

Etiologic agent of tuberculosis. Microscopic morphology of *Mycobacterium tuberculosis*. Metabolic capabilities, nutritional and environmental requirements for growth. MBT generation time. Resistance to physical and chemical challenges.

Tuberculosis (TB) is the disease caused by bacteria of the *Mycobacterium tuberculosis* complex, which includes the clinically relevant species, *M. tuberculosis*, *M. bovis*, and *M. africanum*. These organisms are also known as tubercle bacilli or as acid-fast bacilli (AFB). Although *M. tuberculosis* is the most common cause of TB worldwide, both *M. bovis* and *M. africanum* can produce clinically indistinguishable forms of disease.

Etiologic agent

M. tuberculosis is a rod-shaped, non-spore-forming, thin aerobic bacterium measuring 0,5 µm by 0,3 µm. They have a high lipid content in the wall, probably the highest among all bacteria.

Table 1.1 – Lineage of the agents of TB.

Kingdom	Bacteria
Phylum	Actinobacteria
Class	Actinobacteria
Order	Actinomycetales
Suborder	Corynebacterineae
Family	Mycobacteriaceae
Species	<i>M. tuberculosis</i> <i>M. bovis</i> <i>M. africanum</i>

The taxonomic status of some members of the complex is still uncertain.

Mycobacteria are neutral on Gram's staining. Acid fastness is due mainly to the high content of mycolic acids, long-chain cross-linked fatty acids, and other cell-wall lipids. These lipids may act as carbon and energy reserves. Lipids constitute more than half of the dry weight of the mycobacteria.

The waxy coat confers:

- ✓ acid fastness, extreme hydrophobicity, resistance to injury, including that of many antibiotics
- ✓ slow growth rate of some species by restricting the uptake of nutrients.

The microscopic appearance does not allow the differentiation of the pathogenic agents of TB, mainly *M. tuberculosis*, from other mycobacteria although some

characteristics may be indicative. In smears MBT typically appear as straight or slightly curved rods.

Bacterial cell contents as well ribosomes and DNA filaments. The envelope is composed of the *plasma membrane*, a *cell wall*, and an *outer capsule like layer*.

The ***cytoplasmic membrane*** provides osmotic protection, regulates the traffic of specific solutes between the cytoplasm and the environment. The membrane contains proteins involved in metabolic processes and energy generation. The enzymes intervene in cell wall and membrane synthesis, cell division and DNA replication.

The membrane is surrounded by a ***cell wall*** that protects the cell contents, provides mechanical support and is responsible for the shape of the bacterium. The mycobacterial cell wall is unique among prokaryotes. In the cell wall, lipids (high molecular weight fatty acids called *mycolic acids*) are linked to underlying *arabinogalactan* and *peptidoglycan* which is responsible for the shape-forming. Another molecule in the mycobacterial cell wall, *lipoarabinomannan*, is involved in the pathogen-host interaction and facilitates the survival of *M. tuberculosis* within macrophages. There are also proteins (*porins*) forming hydrophilic channels that permit the passive passage of aqueous solutes through the mycolic acid layer.

While growing in a static liquid culture or within a human cell, MBT accumulates a bioactive ***pseudo-capsule*** which promotes the better cell wall permeability. When the medium is disturbed, the capsule separates, leaving the lipophilic surface with high protective characteristics exposed.

Cell wall of virulent MBT contains a special glycolipid ***cord factor*** due to which bacteria arrange in braided bunches in smear and produce rough textured colonies on solid media. In contrast, non-virulent mycobacteria and tubercle bacilli attenuated by prolonged cultures usually develop smooth colonies on solid media and lie separately randomly when smeared. The recognition of these two peculiarities, ***cording and crumbly colony formation***, allows the presumptive distinction of *M. tuberculosis* from other mycobacteria in cultured specimens and even in sputum smears. Cord factor provides the pathogenicity, toxicity and protection against the host response.

Genome structure

Genome of MBT has been sequenced in 1998. Genome size of *M. tuberculosis* is more than 4mln base pairs long with more than 4 thousands (4043) genes encoding (3993) proteins and (50 genes encoding) RNAs; its high guanine+cytosine content (65,6%) is indicative of an aerobic lifestyle. *M. tuberculosis* is one of the largest known bacterial genomes.

About 250 genes are involved in fatty acid metabolism, with 39 of these involved in generating the waxy coat. Such large numbers genes show the evolutionary importance of the waxy coat to pathogen survival. Over half of the genes have arisen as a result of gene duplication which plays an important role in genome plasticity. Unlike most bacteria which have multiple copies of the rRNA genes, MBT contains a single one. This explains why single mutations in the ribosomal RNA genes result in resistance to protein synthesis inhibitors (rifampicine).

Nutritional and environmental requirements for growth

M. tuberculosis is *mesophile* and *neutrophile* as its multiplication is restricted to conditions offered by warm-blooded animals: about 37°C and a neutral pH.

MBT is *obligate aerobe* but in unfavorable conditions metabolism may shift from an aerobic to one that is more microaerophilic and utilizes lipids (which leads to cell wall disorganization and L-form formation). This is a highly resourceful strategy for pathogenicity and persistence. In nature, the bacillus grows most successfully in tissues with high oxygen partial tension, such as the lungs, particularly the well-aerated upper lobes.

In vitro, the members of the M. tuberculosis complex are not fastidious; the medium used by Koch to cultivate M. tuberculosis was simply sterile coagulated blood serum. Albumin, which is normally provided by adding eggs or bovine serum albumin to the culture media, promotes the growth.

Generation time

Under favorable laboratory conditions, M. tuberculosis divides every **12 to 24 hours**. This pace is extremely slow compared to that of most cultivable bacteria, which duplicate at regular intervals ranging from about 15 minutes to one hour. The slow growth rate might be partially determined by the cell wall impermeability that limits nutrient uptake.

But ribonucleic acid synthesis was identified to be a major factor associated with the long generation time of the tubercle bacillus. The low multiplication rate explains the typically subacute to chronic evolution of the disease and the long time required to attain visible growth in vitro.

The main achievements for diagnosis have been made through the use of tools that enable the detection of a minimal quantity of bacilli in the media.

Metabolic and biochemical markers

In the laboratory the investigation of *niacin accumulation*, *catalase-peroxidase*, *nitrate reductase* and *urease* activity allows the distinction of M. tuberculosis complex.

The thermal-labile *catalase-peroxidase* is a marker of the M. tuberculosis complex. Paradoxically, the catalase is not only self-protective but can also be self-destructive as it activates the anti-TB pro-drug isoniazid. Mutations in the genes encoding the enzyme result in resistance to isoniazid.

Resistance to physical and chemical challenges

Although the tubercle bacillus is not a spore-forming bacterium, it has a remarkable capacity to endure unfavorable conditions. The bacillus is resistant to the macrophages and to antiseptics and antibiotics.

The microorganism also withstands very low temperatures. On the other hand, the bacilli are very sensitive to heat, sunlight and ultraviolet irradiation. Exposed to direct UV irradiation, moderate loads of tubercle bacilli die in a few minutes.

M. tuberculosis tolerates low oxygen tension. The bacilli may survive for many years in this condition but need a minimal concentration of oxygen to induce the switch into a fermentative metabolism.

Bacteriological diagnostics.

Induced sputum. Sputum smear microscopy and culture. Ziehl-Neelsen staining and AFB microscopy. Fluorescent auramine staining. Classic method of cultivation on Löwenstein-Jensen media. Automated culture methods. Genotypic methods of MBT identification and drug resistance detection.

The principal method of pulmonary TB diagnosis is microscopic examination of Ziehl-Neelsen stained sputum samples for AFB. For bacteriological examination, the quality of the samples sent to the laboratory is of fundamental importance.

For pulmonary tuberculosis: the specimen that should be collected for examination is sputum obtained from the patient after coughing (*more rarely the sample is obtained by gastric aspiration or bronchoscopy*).

For extrapulmonary tuberculosis: cerebrospinal fluid (CSF) or biopsied fragments can be sent to the laboratory for culture.

Sputum smear microscopy and culture

AFB Microscopy

There are several staining methods used for the tubercle bacillus; it is important for the method or methods used to be standardized for each country. The stains that are the most effective are hot Ziehl-Neelsen (ZN) staining and auramine staining.

Ziehl-Neelsen staining

The smear is covered with carbol fuchsin, and then heated. The smear is then destained by sulfuric acid and alcohol. All of the smears must be almost totally destained, and then restained with methylene blue. The bacilli are stained red by the fuchsin are resistant to the acid and alcohol, **acid-fast bacilli (AFB)**.

On microscopic examination of the stained smear, the tubercle bacilli look like fine, red, slightly curved rods that are more or less granular, isolated, in pairs or in groups, and stand out clearly against the blue background.

The stained smear is examined using a binocular microscope with an immersion lens (*magnification x100*). The number of AFB per 100 fields (*about one length and one width of a slide*) are counted. This technique is simple, rapid and fairly inexpensive.

Fluorescent auramine staining

The fuchsin is replaced by auramine; the bacilli fix the fluorescent stain and retain it after the acid and alcohol staining.

The stained smear is examined by fluorescence microscopy with a dry lens of low magnification (*25 or 40). This microscope has an ultraviolet lamp to enable the fluorescent bacilli to be seen: they are clearly visible in the form of greenish-yellow fluorescent rods.

The sensitivity and specificity of examination by fluorescence microscopy are comparable to those of microscopy after ZN staining. The main advantage is the ease and rapidity of reading: *on the same slide surface, the results of 10 minutes' reading by optic microscope are obtained in 2 minutes on fluorescence microscopy.*

As this technique requires more costly equipment (the microscope itself, and the lamps, which need to be replaced frequently — on average after 200 hours of use), it is cost-effective only if more than 30 slides are examined each day. A constant electricity supply and trained technicians are also indispensable.

Sputum smear microscopy for tubercle bacilli is positive when there are at least 10000 organisms present per ml of sputum. Such a high number of bacilli is found only in the lesions of patients with cavitary pulmonary tuberculosis.

Classic culture methods

When *M. tuberculosis* is cultured from clinical specimens (e.g. sputum, lymph node aspirate, cerebrospinal fluid) this provides the gold standard for the definitive diagnosis of TB. Tubercle bacilli that have grown in culture can also be tested in vitro

for sensitivity to anti-TB drugs. The usual culture medium is Löwenstein-Jensen, although liquid culture media and automated systems (e.g. Bactec) can also be used in more sophisticated laboratories.

Most pathological specimens, except those that are obtained from closed lesions (serous membranes, joints, samples obtained from surgery), are contaminated by other bacteria. In order to destroy these bacteria, which can contaminate the culture media; it is important to decontaminate the sample with basic antiseptics, which kill the contaminants much more rapidly than the mycobacteria. Decontamination also homogenizes the specimen.

The inoculated tubes are placed in an incubator at 37 °C for 4–12 weeks. As tuberculous mycobacteria grow very slowly (an average period of doubling of 13–20 hours), colonies will be visible to the naked eye after at least 3 weeks' incubation.

When growth has occurred on culture, large, rounded, buff-coloured “cauliflowerlike” colonies are visible to the naked eye on the surface of the culture medium; they have a dry, rough surface, and are isolated or confluent, depending on the number of bacilli present in the original sample.

When colonies appear, they must be identified according to criteria based on their macroscopic aspect (rough colonies) and by their response to biochemical tests: *M. tuberculosis* colonies have a thermolabile catalase activity (positive at 22°C, destroyed by heat at 68 °C), and a nitrate reductase activity, and they accumulate niacin, which can be demonstrated by the niacin test. In other cases another mycobacterium must be identified (*M. bovis*, BCG or atypical mycobacteria).

The number of colonies present in the culture tubes is in direct relation to the number of bacilli in the lesions. This is why the colonies are counted and the results are expressed as the number of colonies per tube, except if their number is so high that they are confluent (in this case the result will be expressed as innumerable confluent colonies).

Other culture methods (Automated culture methods)

Growth in the liquid media is faster than that in solid media, and automated systems allow for growth detection within 1–3 weeks compared with solid media, where growth takes 3–8 weeks.

The BACTEC MGIT 960 system uses the technology of the radiometric system. If viable mycobacteria are present in the tube, oxygen is consumed due to their metabolism and the bottom of the tube fluoresces when exposed to ultraviolet light.

The BACTEC system monitors the tubes at one-hour intervals, alerts when they become positive and signals the end of the incubation period.

Molecular genetics or PCR

The polymerase chain reaction is used for detecting *M. tuberculosis*. PCR can detect and identify the presence of *M. tuberculosis* in a pathological specimen within 24 to 48 hours. However, it is of less sensitivity compared with culture (80% on average), and its specificity is from 97–98%. This delicate technique requires sophisticated, costly equipment.

Genotypic methods to Diagnose M(X)DR TB

Genotypic methods look for genetic determinants of resistance rather than the resistance phenotype and have the advantage of a shorter turnaround time.

PCR sequencing is the gold standard method for mutation detection. It is accurate and reliable and has been widely used for detecting mutations responsible for resistance to anti-tuberculosis drugs.

- ***Solid-phase Hybridization Techniques***

GenoType MTBDR assay (the Line Probe Assay - INNO-LiPA, Hain's test) for the simultaneous detection of isoniazid and rifampicin resistance based on the detection of the most common mutations in the *katG* and *rpoB* genes respectively.

- ***Real-time PCR Techniques.***

Real-time PCR techniques (GeneXpert® MTB/R) have also been introduced for rapid detection of drug resistance using different probes. The main advantages of real-time PCR techniques are the speed of the test and a lower risk of contamination.

PCR detects:

- ✓ mutations in codon of *katG* gene indicating resistance to Isoniazide;
- ✓ mutations in codons of *rpoB* gene indicating resistance to Rifampicin.

Line Probe Assay (INNO-LiPA) is based on reverse hybridization of amplified DNA from cultured strains or clinical samples. The presence or absence of mutated or wild regions is visualized by a colorimetric reaction and the strain can be considered as resistant or susceptible to Rifampicin.

Advantages:

- High sensitivity (97%), specificity (100%);
- allows rapid detection of resistance to RIF directly from sputum samples.

The GenoType MTBDR plus is based on the DNA-Strip technology and permits the simultaneous molecular genetic identification of:

- ✓ the *M. tuberculosis* complex;
- ✓ its resistance to rifampicin and isoniazid by the detection of the most common mutations from smear-positive pulmonary clinical specimens or cultivated samples.

Advantages:

- Results are obtained in 4–5 hrs only.
- Combination of specific amplification and hybridization guarantees diagnostic reliability, high sensitivity and specificity (sensitivity of 88.9% for MDRTB detection, with a specificity of 100%).

Hains Test is based on the DNA-Strip technology and permits the simultaneous molecular genetic identification of the:

- ✓ *M. tuberculosis* complex
- ✓ its resistance to fluoroquinolones like ofloxacin and moxifloxacin *by the detection of the most common mutations in the gyrA gene*
- ✓ its resistance to the injectable antibiotics (viomycin, kanamycin, amikacin and capreomycin) *by detection of the most common mutations in the rrs-gene*
- ✓ its resistance to the first-line drug ethambutol *by detection of the most common mutations in the embB gene from smear-positive pulmonary clinical specimens or cultivated samples.*

Advantages:

- Detection of XDR-TB in patients previously diagnosed with MDR-TB.
- Confirmation of DST results.
- Results are obtained in 4–5 hrs only.
- High sensitivity, specificity and diagnostic reliability

GeneXpert®MTB/RIF

The rapid and fully automated Xpert®MTB/RIF test is a TB-specific real time cartridge-based automated DNA amplification test. It is highly sensitive for confirmation of both smear positive and smear negative samples.

Advantages:

- Deliver a highly accurate result in less than 2 hours.
- Simultaneous detection of both MTB and rifampicin resistance, a marker for MDR strains, as up to 95% of rifampicin resistance strains are INH resistance.
- sensitivity for detecting MTB even in smear negative, culture positive specimens

Disadvantages:

- Xpert® MTB/RIF cannot be used for treatment monitoring, as it detects both live and dead bacteria.
- Microscopy culture and drug sensitivity testing are still required to monitor treatment progress and to detect other types of drug resistance.

The cost of the test and high technical requirements are the disadvantages.

The main advantages of the test are reliability when compared to sputum microscopy and the speed of getting the result when compared with culture.

For diagnosis of TB, although sputum microscopy is both quick and cheap, it is often unreliable. Although culture gives a definitive diagnosis, to get the result usually takes weeks rather than the hours of the Xpert test. The main advantage in respect of identifying rifampicin resistance is again the matter of speed.

Benefits of Genotypic Methods

Culture sensitivity remains the “gold standard” for screening of MDR-TB. However, Lowenstein Jensen method is highly time consuming (6-8 weeks). Radiometric methods such as BACTEC 460 have enabled to reduce the detection time to 2 weeks.

Phenotypic methods do not satisfy the requirement of rapid and sensitive results. Thus, genotypic methods for screening of mutations responsible for drug resistance forms a better alternative before starting the treatment empirically

Drug Susceptibility testing

Susceptibility testing may be conducted directly (with the clinical specimen which takes 3 weeks) or indirectly (with mycobacterial cultures which takes 8 weeks) on solid or liquid medium. Molecular methods are used for the rapid identification of genetic mutations.

In a wild susceptible bacillary strain (which has never been in contact with anti-tuberculosis drugs) from a case with cavitary pulmonary tuberculosis, the majority of bacilli are susceptible, but some rare bacilli are resistant to the different anti-tuberculosis drugs: these are resistant mutants. These bacilli appear in a susceptible strain, without having been in contact with an antituberculosis drug.

This phenomenon of mutation is:

- Spontaneous: *mutation occurs in a bacillary strain without the strain's having come into contact with anti-tuberculosis drugs.*

- Rare and specific: *in a population of 10⁸ bacilli, the probability of finding resistant bacilli varies depending on the anti-tuberculosis drug: a single mutant resistant to rifampicin, 10³ to isoniazid, 10³ to streptomycin, 10⁴ to pyrazinamide.*

- Hereditary: this mutation is transmitted to all the bacilli that result from the multiplication of the resistant mutant.

On the other hand, when a strain is resistant to an anti-tuberculosis drug, most of the bacilli are resistant to this drug, and the rest of the strain is composed of susceptible bacilli and some mutants resistant to the other drugs.

Therefore, when a patient presents with a strain that is resistant to an antituberculosis drug, the whole bacillary population will contain a very high proportion of resistant bacilli. To determine the resistance of a strain to antituberculosis drugs, the classic method used is the “proportion method”, based on the determination of a sufficiently high proportion of colonies of resistant bacilli in the entire bacillary population, in order to confirm the resistance of the strain.

Phenotypic testing in the laboratory

Minimum inhibitory concentration is the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism. *Visibility of growth starts at 5x10⁵ cells/ml. It was found that drug-susceptible strains of M. tuberculosis that have not been exposed to anti-TB drugs (wild-type strains) do not exhibit much variation in minimum inhibitory concentration to those drugs.*

Critical concentration is defined as drug concentration that inhibits the growth of wild-type strains, without affecting the growth of strains with alterations in drug susceptibility. *This categorizes a clinical M.tuberculosis isolate as either susceptible or resistant.*