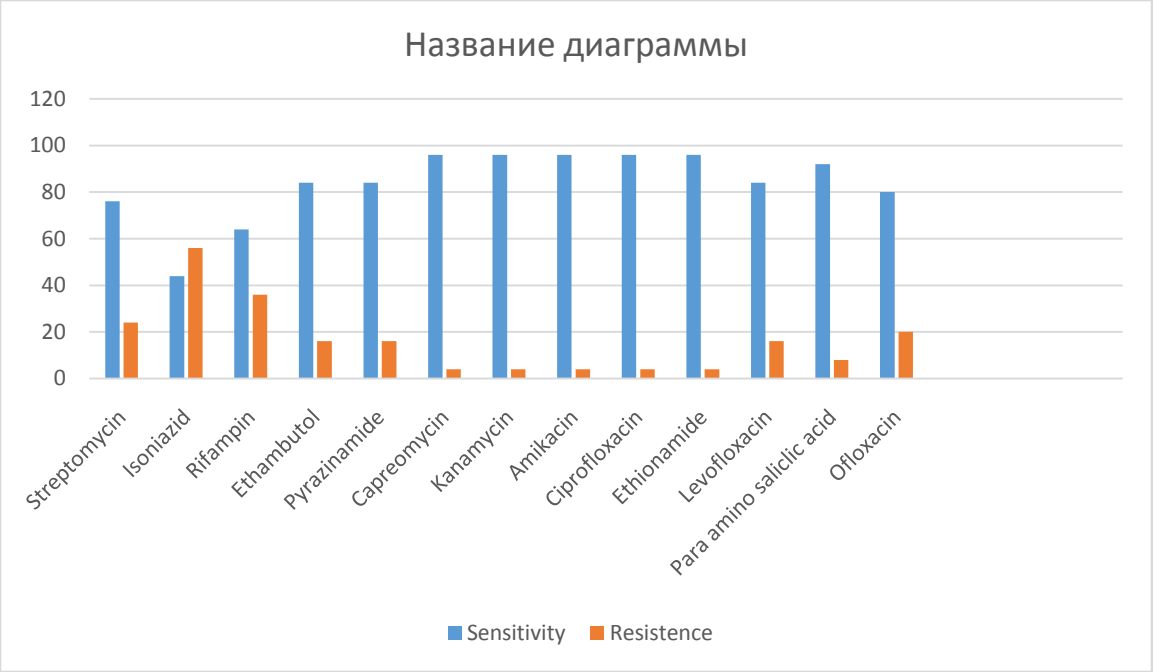

Fig.6.4 Graph shows anti- tuberculosis drugs pattern in percentage(%) from *Mycobacterium tuberculosis* isolated patients.

Discussion

Tuberculosis is now a day global challenging due to prevalence of spreading day by day and infect huge numbers in human diversity. So, earlier diagnosis is most important to control this disease. The new technique is employed for the detection of tuberculosis associated microorganism by BACTEC MGIT960 this technique is very good for rapid diagnosis. This technique is basically based upon fluorescent dye.

The reason for spreading of this disease due lack of proper safety measure, unsuccessful government policy in TB related program like low quality anti-tuberculosis drugs distribution, lack of quality as well as skilled health worker, absent of knowledge like public awareness toward such epidemic. Many others factors also play a vital role unhygienic, comes contact to crowded area, socioeconomic factor, life style, malnutrition, immunological factor, social wrong concept etc. This microorganism are very complex that may exist in extreme as well as in host body even in presence of immune response. They have ability to tolerate the nonspecific immune response because of their strategies are

I. The inhibition of phagosome – lysosomes fusion.
II. The inhibition of phagosome inhibition.
III. The recruitment and retention of tryptophan aspartate containing coat protein phagosome to prevent their delivery to lysosome and
IV. The expression of membrane of the host- induced repetitive glycine- rich family of protein.

The approach to chemotherapy for *tuberculosis* is very different from that for other bacterial infections. The organism has long generation time and a capacity for dormancy, when it's low metabolic activity make it a different therapeutic target. *Tuberculosis* may be located in pulmonary cavity, empyema pus, material were penetration of antibiotic is difficult or the PH is sufficiently low or inhibit the activity of most antibiotics.

A series of animal and human clinical trials has led to the concept that there are different population of bacteria present within the host. Organisms in pulmonary cavity are thought to be multiplying in an aerobic environment and consequently behave in a way that can be mimicked by in vitro tests. Organism is located in environment where the low PH likely to inhibit the activity of agent such as **aminoglycosides.** Each of the anti-tuberculosis drugs has a major role in dealingwith one of these population for example **isoniazid**, is critical early in therapy its bactericidal activity rapidly reduced the sputum. Viable count because it is active mainly against

the organism growing aerobically in pulmonary cavity.

Pyrazinamide is only active at low PH, making it ideally suitable for killingorganism inside necrotic foci. This example the finding that **pyrazinamide** appear to have no benefit after the second month of therapy. **Rifampin** is important in killing organism that are metabolizing slowly, killing the persisters and sterilizing the patients. Sputum as demonstrated by animal studies and clinical trials. Drugs against this organism is combination of many anti tuberculosis drugs to reduce the resistance. Even microorganism gain the resistance. The high prevalence for resistance of anti- tuberculosis drugs is 1st line drugs specially isoniazid and rifampin enhance 44% and 76% sensitive, and 56% and 24% resistance respectively. Intrinsic drug resistance of *M. tuberculosis* has traditionally been attributed to the unusual structure of its mycolic acid-containing cell wall that gives the bacteria a low permeability for many compound such as antibiotics and other chemotherapeutic agents. More recently, the role of efflux mechanism has also been recognized as important factor in the natural resistance of mycobacteria against antibiotic such tetracycline, fluroquinolones and aminoglycosides.

M.tuberculosis acquired drug resistance is caused mainly by spontaneous mutation in chromosomal gene, producing the selection of resistance strains during sub optimal drug therapy. Although no single pleiotropic mutation has been found to cause multiple drug resistance phenotype in *M. tuberculosis*, a possible complex association between classical mutations associated with resistance to one drug could be related to other drugs. In prokaryotes, spontaneous mutation occur at a low rate of 0.0033 per replication. The mutation rate per bp is inversely proportional to the genome size. Previous studies have shown that the rate of mutation depends on the nature of drug selection, but for most main anti-tuberculosis drugs, this occur at a rate of 1/1000000000 mutation per cell division. This is the main reason why anti-TB drugs, are given as a combination, as the risk of a mutant containing two resistance mutation is < 1/1000000000000000000.

Development of multiple drugs resistance *tuberculosis* (MDR-TB)

I. Incomplete and inadequate treatment: - Multiple drug resistance tuberculosis, though some individual who did not have previous tuberculosis treatment can be infected by multiple drugs resistance tuberculosis. Many new cases of multiple drug resistance tuberculosis are created each year by physician errors (drug, dosing intervals, duration)
II. Inadequate treatment adherence: - Non-adherence to prescribed treatment is often

underestimated by the physician and is difficult to predict. Certain factor such as psychiatric illness, alcoholism, drugs addiction, and homelessness do predict non-adherence to treatment.

III. Some other factor also play important role in the development of multiple drug resistance tuberculosis such as poor administrative control on purchase and distribution of the drugs with no proper mechanism on quality control and bioavailability test.

Conclusion:

My studies is a determination to estimate some of the elementary matters involving the decision making for TB patients. Most of number of patients at age group 40-55 in both the cases male and female it indicates that *Mycobacterium tuberculosis* have ability to escape as well as victory over the immune system of this age group. This studies show also high prevalence of MDR-TB in both case male and female. So, it gives the impression essential to carry out DST before the re-treatment of such patients in order to avoid the spread of MDR-TB in the human diversity. My data shows organism has been early resistance of first line drugs and effective drugs are some of second line which is effective, it may be resistance in future if management will be not properly. Therefore, now a days it is critical issues to control the spread of MDR-TB in human diversity.

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(iii) http://jcm.asu:org /content /41/6/2741

Appendix

Preparation of 2.9 % sodium citrates

- Distilled water 500 ml
- sodium citrates 14.5 gms

Preparation of 4% NAOH

- Distilled water 500 ml
- NAOH 20 gms

Preparation of phosphate buffer

- Potassium di hydrogen phosphate 4.53gms
- di-sodium hydrogen phosphate 4.73 gms
- Distilled water 500 ml