

Mycobacteria

General characters:

They are acid-fast, slender, rod, aerobic, non-motile, non-capsulated and non-sporing. Growth is generally slow. They do not grow on ordinary media. They require enriched media with egg albumin, e.g. Lowenstein-Jensen's medium.

Classification of Mycobacteria

A. *Tubercle bacilli*

1. *M. tuberculosis* (human).

B. *Lepra bacilli*

1. Human-*M. leprae*.

C. *Mycobacteria cause skin lesion*

1. *M. ulcerans*.

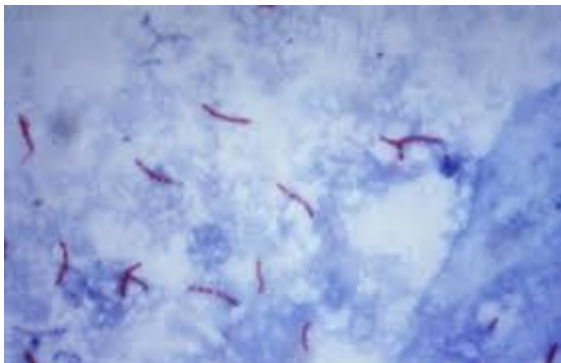
2. *M. balnei*

MYCOBACTERIUM TUBERCULOSIS

Morphology:

M. tuberculosis is straight or slightly curved rod 1 to 4 μ long and 0.2 to 0.8 μ wide. It may be arranged singly or in groups.

It is non-motile, non-sporing and non capsulated. *Mycobacterium bovis* is usually straighter, stouter and shorter. It is Gram positive. It is acid fast (acid fastness is due to mycolic acid). Beaded or barred staining is seen in *M. tuberculosis*. It is more uniform without any bead in the *M. bovis*. Non-acid fast rods and granules from young culture are also reported and when they are injected into susceptible animal they produce tuberculosis. Perhaps these granules are nonacid fast form of tubercle bacilli. These bacilli are called Much's granules.



Culture Characters

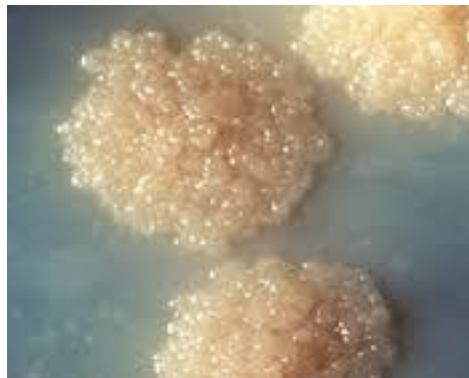
It is aerobic. It grows slowly (generation time 14 to 15 hours). Colonies appear in 2 to 6 weeks. Optimum temperature is 37°C and optimum pH is 6.4 to 7. *M. tuberculosis* grows luxuriantly in culture (eugonic). Addition of glycerol improves the growth of human types. The enriched media is prepared by adding eggs, glycerol, potatoes, meat, bone marrow infusions or asparagine.

Liquid media:

In liquid media without dispersing agent growth creeps up the side from the bottom, forming surface pellicles extending along the side of tube. Diffused growth is obtained in Dubo's medium (contain Tween 80). Virulent strains tend to form serpentine cords in liquid media. Liquid media are generally required for sensitivity test, biochemical tests, and preparation of antigen and vaccine.

Solid media:

M. tuberculosis forms dry, rough, raised and irregular colony. It is creamy white first and becomes yellowish or buff colored later on. It is not emulsified easily. Egg based solid media like L.J, Petraghani or American Tradeau Society medium have been used for primary isolation of *Mycobacterium tuberculosis* from clinical samples and have been found more sensitive than agar based media. Otherwise agar based media are more transparent and permit early microscopic detection of colonies. Culture techniques are more sensitive, than staining procedure, but the major limitation are long time taken for their growth. Solid media used are Dorset egg, Lowenstein- Jensen's medium and Loeffler serum slope. Lowenstein-Jensen's medium is most widely used which contains coagulated hen's eggs, salt solution, malachite green, glycerol and asparagine. Tubercle bacilli may also be grown on chick embryos and in tissue culture.

**Biochemical reactions:**

The important biochemical tests for its identification are:

1. **Niacin test:** Human tubercle bacilli form niacin when grown on egg medium. Ten percent cyanogen bromide and 40 percent aniline in 96 percent ethanol are added to a suspension of culture. Yellow color indicates positive test.
2. **Aryl sulfatase:** Organism is grown, for 2 weeks in media, containing tripotassium phenolphthalein disulphate. Detection of free phenolphthalein is detected by addition of alkali. Red color indicates positive test. It is positive in atypical mycobacteria.
3. **Neutral red:** The virulent strain of tubercle bacilli may bind the neutral red in alkaline buffer more readily and firmly than bovine types.
4. **Catalase test:** A mixture of equal volume of 30 Vol H₂O₂ and 0.2 percent catechol in distilled water is added to 5 ml of test culture. Effervescence indicates positive catalase test and browning of colonies indicates peroxidase activity.

Most atypical mycobacteria are strongly catalase positive. Tubercle bacilli is peroxidase positive. Catalase and peroxidase activities are lost when tubercle bacilli develop resistance to INH.

5. **Nitrate reduction:** This is positive in *M. tuberculosis* and negative in *M. bovis*.
6. **Amidase test:** Five amides are used, i.e. acetamide, benzamide, nicotinamide, carbamide and pyrazinamide. A 0.00164 M solution of amide is incubated with bacillary suspension at 37°C. 0.1 ml of MnSO₄ 4H₂O, 1 ml of phenol and 0.5 ml of hypochlorite solutions are added. These tubes are placed in boiling water for 20 minutes. A blue color means positive test. The ability to split amides has been used to differentiate atypical mycobacteria.

Laboratory Diagnosis

A. Hematological investigations:

1. *Total leukocyte count*: In early acute cases there is leukocytosis. In miliary infection there may be leucopenia.
2. *Differential leukocyte count*: In early cases there is increase in neutrophils. Later on there is an increase in monocytes and lymphocytes.
3. *Erythrocytic sedimentation rate* is usually raised which is an index of progress of the disease.

B. Bacteriological investigations:

Microscopy: Smears may be prepared from specimen like sputum, laryngeal swab, pleural fluid, peritoneal fluid, cerebrospinal fluid, pus, urine, gastric lavage, feces and other infected material.

New glass slides should be used for smear. Smear should be made from thick purulent portion of sputum. The smear is dried, fixed and stained by Ziehl-Neelsen technique. The slide is studied under oil immersion objective. Acid fast bacilli are seen as pink brightened rods, while background is blue. It has been estimated that at least 50,000 to 1,00,000 acid fast bacilli should be present per ml of sputum to get positive report. A negative report should not be given till at least 100 fields are examined taking about 10 minutes time.

Where several smears are to be examined daily, it is more convenient to use fluorescent microscopy. Smears are stained with auramine phenol or auramine rhodamine fluorescent stain. They are examined under ultraviolet light and bacilli appear bright rods against dark background.

Concentration Methods

Following are the methods which concentrate the bacilli without inactivating the bacilli and hence can be used for culture and animal inoculation:

1. *Petroff's method*: Sputum is incubated with equal volume of 4 percent solution of sodium hydroxide. Shake till it becomes clear. It takes about 20 minutes. Centrifuge it at 3000 rpm for 30 minutes and sediment is neutralized with N/10 HCl. Sediment is used for smear culture and animal inoculation.
2. Homogenization may be achieved with dilute acid (6% H₂SO₄, 3% HCl or 5% oxalic acid).
3. *Flocculation method*: Specimen is treated with digester containing sodium hydroxide and potash alum. It is neutralized with acid. Floccules start appearing. These floccules are sedimented by centrifugation.
4. *Jungmann's method*: Acidic ferrous sulfate and H₂O₂ is used. A bulky deposit is obtained and used for inoculation.
5. Trisodium phosphate is lethal for many contaminating bacteria but tubercle bacilli are unaffected.
6. N-acetyl-L-cysteine with NaOH is considered rapid and effective method for homogenization of mucopurulent specimen. Pancreatin, desogen, zephiran and cetrimide are other homogenizing agents.
7. Addition of 20 percent clorox to sputum but this method is not suitable for culture.
8. Lauryl sulfate method: Here 1.5 percent sodium hydroxide and 4.5 percent lauryl sulfate is used.

Culture: Culture techniques have been estimated to detect 10 to 1000 viable mycobacteria per ml of specimen. The most common media are based on egg and also contain high concentration of malachite green to overcome contamination with other bacteria. The concentrated material is inoculated into 2 bottles of L.J. media.

The tubercle bacilli grow in about 4 to 8 weeks of incubation at 37°C. However, rapid growing mycobacteria may appear in 4 days' time. A negative report is sent if no growth appears by the end of 8th week to 12th week.

Ogawa medium: It is egg based medium. It is most economical because it replaces asparagine by sodium glutamate. Actually it contains mineral salts (potassium dihydrogen phosphate anhydrous, sodium glutamate), distilled water, malachite green dye and homogenized eggs. The modified Ogawa medium in addition contains magnesium citrate and glycerol. The pH of modified medium is 6.4. The ingredients are mixed well and dispensed in 6 to 8 ml volume in MacCortneys bottles. Now medium in MacCortneys bottle is inspissated at 80°C to 85°C for 45 minutes. The inspissation is repeated for 2nd and 3rd time. This medium may be stored in refrigerator for several weeks.

In laboratories where centrifuges are not available, sputum specimens are decontaminated with 4% of sodium hydroxide and inoculated directly on modified Ogawa medium.

Middlebrook 7H 10 and 7H 11 are agar based media. They achieve slightly higher isolation yield than egg based media but are quite expensive.

Blood agar is an alternative culture medium for the isolation of mycobacterium.

Mycobacterium tuberculosis grows within one or two weeks on blood agar plates. Average number of colonies isolated from clinical specimens on blood agar is significantly higher than the number of colonies on egg based medium. It can also be used as alternative medium for drug sensitivity testing of *Mycobacterium tuberculosis* against antitubercular drugs like isoniazid, rifampicin, streptomycin and ethambutol.

Sensitivity tests: Drug resistance is a big problem. Drug sensitivity is done by:

1. *Absolute concentrate method:* Media contains serial dilutions of drug is inoculated. Minimum inhibiting concentration is the least drug concentrate that inhibits growth.
2. *Resistance ratio method:* Two sets of media are used containing graded concentration of drug. One set is inoculated with standard strain and other with test strain and resistance ratio is worked out.
3. *Proportion method:* It indicates average sensitivity of strain.
4. *Rapid drug susceptibility test using Resazurin:* Here metabolic activity of *Mycobacterium tuberculosis* is indicated through the use of oxidation reduction indicator Resazurin.
5. A new test, developed at the Albert Einstein College of Medicine in New York involves introducing a gene from firefly into *Mycobacterium tuberculosis* exposed to drugs. Only the drug resistant to *Mycobacterium tuberculosis* will glow. To make the bacteria glow, a gene is introduced for the luciferase enzyme from the firefly into *Mycobacterium tuberculosis*, the genetic engineered microorganism then emits light. When exposed to antituberculosis drugs, the dead *Mycobacterium tuberculosis* is incapable of producing light but drug resistant bacteria will continue to glow. This test is reported to identify drug resistance within two or three days which is likely to be further reduced to few hours.

Newer Drug Screening Methods of *Mycobacterium Tuberculosis*

1. PCR method has special promise for future.
2. E. test is an emerging method for MIC determination anti-microbial agents. All

Biodiscs employing various drugs have been found to be promising for *Mycobacterium tuberculosis* and others.

3. FDA staining fluorocytometry is based on differentiating the live and dead organisms by fluorescent dyes like fluororescentdiacetate and ethidium bromide. This method is also used in automated method for *Mycobacterium tuberculosis*.

4. Hybridization protection assay based on quantitative assessment of gene hybridization has been reported promising.

Animal inoculation:

Two healthy guinea pigs are taken. Tuberculin test is done to ascertain that they are not tuberculous. Concentrated material is injected intramuscularly into the thigh of both animals. Progressive loss of weight is an indication of tuberculosis. One animal is killed at 4 weeks and if no evidence of tuberculosis is noticed at autopsy the other is killed after 8 weeks. At autopsy infected animal shows:

1. Caseous lesion at the site of inoculation
2. Enlarged caseous draining lymph nodes.