

Mycobacterium

The name Mycobacterium (plural: Mycobacteria) is derived from its "fungus-like" nature. Branching filamentous forms are sometimes seen.

May occur as saprophyte, animal pathogen, opportunistic pathogen or as obligate human pathogen.

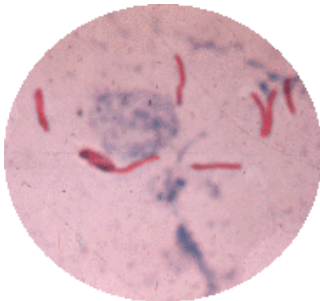
Several species of Mycobacteria occur, *M. tuberculosis* is an important human pathogen. Other pathogens include *M. bovis*, *M. avium-intercellulare* complex, *Mycobacterium africanum* etc.,

First member of Mycobacteria discovered was *M. leprae* by Hansen in 1868, hence called Hansen's bacilli. *M. tuberculosis* was discovered by Robert Koch in 1882.

All Mycobacteria are acid-fast, aerobic, non-motile, non-capsulated and non-sporing. Tubercle bacilli belong to the family Mycobacteriaceae and the order Actinomycetales.

Mycobacteria are difficult to stain, but once stained they are not readily decolorized even by weak mineral acids. Hence they are termed acid fast bacilli.

M. tuberculosis



It can not be stained by Gram stain, yet is considered a gram positive bacilli based on cell wall structure.

It is slightly curved bacilli (1-4µm long), which may show beaded forms, occasional branching, occurring singly, pairs or in small clumps. They are both alcohol and acid fast.

Acid fastness is due to presence of unsaponifiable wax (**mycolic acid**)



They are aerobic as well as fastidious bacteria. They have a long generation time (~16 hrs), hence grow very slowly on culture medium, taking 2-8 weeks for growth to occur. Commonly used medium is Lowenstein Jensen (LJ) medium. It is made selective by addition of malachite green. Other components include egg yolk, mineral salt solution & asparagine.

Differences between *M. tuberculosis* and *M. bovis*

	<i>M. tuberculosis</i>	<i>M. bovis</i>
Morphology	Straight-slightly curved, with beaded forms	Short, straight rods that stain uniformly
Cultural characteristics	Obligate aerobe	Initially microaerophilic, later become aerobic

Growth characteristic	Grows luxuriently (eugonic)	Grows sparsely (dysgonic)
0.5% Glycerol addition	Stimulates growth	Inhibit or no effect
Colony characteristics	Dry, rough, irregular, wrinkled, yellowish-buff coloured	Smooth, white, moist, flat colonies
Emulsification of colony	Difficult	Easy
Biochemical tests	Niacin test positive Nitrate test positive	Niacin test negative Nitrate test negative

M. tuberculosis forms long serpentine cords in liquid medium due to **Cord factor** (6,6' trehalose dimycolate), which was earlier thought to be responsible for its virulence.

- Bacilli are killed at 60°C in 15-20 minutes
- Bacilli in sputum survive for 20-30 hours
- Bacilli remain viable in droplet nuclei for 8-10 days
- Survive exposure to 5% phenol, 15% sulphuric acid, 4% NaOH
- They are resistant to malachite green and penicillin
- Bacilli are destroyed by tincture iodine in 5 minutes & 80% ethyl alcohol in 2-10 minutes
- It is a very hardy bacillus that can survive under adverse environmental conditions.

Important biochemical tests:

Niacin test: *M. tuberculosis* (positive); *M. bovis* (negative)

Aryl sulphatase test: Positive by atypical mycobacteria only

Neutral red test: Virulent mycobacteria are positive

Nitrate test: *M. tuberculosis* (positive); *M. bovis* (negative)

Catalase test: *M. tuberculosis* (weakly positive); atypical mycobacteria (strongly positive)

Peroxidase test: *M. tuberculosis* (positive); atypical mycobacteria (negative)

Note: Isoniazid (INH) resistant strains of *M. tuberculosis* are negative for catalase & peroxidase test.

Antigenic properties:

Protein antigens: Tuberculin protein is shared with few other mycobacteria such as *M. bovis*, *M. microti* etc. It induces tuberculin sensitivity. Also elicit antibody production.

Lipids: These are bound to proteins & polysaccharides. Muramyl dipeptide with mycolic acid induces granuloma formation. Phosphatides induce caseation necrosis. Analysis of lipids by gas chromatography aids in species identification. Cord factor inhibits leucocyte migration.

Polysaccharides: Many varieties present, but role uncertain. Induce antibody formation.

Both lytic and lysogenic bacteriophages isolated from *M. tuberculosis*. It is divided in four phage types: A, B, C and I, which is intermediate to A and B types.

Type A is commonest and present worldwide. Type B is seen in Europe and North America. Type C is rare. Type I is seen in India and neighbouring countries.

Note:

Phage 33D lyses all variants of *M. tuberculosis* but not BCG.

Some strains harbour lysogenic phage's DNA in free form (like a plasmid) instead of integrating with its chromosome. This is termed **pseudolysogeny**.

Epidemiology

- Tuberculosis a deadly disease, is on the rise and is revisiting both the developed and developing world. Tuberculosis (TB) kills about two million people each year, making it one of the world's leading infectious causes of death among young people and adults.
- Around 1 billion people catch TB each year. In 1993, the World Health Organization (WHO) declared TB to

be a global public health emergency.

- One-third of the world's population is infected with TB. Five to 10 percent of people who are infected with Mycobacterium become sick with TB at some time during their life.
- Each year, more than 8 million people become sick with TB.
- A single patient can infect 10 or more people in a year.
- Due to a combination of economic decline, the breakdown of health systems, insufficient application of TB control measures, the spread of HIV/AIDS and the emergence of multidrug-resistant TB (MDR-TB), TB is on the rise in many developing and transitional economies.
- Tuberculosis is considered "captain of all men of death".

Pathogenesis:

- Tuberculosis is acquired by inhalation of infective aerosols, less so by ingestion of milk (*M. bovis*). Humans are the only known reservoirs for *M. tuberculosis*.
- All infections do not necessarily end in tuberculosis disease.
- A person with active pulmonary tuberculosis can spread the disease by coughing or sneezing. 5-200 inhaled bacilli are usually necessary for infection.
- The process of catching tuberculosis involves two stages: first, a person has to become infected; second, the infection has to progress to disease.
- Progression to disease depends on personal nutritional and immune status, virulence of strain, infective dose.
- TB can develop more easily if the immune system weakens, as happens with malnutrition, AIDS, diabetes, cancer, or treatment with immunosuppressant drugs.

Following inhalation, mycobacteria reach lungs where they are ingested by alveolar macrophages. Virulent strains establish themselves within macrophages and multiply.

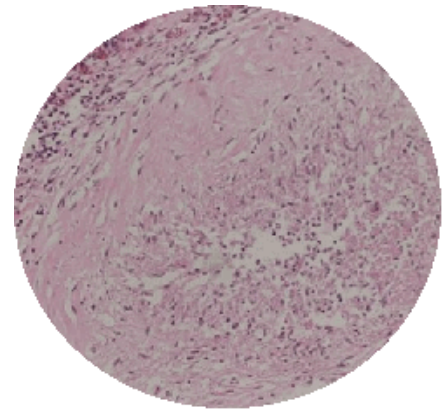
Two types of lesions are encountered in tuberculosis: exudative & productive

Exudative type: Consists of acute inflammatory reaction with edema fluid and polymorphonuclear leucocytes, later monocyte around mycobacteria.

In lung tissue, it resembles bacterial pneumonia. It may heal by resolution, undergo massive necrosis or develop into productive type

Productive type: It is a chronic avascular granuloma consisting of 3 regions; central zone of large multinucleated giant cells containing mycobacteria; a mid-zone of radially arranged pale epithelioid cells and a peripheral zone of fibroblasts, lymphocytes and monocytes.

Later, peripheral fibrous tissue develops and central area undergoes caseation necrosis. Such a lesion is called **tubercle**.



The name tuberculosis is because of this typical lesion. A caseous tubercle may break into a bronchus and empty its content there, and form a cavity. It may subsequently heal by fibrosis or calcification.

Spread of infection:

- Mycobacteria inside the phagocyte may multiply, lyse the cell and spread to adjacent area by direct extension.
- Phagocytes with ingested mycobacteria may act as vehicles transporting the infection to various parts.
- Infection may spread to regional lymph nodes from the initial site through lymphatics.

- Bacilli may then reach the bloodstream. Erosion of vein by caseating tubercle or lymph node too can induce mycobacteremia.
- Mycobacteremia disseminates bacilli to all organs (miliary distribution).
- If a caseating lesion discharges its contents into a bronchus, they may be aspirated and distributed to various parts of lung. If this discharge is swallowed, it would infect the stomach and intestine.

Virulence factors:

Mycobacteria do not produce any toxins. Its ability to survive and multiply within macrophage is its main virulence factor. The organism is slow growing and tolerates the intracellular environment, where it may remain metabolically inert for years before reactivation and disease. They remain in an endocytic vacuole and block transport machinery that enables the phagosome to fuse with the lysosome. It is known to prevent the acidification of the phagosome that is needed for effective killing of microbes by lysosomal enzymes. Lipoarabinomannan is a heteropolysaccharide that inhibits macrophage activation by IFN-gamma.

Types of infection:

- Primary and secondary (reactivation, post-primary, adult)
- Pulmonary and extra-pulmonary

Primary infection:

- Seen when a person gets infected for the first time; usually seen in children.
- Sub-pleural focus of tuberculous pneumonia in lung parenchyma usually in the lower lobe or lower part of upper lobe (**Ghon focus**).
- Characterized by acute exudative lesion that spreads rapidly to lymphatics and regional lymph nodes. Lymph node (hilar) enlarge.
- These features together is known as "**primary complex**".
- The lymph node may undergo massive caseation, which usually calcifies.
- Tuberculin test becomes positive at this stage.
- Usually passes off as asymptomatic infection with spontaneous resolution.
- Rarely, the primary infection may lead to hematogenous spread leading to tubercular meningitis, miliary tuberculosis or lesions in organs such as liver, kidney, spleen etc.

Secondary/Reactivation infection:

- Occurs due to reactivation of tubercle bacilli that have survived in the primary lesion. Also known as post-primary or endogenous infection, it is often seen in adults.
- Reactivation type almost always begins at the apex of the lungs.
- It is characterized by chronic tissue lesions, formation of tubercles, caseation and fibrosis. Regional lymph nodes are rarely involved and do not caseate.
- Usually resolve by resorption, fibrosis and occasional calcification. It may progress to chronic caseation, tubercle formation and cavitation. Shedding of bacilli in sputum in large numbers occur (**open cases**).
- In individuals who are immunocompetent, the lifetime risk of developing disease is 5-10%. In certain instances, such as extremes of age or defects in CMI, HIV infection, malnutrition, administration of chemotherapy, prolonged steroid use), TB may result.

Clinical presentations:

- The primary stage of the disease may be asymptomatic, or the individual may experience a flu-like illness.
- In the secondary stage (active tuberculosis), there might be a slight fever, evening rise in temperature, night sweats, weight loss, fatigue and various other symptoms, depending on the part of the body affected.
- Pulmonary involvement gives rise to chronic cough, a dry cough that eventually leads to a productive cough with blood-stained sputum. There might also be chest pain and shortness of breath.
- Extra-pulmonary tuberculosis may show up as meningitis, renal tuberculosis etc.

Note: The disease is more the result of immune reactions of the host rather than the invasive power of the pathogen.

Immunity:

- Host responds to infection by non-specific way (by way of macrophages) and specifically by humoral and cell mediated immunity.
- CMI is more effective than humoral immunity in checking the proliferation of mycobacteria. A CMI response terminates the growth of the M. tuberculosis 2-3 weeks after initial infection.
- Mycobacterial antigens are presented by macrophages to T helper cells, which gets activated, produce cytokines and clone themselves.
- CD4 helper T cells activate the macrophages to kill the intracellular bacteria with resultant epithelioid granuloma formation.
- CD8 suppressor T cells lyse the macrophages infected with the mycobacteria, resulting in the formation of caseating granulomas.
- Formation of granuloma by activated lymphocytes and monocytes helps to arrest spread of infection.
- Cytokine activated macrophages can eliminate engulfed mycobacteria.
- Cytokines produced by activated cells such as lymphocytes, monocytes and macrophages play important role.
- Cytokines such as IL-1, IL-2, IL-12, IL-18, IFN-gamma & TNF-alpha stimulates CMI, whereas IL-4, IL-5, IL-6, IL-10 and IFN-beta stimulate humoral immunity.
- Higher CMI heightens immunity while higher humoral immunity lowers protection. These two are inversely proportional.
- Due to failure or inadequate CMI, B lymphocytes are stimulates resulting in antibody production.
- Antibodies have no protective value against intracellular mycobacteria.

Hypersensitivity:

- In the course of primary infection, the host also acquires hypersensitivity to tubercle bacilli.
- Immunity and hypersensitivity are two important parts of cell mediated response.
- It was demonstrated in guinea pig by Robert Koch and is known as "**Koch phenomenon**".
- Subcutaneous injection into normal guinea pig doesn't produce any immediate effect, but after 10-14 days a nodule develops at the site, which breaks down to form an ulcer and persists till the animal dies of progressive tuberculosis. The draining lymph nodes are enlarged and caseous. On the other hand if an intradermal injection is given to guinea pig that has already been infected with tubercle bacilli 4-6 weeks earlier, an indurated lesion appears at the site in 24-48 hours, which undergoes necrosis, forms a shallow ulcer and then heals rapidly without involvement of lymphatics. This is the result of both immunity as well as hypersensitivity.
- In humans, hypersensitivity can be demonstrated by tuberculin test.

Tuberculin test:

The original preparation of tuberculin (OT; old tuberculin) was made by Robert Koch by growing the tubercle bacilli in 5% glycerol broth for a duration of 6-8 weeks. The culture filtrate was concentrated ten-folds by evaporation and protein extracted. Koch intended to use this in treatment.

Since the extract contained components of the culture medium and the potency varied from batch to batch, purified components were taken and standardized. PPD (purified protein derivative) was derived by chemical fractionation of OT from bacteria grown in semi-synthetic medium. A batch of purified tuberculin made by Seibert (Lot no. 49608) was recognized by WHO as standard. This is PPD-S, 1 mg of which was arbitrarily assigned to contain 50, 000 TU (tuberculin units). 1 TU of PPD-S should weigh 0.00002 mg.



Mantoux test: 0.1 ml of the preparation (stabilized with polysorbate 80) is administered intradermally on the flexor aspect of the forearm using a tuberculin syringe.

1 TU is used when extreme hypersensitivity to tuberculin is suspected. 5 TU is normally used. If test is negative, it may be repeated with higher units (10 TU, 100 TU or 250 TU).

Person sensitized to tubercle bacilli will ordinarily develop erythema, edema and induration at the site in 24-48 hours, which may progress to central necrosis.

Diameter of induration at the site of injection (not the erythema) must be measured after 24-48 hours. A measurement of 10mm or more is considered positive, 6-9mm equivocal and less than 5mm negative.

Persons who were PPD positive years ago may fail to give test positive in the absence contact with bacilli. When such persons are re-tested 2 weeks later, they give positive PPD test. This is known as "booster effect" or "2-step test".

Reasons for false positive test:

- Measurement of erythema instead of induration
- Recent BCG vaccination
- Infection by *M. avium* or atypical mycobacteria

Reasons for false negative test:

- Error in proper measurement
- Improper storage of PPD (lost or decreased potency)
- "Anergy", patient suffering from miliary tuberculosis, lymphoreticular malignancy, Hodgkin's disease, Sarcoidosis, AIDS, convalescence from measles, severe malnutrition, immunodeficiency disorders etc.
- Positive tuberculin test indicates that the person had been infected in the past and continues to carry viable bacteria in some tissue.
- It does not differentiate past from current infections.
- A negative test does not rule out tuberculosis and a positive test does not confirm tuberculosis, especially in places where TB is endemic.
- However, an unvaccinated child turning PPD positive may indicate new infection.
- After BCG vaccination, a test may remain positive for 3-7 years.
- A positive tuberculin test may occasionally revert to negative upon isoniazid treatment of a recent converter.
- Tuberculin test become positive 4-6 weeks after infection or injection of avirulent bacilli.
- Tuberculin positive persons are at risk of developing disease from reactivation of primary disease. Tuberculin negative persons, who have never been infected are not at such risk, but can suffer primary infection.

Laboratory diagnosis of pulmonary tuberculosis:

Specimen collected: 3 samples of sputa (spot-morning-spot). Alternative samples include laryngeal swab, gastric lavage (in children), induced sputum, invasive techniques (bronchial brush sampling, transtracheal aspirate, lung biopsy) etc.,

Microscopy: Acid fast staining of smears or fluorescent stained (auramine-rhodamine) smears. Acid fast staining could be hot (Ziehl Neelsen) or cold (Gabbett's or Kinyoun's) method. Minimum of 100 fields must be scanned before issuing a negative report.

Smear must be graded as follows (RNTCP recommendations, 1998)

Number of AFB	Result	Grading	No. of fields to examine
More than 10 AFB per oil immersion field	Positive	3+	20
1-10 AFB per oil immersion field	Positive	2+	50
10-99 AFB per 100 oil immersion field	Positive	1+	100

1-9 AFB per 100 oil immersion field	Scanty	Exact number	200
No AFB in 100 oil immersion field	Negative	-	100

Grading of smear is helpful in monitoring prognosis.

Its specificity is almost 100% but sensitivity is 45-75% because the specimen must contain 5000-10,000 bacilli/ml. If numbers are scanty, the sample may be concentrated by one of several techniques (Petroff's, NALC, Zepharin chloride etc).

Microscopy can not differentiate between live and dead bacteria.

Culture:

Direct or concentrated specimens are inoculated after decontamination (antibiotic treatment to kill commensals) into LJ medium and incubated in 5-10% carbon dioxide for 2-8 weeks at 37°C . Cultures can be positive with as few as 10-100 bacilli/ml of sputum. Slopes must be observed daily until growth occurs and discarded after 8 weeks if no growth is seen. Other culture media used are Middlebrook 7H11 agar, Middlebrook 7H9 broth, Dubos oleic-albumin liquid medium and Kirchner's liquid medium.

Rapid culture methods:

- Microcolony counting method
- Septi-check AFB system
- Mycobacterial growth indicator tube (MGIT) system
- Radiometric BACTEC method

Animal inoculation: Not routinely performed for diagnosis. Two guinea pigs (12-week old) are inoculated intramuscularly into thigh. Animals are weighed before and after inoculation regularly; weight loss is indication of TB. Infected animals give positive tuberculin test. One animal is killed after 4 weeks and autopsied for typical lesions. If typical lesions are absent the other animal is killed after 8 weeks.

Note: Catalase negative (INH resistant) strains do not produce progressive disease in guinea pigs.

Serology: Detection of anti-mycobacterial antibody is not of much significance especially in endemic areas. Several types of tests have been employed in antibody detection. ELISA based tests detecting antibody to select antigen (Ag-5, 85 complex, PPD and lipoarabinomannan) are currently in use, although their significance is debatable.

Molecular techniques: Molecular techniques such as DNA hybridizations, polymerase chain reaction, ligase chain reaction are currently being employed for rapid detection of mycobacteria in clinical specimens. Apart from detecting in short period, these techniques can also detect drug resistance.

Treatment:

Typical duration of anti-tuberculosis therapy in new cases lasts for 6 months (**directly-observed short course chemotherapy**). This comprises of an intensive phase of two months and continuation phase of four months. However, for retreatment cases (relapses & treatment failures) the short course chemotherapy is of 8 months duration.

Commonly used drugs are Isoniazid (H), Rifampicin (R), Pyrazinamide (Z), Streptomycin (S), Ethambutol (E) and Thiacetazone (T)

Mycobacteria strains undergo spontaneous mutation during chemotherapy giving rise to resistant mutants. Such mutant strains may eventually replace the sensitive strains and result in treatment failure. It is for this reason that drugs are used in combination. Besides, these drugs have synergistic effect.

Treatment of new cases:

Recommended regimen for **intensive phase** is $2(\text{HRZE})_3$, i.e. isoniazid, rifampicin, pyrazinamide and ethambutol administered 3 times a week for 2 months. If the patient remains sputum positive at the end of intensive phase, the same is continued for another month. At the end of this period the patient is given continuation phase whether the smear is positive or not.

The regimen for **continuation phase** is $4(\text{HR})_3$, i.e rifampicin & pyrazinamide administered 3 times a week for 4 months. For patients with tuberculous meningitis or disseminated disease, drugs should be given for 6-7 months.

Retreatment regimen:

The **intensive phase** is $2(\text{HRES})_3/1(\text{HRZE})_3$, i.e. rifampicin, isoniazid, pyrazinamide and ethambutol, supplemented with streptomycin for first 2 months followed by same drugs without streptomycin for 1 month given 3 times a week.

The **continuation phase** is $5(\text{HRE})_3$, i.e 5 months of isoniazid, rifampicin & ethambutol 3 times a week. If the patient remains smear positive despite this treatment, the patient is managed as a chronic case.

Drug resistance:

Antibiotic resistance in *Mycobacterium tuberculosis* are of two types, primary and secondary. Primary resistance is the resistance pattern seen in new patients, who have not been exposed to anti-TB drugs previously. Secondary resistance is the resistance pattern in patients with previous history of anti-TB treatment and is due to ineffective chemotherapy. Multi-drug resistance in *M.tuberculosis* refers to simultaneous resistance to at least Rifampicin and Isoniazid (INH), with or without resistance to other drugs.

Mechanism of drug resistance:

Resistance is usually acquired by the bacilli either by alteration of the drug target through mutation or by titration of the drug through overproduction of the target. **MDR-TB** results primarily from accumulation of mutations in individual drug target genes. Multiple-drug resistance in mycobacteria is the result of the step-wise accumulation of resistance to individual drugs.

Chance of a random mutation conferring drug-resistance:

- Isoniazid 1 in 10^{-5} to 10^{-6}
- Streptomycin 1 in 10^{-8} to 10^{-9}
- Ethambutol 1 in 10^{-7}
- Rifampin 1 in 10^{-10}

The probability of resistance is very high for less effective antitubercular drugs such as thiacetazone, ethionamide, capreomycin, cycloserine, and viomycin; intermediate for drugs such as INH, Streptomycin, Ethambutol, Kanamycin, and p-amino salicylic acid; and lowest for Rifampicin. The probability of a mutation is directly proportional to the bacterial load.

A bacillary load of 10^9 will contain several mutants resistant to any one antitubercular drug.

Prophylaxis:

There are no sure ways of preventing infection by *Mycobacterium*. Some degree of immunity can be achieved by immunization with BCG vaccine.

BCG (Bacille Calmette-Guérin, named after the two Frenchmen) consists of a live attenuated strain derived from *Mycobacterium bovis*, which has been attenuated by 239 successive in vitro subcultures in glycerol-bile-potato medium for over 13 years.

Within a few weeks of inoculation, the bacilli stop multiplying although they survive in tissue for an indefinite period of time. The lesions do not spread and disappear slowly. The immunity induced by BCG is similar to the immunity following natural infection with tubercle bacilli. There is however, no risk of reactivation.

0.1 ml of the preparation is injected intradermally into the deltoid area of the arm. In India it is usually given at birth. Complications of BCG vaccine are local (ulcers, keloid), regional (enlargement & abscess formation of lymph nodes) and general (fever, mediastinal adenitis).

The degree of immunity conferred by BCG has been controversial. Various field trials have shown immunity ranging from 0-60%. Controversy exists about the efficacy of BCG against pulmonary TB but various trials concluded that the vaccine is efficacious against miliary and meningeal TB.

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