Tuberculosis Lab

CLS 417: Clinical Practice in Microbiology

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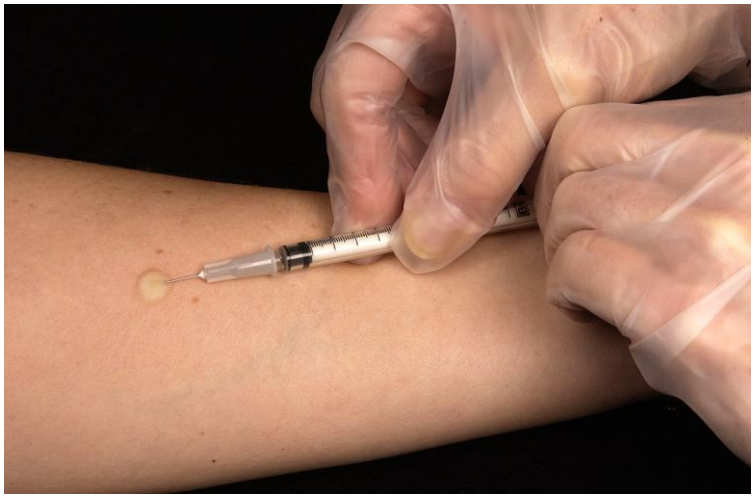
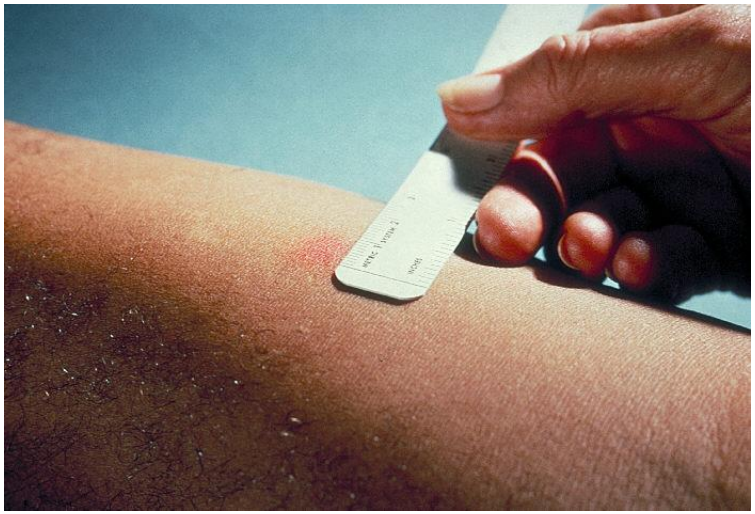
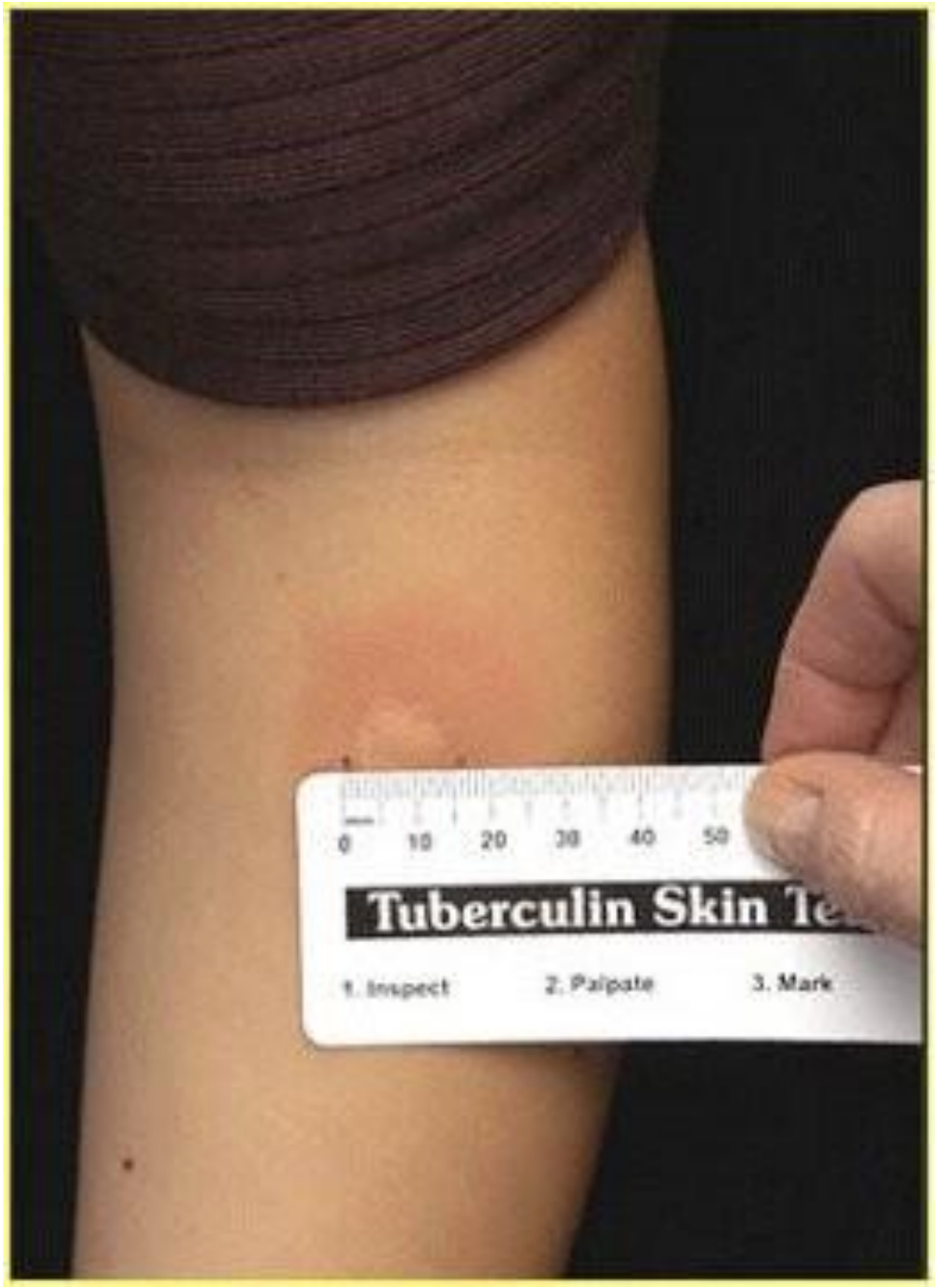
Manitoux Tuberculin Skin Test (TST)

- A screening test to detect Tb infected patients.
- Intradermal injection of purified protein derivative (PPD).
- Measure diameter of induration in the forearm after 48-72 hours. Redness is not measured.






0
10
20
30
40
50
Tuberculin Skin Te
1. Inspect
2. Palpate
3. Mark

TST Positive Results

Diameter of Induration	5-9 mm Induration	10-14 mm Induration	15 mm or more Induration
	•HIV-infected persons •A recent contact of a person with TB disease •Persons with fibrotic changes on chest X-ray consistent with prior TB •Patients with organ transplants •Persons who are immunosuppressed for other reasons	•Recent immigrants (< 5 years) from high-risk countries •Injection drug users •Residents and employees of high-risk congregate settings •Mycobacteriology laboratory personnel •Persons with clinical conditions that place them at high risk •Persons exposed to adults in high-risk categories.	Any person

Types of Tb Lab Specimens

SPUTUM

Three early morning specimens obtained on different days should be submitted. A volume of 5-10 ml is adequate. The sample should contain recently discharged material with minimal saliva content.

INDUCED SPUTUM

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.

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.

Types of Tb Lab Specimens

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.

BLOOD

Specimens should be collected in Isolator tubes available from the kit room of this laboratory on request.

FLUIDS

Body fluids (CSF, pleural, pericardial, synovial, ascitic, pus, bone marrow, gastric aspirate, bronchial wash, larygeal swabs) must be aseptically collected and submitted in sterile containers. Keep refrigerated until transport.

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.

Collection of Sputum

Transportation & Storage

- Specimens should be transported to the laboratory as quickly as possible. Delays in transportation, especially in hot weather, result in an increase in contaminating bacteria that result in higher contamination rate of the medium.
- Specimens should be transported in a container, such as an ice box, in which temperature is maintained as low as possible. This is especially important in countries with high ambient temperatures.
- Upon receipt, the specimens should be refrigerated and processed as soon as possible.

Pathogens Isolated in Tb Lab

***M. tuberculosis* complex (MTBC):**

- *M. tuberculosis*
- *M. bovis*
- *M. canetti*
- *M. microti*
- *M. africanum*

Mycobacteria other than Tuberculosis (MOTT):

- *M. leprae*
- *M. avium*
- *M. kansasii*
- *M. scrofulaceum*
- *M. marinum*

Precautions in Tb lab

- *M. tuberculosis* is a highly infectious (hazard Risk group 3) pathogen, therefore handle specimens with care.
- It is particularly important to minimize the creation of aerosols and to ensure the laboratory is well ventilated.
- The use of personal respirators should be considered to protect staff working with *M. tuberculosis.*
- Using a bleach centrifugation concentration technique not only improves the sensitivity of smear examination but also kills *M. tuberculosis.*
- When a Safety Cabinet is used this must be fitted with a high efficiency particulate air filter (HEPA) and installed, used, and maintained correctly.
- Smears however, can be more easily made using a wooden stick which can be easily discarded and incinerated after use.

Decontamination of Sputum

I. Sodium hypochlorite centrifugation technique

1. Work under safety cabinet, transfer 1–2 ml of sputum (particularly that which contains any yellow caseous material) to a screw-cap *Universal bottle or other container of* 15–20 ml capacity.
2. Add an equal volume of concentrated NaOC1 (bleach) solution and mix well.
3. Leave at room temperature for 10–15 minutes, shaking at intervals to break down the mucus in the sputum.
4. Add about 8 ml of distilled water. Mix *well.*
5. Centrifuge at 3000 g for 15 minutes or at 250–1000 g for 20 minutes.
6. Using a pipette, remove and discard the supernatant fluid. Mix the sediment.

Decontamination of Sputum

II. Sodium hydroxide (modified Petroff) method

1. To 2ml of sputum in the centrifuge tube, add an equal volume of 4% NaOH and tighten the screw-cap. Vortex to digest.
2. Allow to stand for 15 minutes at room temperature.
3. Fill the tube to within 2 cm of the top with phosphate buffer.
4. Centrifuge at 3000*g* for 15 minutes.
5. Carefully pour off the supernatant through a funnel into a discard can containing 5% phenol or other mycobacterial disinfectant.
6. Resuspend the deposit in approximately 0.3 ml phosphate buffer.

III. Decontamination using NALC–NaOH (N-acetyl L-cysteine)

IV. Decontamination using Trisodium Phosphate Solution

V. Decontamination using 5% oxalic method

VI. Decontamination using CPC/NaCl method

Zeihl-Neelsen Stain

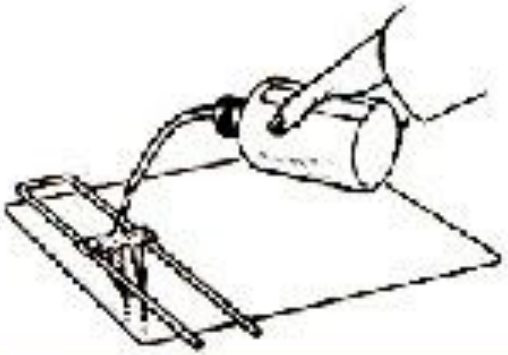
Hot and cold Zn Techniques

- **The 'hot' Zn technique:** the phenolic-carbol fuchsin stain is heated to enable the dye to penetrate the waxy mycobacterial cell wall.

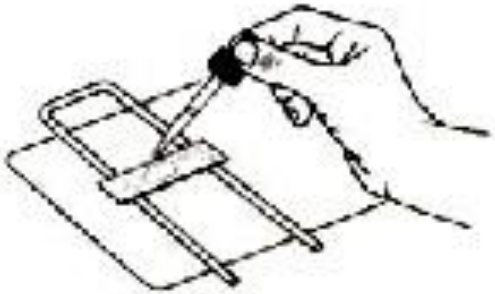
- **The 'cold' Zn technique: Also called Kinyoun's staining** techniques which do not heat the stain. In these, penetration of the stain is usually achieved by increasing the concentrations of basic fuchsin and phenol and incorporating a 'wetting agent' chemical.

1 Cover smear with carbolfuchsin.Steam over boiling water for 8 minutes. Add additional stain if stain boils off.

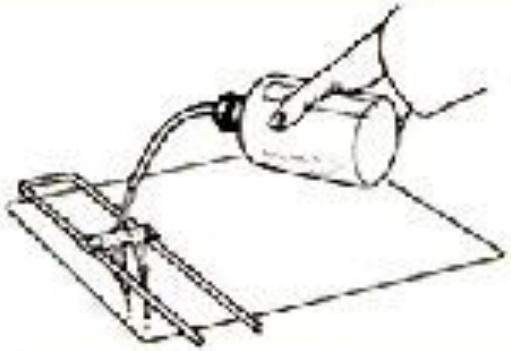
2 After slide has cooled decolorize with acid-alcohol for 15 to 20 seconds.



3 Stop decolorization action of acid-rinsing briefly with water.



4 Counterstain with methylene blue for 30 seconds.



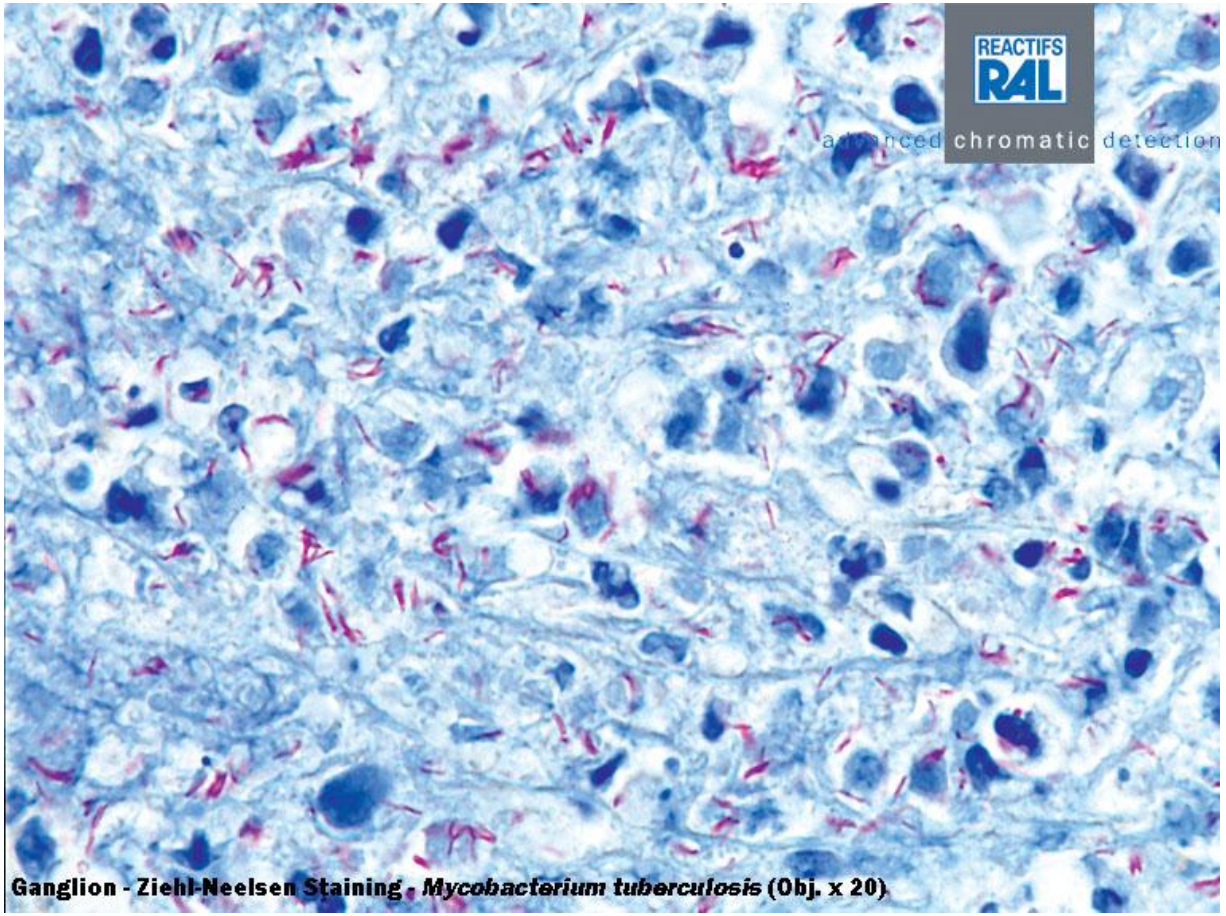
5 Rinse briefly with water to remove excess methylene blue.

6 Blot dry with bibulous paper. Examine directly under oil immersion.

Ziehl-Neelsen acid-fast staining procedure

Zeihl-Neelsen Staining

The acid-fast bacilli (AFB) appear red and any other bacteria blue.



Ganglion - Ziehl-Neelsen Staining - *Mycobacterium tuberculosis* (Obj. x 20)

Fluorescence Stain

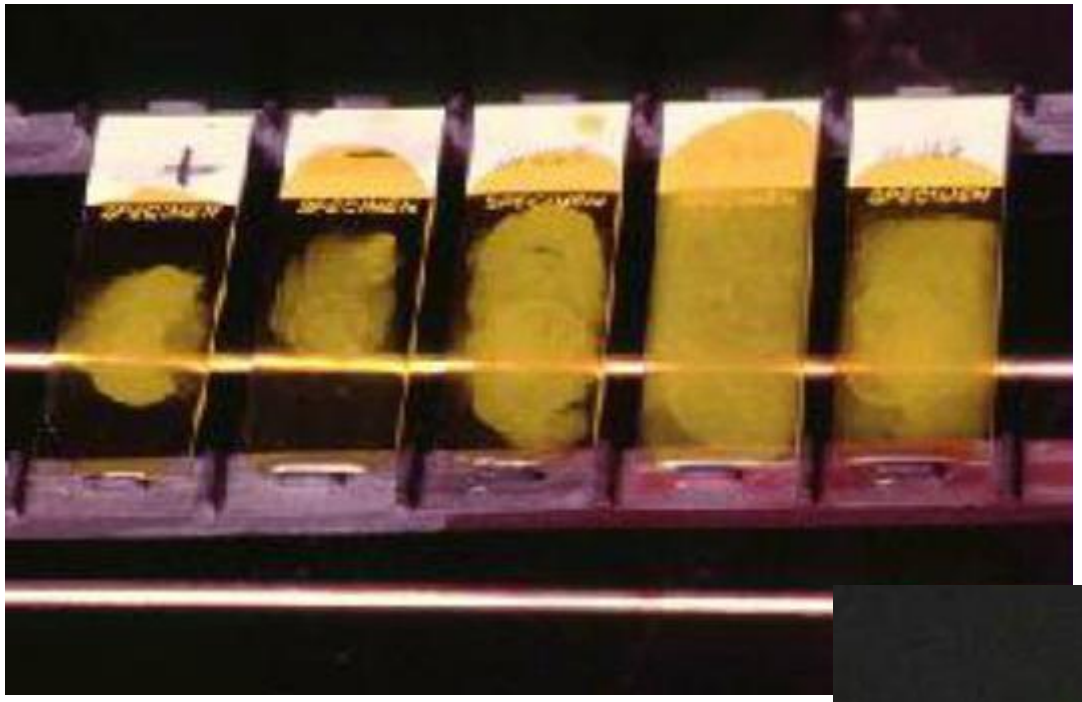
- This technique increases the possibility of finding AFB especially when they are few.
- Acid fast bacilli (AFB) look **yellow or yellow-orange rods glowing against a dark background.**

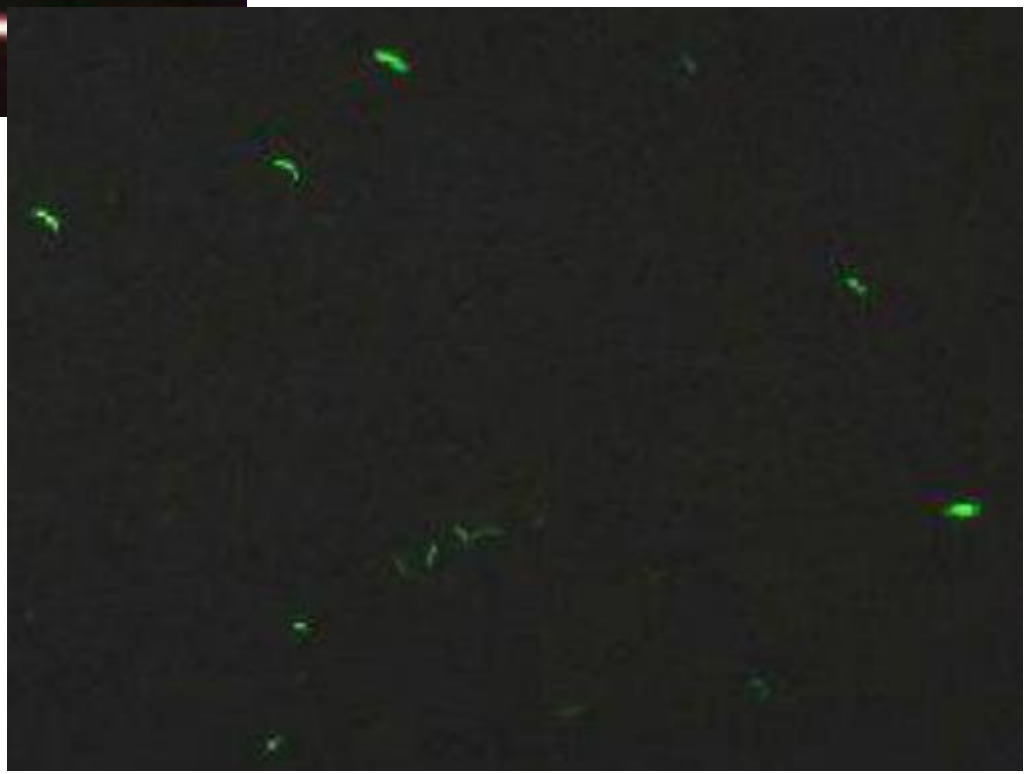
- **Types of fluorescent dyes:**

1. Auramine O.
2. Auramine-Rhodamine.
3. Acridine Orange.

Auramine-phenol Fluorochrome Staining Technique

1. Transfer a drop of the well-mixed sediment to a clean glass slide. Spread the sediment to make a thin preparation and allow to air-dry. Heat-fix the dried smear.
2. Cover the fixed smear with the **auramine-phenol stain** for 10 minutes.
3. Wash off the stain with clean water.
4. Decolorize the smear by covering it with **1% v/v acid alcohol** for 5 minutes.
5. Wash off the acid alcohol with d. water.
6. Cover the smear with the **potassium permanganate solution** for about 10 seconds, followed by several rinses with d. water.
7. Wipe the back of the slide clean and place it in a draining rack for the smear to dry.
8. Systematically examine the smear for AFB by fluorescence microscopy using the 40objective.





Reporting Sputum Smears

1. When any definite red bacilli are seen, report the smear as **'AFB positive'.**
2. Give an indication of the **number of bacteria present as follows:**

- More than 10 AFB/field . . . . **Report +4**
- 1–9 AFB/field . . . . . **Report +3**
- 1–9 AFB/10 fields . . . . . **Report +2**
- 1–9 AFB/100 fields . . . . . **Report +1**
- 1–9 AFB/Whole smear . . . . . **Make new Smear**

Culturing the Sputum

- In most developing countries, isolation, identification and susceptibility testing of *M. tuberculosis are usually carried out in a* **Reference Tuberculosis Laboratory** to manage treatment failures and patients that have relapsed, to monitor multi-drug resistance, and to identify *M. tuberculosis variants and strains, e.g. M. bovis, for* epidemiological purposes.
- As more rapid and less expensive culture and other techniques are developed for detecting, identifying, and susceptibility testing of *M. tuberculosis, it may be possible to* detect tuberculosis at an earlier stage of infection when AFB are too few to be detected in direct sputum smears.
- Culture is considerably more sensitive than microscopy, detecting 10–100 viable organisms/ml of sputum.

Culturing the Sputum on LJ Medium

LJ Medium (Lowenstein Jensen)

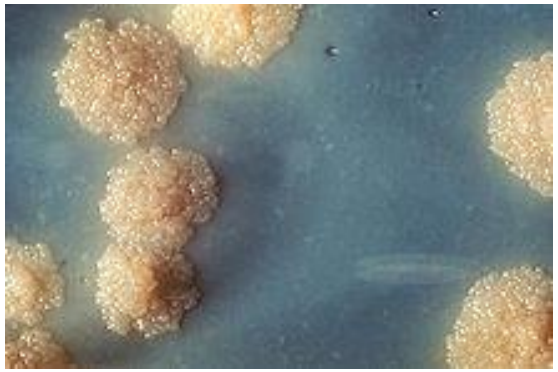
- **Malachite green:** inhibit the growth of most bacteria.
- **With/without Glycerol:** to differentiate *M. tuberculosis* from other *Mycobacteria*.
- **Low levels of penicillin and nalidixic acid:** inhibit growth of Gram positive and negative bacteria.
- Asparagine
- Potato starch
- Coagulated eggs
- Mineral salt solution
  - Potassium dihydrogen phosphate
  - Magnesium sulfate
  - Sodium citrate

Incubation of LJ Medium

- *M. tuberculosis will grow aerobically on a protein enriched* medium @35–37^{o}C. The organism is slow-growing.
- Tubes should be incubated in a slanted position, with screw-caps loose, for at least 1 week to ensure even distribution and absorption of inoculum. After 1 week of incubation, caps are tightened to minimize evaporation and drying of the media. Tubes may then stand upright to save space in incubators.

Colonies on LJ Medium

- *M. tuberculosis produces raised, dry,* cream (buff) colored colonies.



Reporting the LJ Culture

- Check colony formation every week, preferably twice within the first week, to allow rapid detection of contamination and a timely request for another specimen if necessary.
- Contaminated cultures and rapidly growing mycobacteria (colonies apparent in less than 7 days) are removed. *Report results immediately and ask for another specimen.*
- *M. tuberculosis* colonies should be well developed within 3–4 weeks. *Report results immediately after detection and identification*.
- Cultures should be kept for up to 8 weeks before being reported as negative.

MGIT Liquid Media

MGIT: Mycobacteria Growth Indicator Tube

- **The MGIT contains:**

1. 7.0ml of modified Middlebrook 7H9 broth base.
2. Enriched with: Oleic acid, Albumin, Dextrose and Catalase.
3. **PANTA** (Polymyxin B, Amphotericin B, Nalidixic Acid, Trimethoprim, and Azlocillin).
4. An oxygen-quenched fluorochrome.

- MGIT tubes may be incubated at 37^{o}C and **read manually** under a UV light.
- Or entered into a **MGIT 960 instrument** where they are incubated and monitored for increasing fluorescence every 60 minutes. The intensity of fluorescence is directly proportional to the extent of oxygen depletion.

Reporting MGIT Cultures

- Check every day (or following manufacturer's instructions when using an automated system).
- Any tube detected as positive must be checked for purity by acid-fast microscopy and inoculation on solid medium suited to growth of most bacteria (e.g. blood agar, chocolate, brain heart infusion agar plate).
- Cultures should be kept for 6 weeks before being reported as negative. Rather than discarding the vial, it may be advisable to centrifuge the tube and reinoculate a liquid vial and an LJ slope for further examination of possible growth.

Tb Lab Final Report

Conventionally, reports are sent at the following points:

a. Smear from specimen (fluorochrome or ZN) – Report positive or negative and the staining method used. (CDC recommendation within 24 hours of receipt of specimen.)

b. Culture – Positive (with confirmation by AFB smear). Preferably after completion of identification – *M. tuberculosis complex or MOTT bacilli (CDC recommendation* within an average of 14 days) Speciate mycobacteria later and report.

c. Drug susceptibility test – susceptible or resistant to each test drug. (CDC recommendation within an average of 28 days.)

d. Culture – Negative upon completion of the incubation protocol (42 days).

Antibiotic Susceptibility Testing

- Two MGIT tubes are inoculated with the test culture.
- A known concentration of a test drug is added to one of the MGIT tubes, and growth is compared with the MGIT tube without the drug (growth control).
- If the test drug is active against the isolated mycobacteria, it will inhibit the growth and thus there will be suppression of fluorescence, while the growth control will grow uninhibited and will have increasing fluorescence.

Antimicrobial Susceptibility

- Antimicrobial susceptibility testing is particularly required when a relapse occurs during treatment, a patient does not respond to treatment and drug resistance is suspected.
- Drug susceptibility testing must be accurately performed and only **in a Tuberculosis Specialist Laboratory** by adequately trained staff.
- ***The first line drugs:*** *isoniazid,* rifampicin, pyrazinamide, ethambutol.
- ***The second line drugs:*** streptomycin, capreomycin, cycloserine, thiacetazone, and ethionamide.

Environmental *Mycobacteria*

- **Also referred to as atypical, 'anonymous' or 'Mycobacteria other than tubercle' (MOTT).**
- Environmental mycobacteria are being increasingly reported as causing opportunistic infections in those with HIV disease and other conditions associated with immunosuppression.
- They can cause opportunistic pulmonary infections, disseminated disease, lymphadenitis.
- **They can be differentiated from M. tuberculosis (in a Reference Laboratory) by:**

1. **Ability to produce pigment:**

- **Scotochromogen:** produce pigments when cultured in darkness and light.
- **Photochromogen:** produce pigments when only exposed to light.
- **Nonchromogen:** non-pigment producing.

2. **Ability to grow at 25^{o}C**
3. **Ability to grow in 4 (p)-nitrobenzoic acid (PNB) medium.**

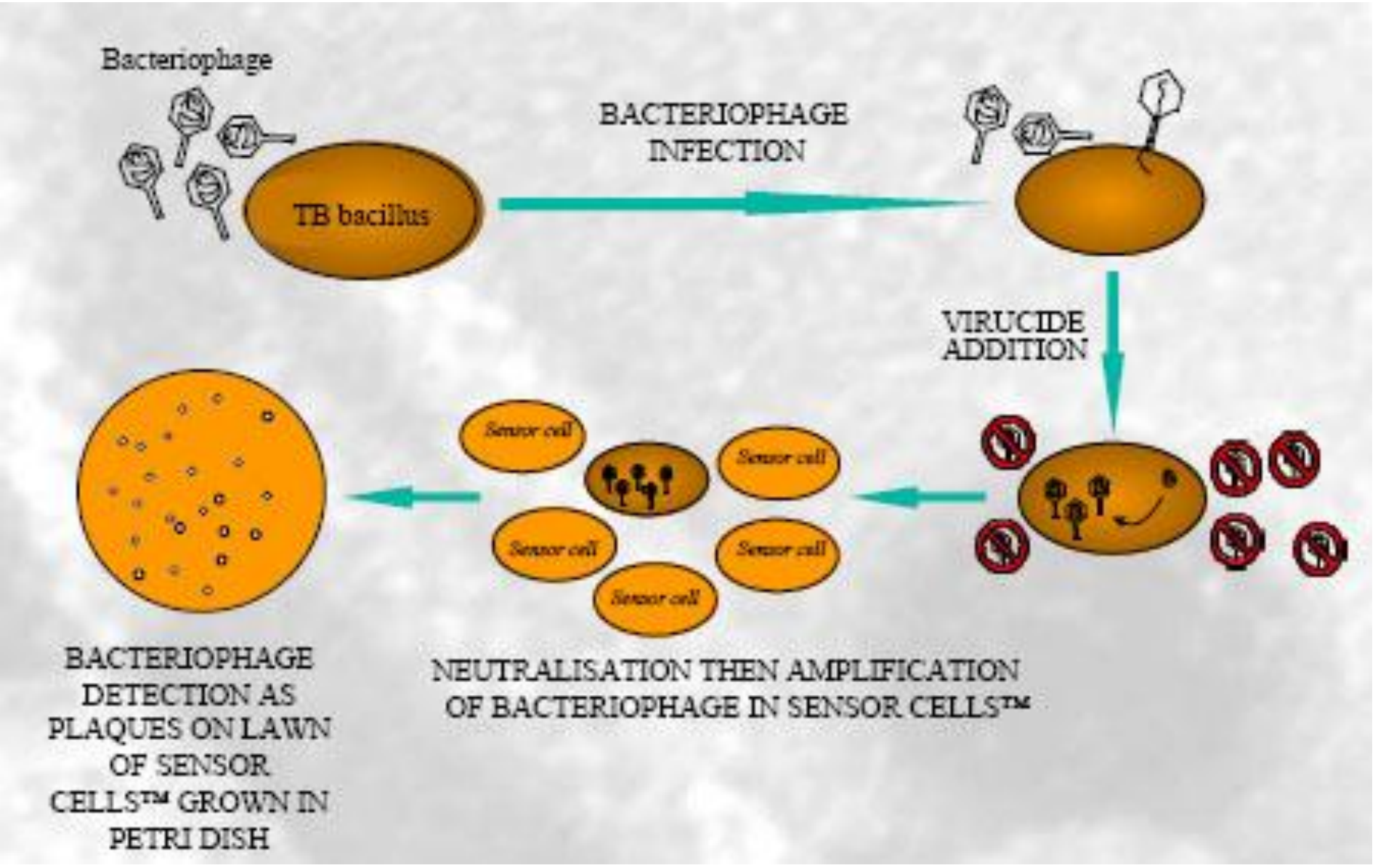
Antibody Tests to Diagnose Tuberculosis

- While several antibody tests are available to assist in the diagnosis of tuberculosis, evaluations in developing countries with high tuberculosis infection rates and high prevalence of HIV infection, have shown the tests to have limited value. The tests lack adequate sensitivity and specificity.

New Technologies for the Rapid Diagnosis of Tb

FASTPlaque Test

- *A bacteriophage-based assay to* detect within 48 hours.
- The sample is mixed with Mycobacteriophages. If the M. tuberculosis is in the sample the phage will infect and grow inside the bacilli.
- Add fast growing non-pathogenic helper cells.
- The mycobacteriophages in turn infect, replicate and lyse these helper cells and the lysis is detected as plaque (Clear Zone).


Bacteriophage
TB bacillus
BACTERIOPHAGE INFECTION
VIRUCIDE ADDITION
Sensor cell
Sensor cell
Sensor cell
Sensor cell
Sensor cell
BACTERIOPHAGE DETECTION AS PLAQUES ON LAWN OF SENSOR CELLS™ GROWN IN PETRI DISH
NEUTRALISATION THEN AMPLIFICATION OF BACTERIOPHAGE IN SENSOR CELLS™

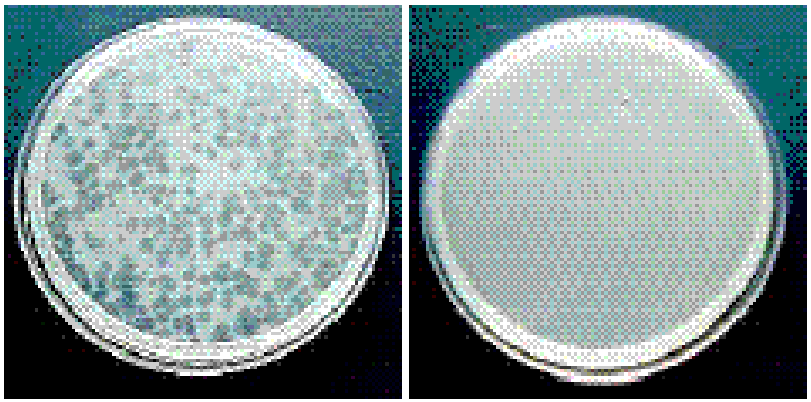
Strain Susceptible to Rifampicin

Strain Resistant to Rifampicin

Incubate without rifampicin

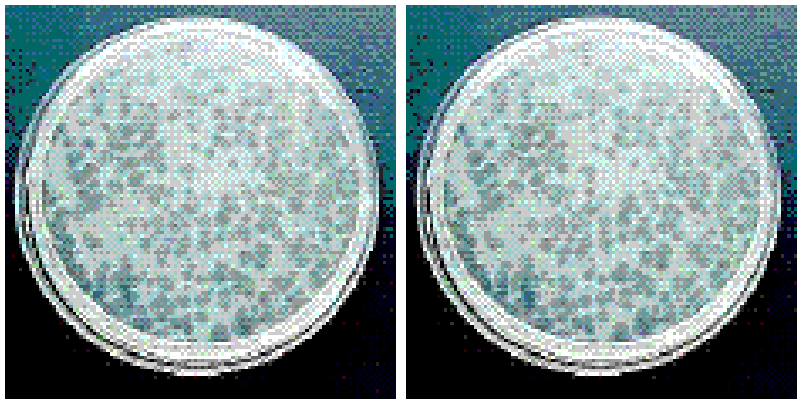
Incubate with rifampicin

Presence of viable MTB cells detected by the ***FAST****Plaque*™**TB-*MDR*i**™ assay and visualisation of plaques on lawns of Sensor™ Cells in Petri dishes



Plaques – viable MTB cells present

No plaques – no viable MTB cells present



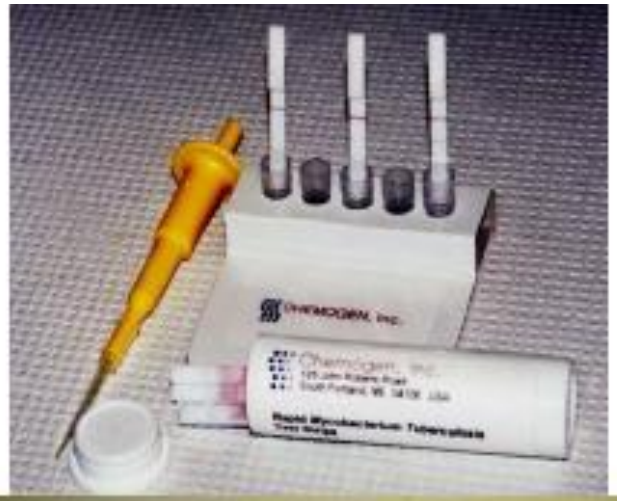
Plaques present in both samples – MTB cells unaffected by rifampicin

TK Medium

- *A mycobacterial rapid and differential solid culture* medium which contains color dye indicators to detect mycobacterial growth at an earlier stage (average 10–18 days) than other culture media.
- When there is mycobacterial metabolic activity, the color of the medium changes from red to yellow. Bacterial contamination is indicated by the development of a green color.

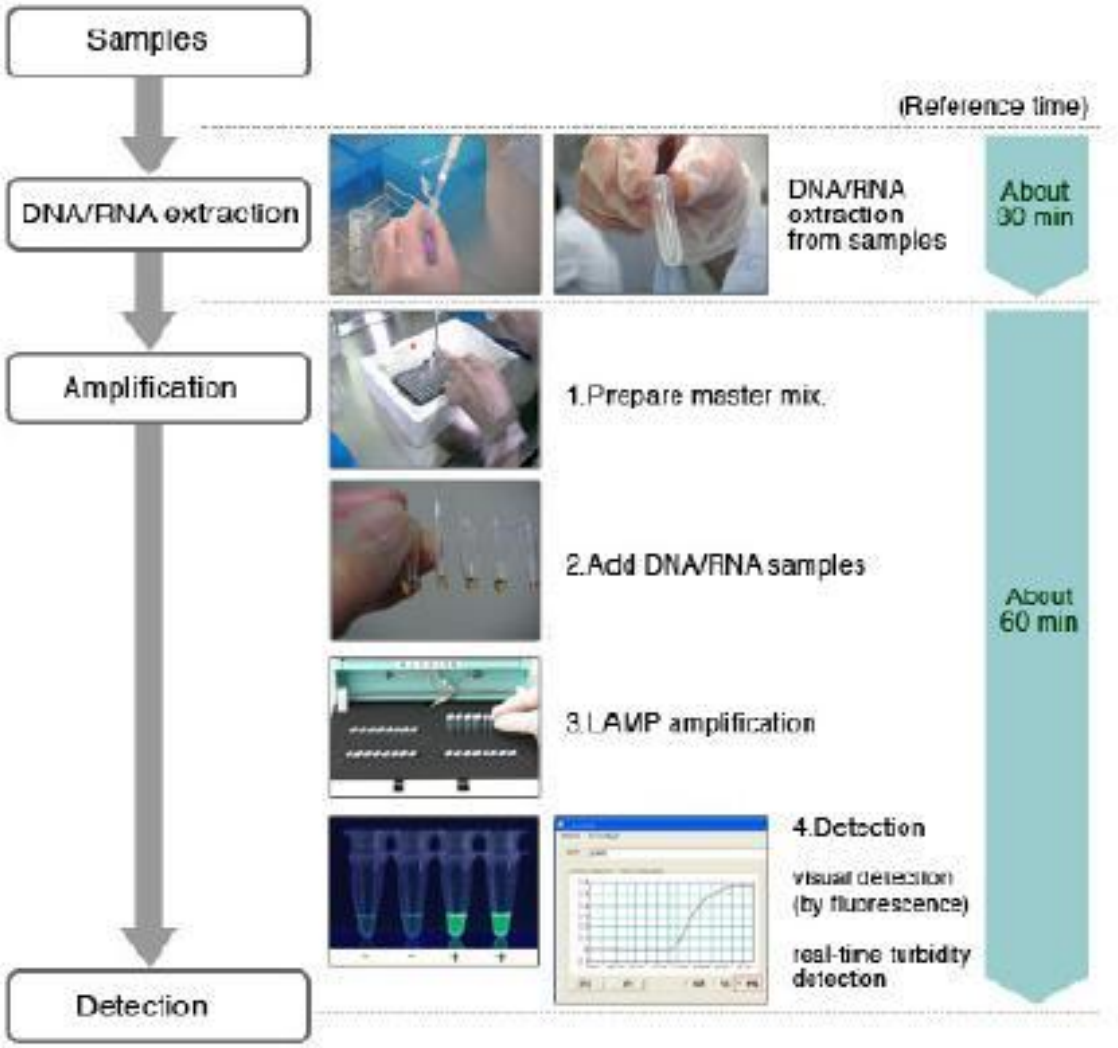
ICT Strip

- *An immunochromatographic* urinary antigen test based on the detection of lipoarabinomannan (LAM) in urine.



LAMP

- *Loop-mediated isothermal amplification test, a sensitive molecular amplification technique* to diagnose tuberculosis by detecting *M. tuberculosis* DNA in clinical samples.



Proteome Systems TB test

- *A rapid technique to* detect antigens produced in active tuberculosis and to measure severity of infection.