CHAPTER II
LITERATURE REVIEWS

General Characteristics

- **Definition**

The genus *Mycobacterium* is currently the only genus in the family Mycobacteriaceae, orders Actionmycetales, and related to other mycolic acid-containing genera. The minimal standards for including a species in this genus are:

- The acid-alcohol fastness (i.e. Resist decolorization by acidified alcohol after being stained with a basic fuchsin dye).
- The presence of mycolic acids containing 60 – 90 carbon atoms, which are cleaved to C_{22} – C_{26} fatty acid methyl esters by pyrolysis.
- The G + C content of the DNA of 61 – 71 mol% [30], with the only exception being *M. leprae* (>57 percent)[31].

Mycobacteria are nonmotile, nonspore-forming, weakly gram-positive, aerobic or microaerophilic, straight or slightly curved rod-shaped bacteria (0.2-0.6 x 1.0-10 μm) [32]. Some mycobacteria display coccobacillary or branched forms. Filamentous growth may occur, but on slight disturbance usually becomes fragmented into rods or coccoid elements. Currently, there are about 100 mycobacterial species that are usually grouped into two major divisions, 'slowly growing' and 'rapidly growing' mycobacteria. The slow growers require more than 7 days producing visible colonies on solid media from a dilute inoculum under ideal culture conditions. Colony morphology varies among the species, ranging from smooth to rough and from nonpigmented (nonphotochromogens) to pigment (yellow to orange, rarely pink). Some of the latter require light to form pigment (photochromogens), while orther, produce pigment in either the light or in the dark (scotochromogens) [33]. These divisions have, however, no formal taxonomic standing but are useful for a preliminary identification. Slowly growing mycobacteria were among the first bacteria to be ascribed to specific diseases. In 1874, Armauer Hansen identified a rod-shaped bacillus (Bacillus leprae) in a tissue biopsy from a patient and suggested that it was the

etiological agent of leprosy [34]. Eight years later, Robert Koch identified another rod-shaped bacillus (Bacterium tuberculosis) as the causative agent of tuberculosis (TB) and formulated Koch's postulates for establishing a causal relationship between a suspected pathogen and a given disease [35]. These two species were subsequently renamed *Mycobacterium leprae* and *Mycobacterium tuberculosis*, respectively, and placed in the genus Mycobacterium ('fungus bacterium,' named to reflect the mould-like pellicle formed by *M. tuberculosis* on liquid media) [36]. At the beginning of the twentieth century, *M. tuberculosis* was the only species of Mycobacterium routinely isolated from, and associated with, human disease. As time went on, more and more species of environmental mycobacteria, called nontuberculous mycobacteria (NTM) (formerly atypical mycobacteria or mycobacteria other than tubercle bacilli (MOTT)), were recognized as causes of human disease.

- **Habitat**

Isolated from a wide variety of environmental sources, including water, soil, dust, and Sphagnum vegetation[37-39] mycobacteria have been classified as obligate pathogens, facultative or opportunistic pathogens, or free-living saprophytes. Obligate pathogens are species that do not appear to multiply outside their hosts and include *M. tuberculosis* complex (i.e. *M. tuberculosis, M. bovis, M. bovis* BCG, *M. africanum, M. microti,* and *M. canettii*), *M. leprae, M. marinum*, and *M. paratuberculosis*. TB manifests both pulmonary and extrapulmonary in humans and warm-blooded animals and has become a new threat in developing countries in the most recent past. Facultative pathogens are able to multiply outside the host as well and cause infections in humans or animals and include, among others, *M. avium complex, M. kansasii, M. malmoense, M. simiae, M. scrofulaceum, M. ulcerans,* and *M. xenopi*. Free living saprophytes such as *M. gastri, M. gordonae*, and *M. nonchromogenicum* do not generally cause progressive infection or disease in immunocompetent individuals.

- **Nutritional requirements and metabolic activities**

- Carbon and nitrogen sources

Important carbon sources are carbohydrates (e.g. glucose, fructose, sucrose, mannose, trehalose, inositol, mannitol), organic acids, including short-chain (pyruvic, acetic, and citric) and long-chain (oleic and palmitic) acids, hydrocarbons (polycyclic aromatic compounds), and even CO_2, which is essential for optimal growth of many species and usually included in media as $NaHCO_3/Na_2CO_3$ or as CO_2 in the gas phase (5-10 percent). Laboratory culture medium traditionally contains glycerol because it is the only carbon source that can be utilized by all members of the genus, although it is not the optimal C-source for many species. For instance, pyruvate is a better C-source for *M. bovis*. Expressing a number of hydrolytic and degradative activities, mycobacteria may be able to utilize additional sources of carbon, such as Tween 80, which can be hydrolyzed to oleic acid. In growth media, the preferred nitrogen source is asparagines or glutamine, although mycobacteria can obtain nitrogen from many inorganic (e.g. NH_4^+ salts) and organic sources (e.g. amides, amines, amino acids, nucleosides). A few species may be able to assimilate nitrate or nitrite via nitrate and nitrite reductases to generate NH_3.

- Acquisition of iron

Iron is essential for the growth of mycobacteria both in vivo and in vitro. In secreting small water-soluble iron-binding siderophores called exochelins, mycobacteria have evolved a sophisticated system for scavenging iron from the environment and their eukaryotic hosts [40]. The MS-type of exochelin (e.g. produced by *M. smegmatis*) remains water-soluble at all stages, whereas the MB-type (e.g.produced by *M. tuberculosis*) becomes chloroform extractable when complexed with Fe (III). Each type of exochelin actually encompasses a family of small molecules whose masses range from approximately 0.7 to 0.8 kDa, which reflects varying numbers of CH_2 groups on an alkyl side chain [41]. Exochelins extract iron from various iron-containing molecules in their vicinity (e.g. ferritin) by virtue of their very high affinity for ferric ions (K_s of approximately 10^{25} M) which is used as a growth supplement for fastidious mycobacteria. The ferriexochelin can then interact with a specific protein receptor on the mycobacterial cell surface and be transported to

the interior of the cell. Alternatively, the ferric iron may be transferred to a related membrane-bound molecule called mycobactin. Mycobactins and exochelins share a common iron-binding core but differ in alkyl side chains, which accounts for the differences in polarity. Mycobactins can also bind free ferric ion and facilitate the transport of it across the membrane to the interior of the bacterium. Release of the iron from either ferriexocheling or ferrimycobactin is accomplished by reduction of the ferric ion to ferrous ion by an NADH-dependent reductase. Because of the efficient iron-scavenging systems, most mycobacteria require only small amounts of iron (1 µg Fe per ml) in culture medium for growth. *M. haemophilum* is unusual in that it requires high iron concentrations (2 percent ferric ammonium citrate or 40 µg hemin per ml) for growth.

- **Metabolic pathways in mycobacteria**

In general, metabolic activities of mycobacteria are similar to those of other bacteria. Of course, mycobacteria do have several unique biochemical activities related to the synthesis of mycobacteria-specific compounds, such as mycolic acids or the phenolic glycolipid I. Also, individual species express characteristic sets of enzymatic activities, probably related to their ecological niches, and these characteristic activities are exploited in species identification tests. Relatively little is known about metabolic activities in two environments that are important in mycobacteria caused diseases. First, the environment encountered by an intracellular pathogen is quite different from that encountered on an agar slant. Second, *M. tuberculosis* can persist in the human host for long periods in an apparently metabolically dormant state. These bacilli cease to replicate, reduce their oxidative metabolism, and survive in anaerobic conditions much longer than actively metabolizing cells. Conversion of lipids into carbohydrates through the glyoxylate-shunt pathway may be essential for 'dormant' *M. tuberculosis* cells, allowing long-time survival in the host [42, 43].

- **Cell-wall structure and components**

Mycobacteria have a complex outer envelope composed of several distinct layers [44, 45]. The innermost layer is the plasma membrane, which displays an asymmetric bilayer structure. Inserted into it are proteins, phosphatidylinositol mannosides, and lipoarabinomannan. Next is the peptidoglycan layer, which determines the shape of the cell and is similar to peptidoglycans of other gram-positive bacteria. It contains repeating disaccharide units of N-acetylglucosaming (β1-4)-N-glycolylmuramic acid cross-linked via L-alanyl-D-isoglutaminyl-meso-diaminopimelyl-D-alanine tetrapeptides. About 10 percent of the N-glycolylmuramic acid residues are covalently attached to a branched-chain polysaccharide, arabinogalactan, via phosphodiester bonds. Distinct arabinose residues of the arabinogalactan molecules are esterified to high-molecular-weight mycolic acids. Finally, the outer surface of the mycobacterium is formed by the intercalation of medium-chain (e.g. mycocerosates) and short-chain (e.g. acylglycerols) lipids, glycolipids, and peptidoglycolipids into the uneven hydrophobic layer of mycolic acids. Proteins (e.g. porins, transport proteins) are found throughout the various layers.Several components of the mycobacterial envelope are strongly immunologically active [46]. It is the peptidoglycan (murein) layer that contains this adjuvant activity. A water-soluble fragment of the peptidoglycan called muramyl dipeptide (MDP) (N-acetylmuramyl-L-alanyl-D-isoglutamine) also acts as an adjuvant. MDP has the advantages over heat-killed mycobacteria in that it can be chemically systhesized, is commercially available, and is not immunogenic (i.e. it does not elicit an immune response to itself). Lipoarabinomannan (LAM) is also highly immunoreacctive.

- Lipid

A prominent feature of the outer surface of mycobacteria is its high content of lipid, which accounts for up to 60 percent of the cell-wall weight and contributes to several biological features, including the hydrophobicity of mycobacteria, their tendency to form clumps and cords, their resistance to common lysis procedures, and their ability to survive for a long period of time. In general, the lipids are long-chain fatty acids (e.g.CH_3 $(CH_2)_{nCOOH}$), which are often modified by the presence of

unsaturated bonds, cyclopropane rings, or side groups such as methyl, methoxy, hydroxyl, and keto groups.

Lipoarabinomannan

LAM is structurally and functionally related to the O-antigenic lipopolysaccharides of other bacteria. LAM is thought to be anchored in the plasma membrane and to extend through the cell wall to the surface of the mycobacteria. The membrane anchor is a diacylglycerol moiety, which in *M. tuberculosis* contains palmitic acid and tuberculostearic acid (TBSA) and which is attached to a phosphatidylinositol residue via a phosphodiester linkage. The polysaccharide backbone is also attached to the phosphatidylinositol and consists of α (1-6)-linked D-mannose residues to which short side chains of α (1-2)-linked D-mannose residues and α (1-5)-linked D-arabinose residues are attached. Thetermini of the LAM molecules may be branched hexa-arabinosides or linear tetraarabinosides [47] and the arabimose termini may be capped with one to three mannose residues [48]. Biological activities of LAM include strong seroreactivity, inhibition of interferon-γ-mediated activation of macrophages, induction of cytokine production and release by macrophages, scavenging reactive oxygen intermediates, and suppression of T-cell proliferation [49-52]. Several parts of LAM are involved in these biological activities. The terminal arabinose units are targets of circulating antibodies, and the degree of mannosylation of the terminal arabinose units influences the biological activities of LAM (e.g.ability to induce tumor necrosis factor alpha (TNF-α) production)[49]. This difference may be related to virulence. The acyl groups are also required for some biological activities, such as the induction of cytokine production, because removal of the acyl groups by mild alkali treatment abolishes the activity, LAM has been identified as a key ligand of dendritic cell (DC)-specific intracellular adhesion molecule-3 grabbing nonintegrin (SIGN). DC-SIGH-mediated entry of *M. tuberculosis* in DCs in vivo is likely to influence bacterial persistence and host immunity [53]. In addition, the cell-surface receptor DC-SIGH discriminates between *Mycobacterium* species through selective recognition of the mannose caps on LAM [54].

Mycolic acids

Mycolic acids are a defining characteristic of members of the genus *Mycobacterium*, although related long-chain fatty acids also are found in the genera *Corynebacterium* (containing 28-40 carbon atoms) and *Nocardia* (containing 40-60 carbon atoms). The mycobacterial mycolic acids are α-branched, β-hydroxy fatty acids with 60-90 carbon atoms in the primary chain. The alkyl branches are attached to the α-position (the CH_2 group adjacent to the terminal carboxylic acid) and typically contain 22-26 carbon atoms. Mycolic acids are categorized according to oxygen-containing modifications found in the primary chain. If not containing any oxygen functions, in addition to the β-hydroxy group they are called α-mycolates. Those containing keto groups are ketomycolates, those with methoxy groups are methoxymycolates, and those with epoxy groups are epoxymycolates. Each species of Mycobacterium appears to synthesize a unique set of mycolic acids, and this has been exploited for identifying mycobacteria[55]. The resulting pattern is compared with a library of reference patterns to identify the species.

- Acetylated trehaloses

In 1950, Bloch Isolated 6.6′-dimycolyl-α-α′-D-trehalose (trehalose dimycolate (TDM)) and suggested it was the cell-surface component responsible for the ability of virulent tubercle bacilli to form serpentine cords and to absorb the cationic phenazine dye neutral red. Subsequent studies have not borne out a role for TDM in either cord formation or virulence. Regardless, biological activities associated with cord factor include systemic toxicity, granulomagenic activity, and cytokine induction [56]. Other trehalose-based lipids found in *M. tuberculosis* are sulfolipids, which are thought to play a role in the intracellular survival of tubercle bacilli by inhibitihg phagosome activation [57]. The sulfolipids are based on trehalose 2′-sulfate acetylated with hydroxyphthioceranic, phthioceranic, and saturated straight-chain fatty acids such as palmitate and stearate. Additional polar [58] and nonpolar [59] acetylated trehaloses have been described and suggested to be virulence factors, because these compounds have been found in virulent strains of *M. tuberculosis* only.

- Mycosides

The outer surface of a mycobacterium contains a heterogeneous group of biologically and immunologiacally active medium- and short-chain lipids, which are analogous structurally and functionally to the O antigens of gram-negative enteric bacteria. The mycosides fall into two major groups:

1. A peptidoglycolipids, which contain mycoserosic acid, sugars and amino acids.

Peptidoglycolipids often contain unusual sugar residues such as O-methyl rhamnose, fucose, or deoxytalose and play roles in determining serotype, colony morphology, and virulence. For example, peptidoglycolipids form the basis of an agglutination serotyping scheme for members of the *M. avium* complex. A peptidolipid also serves as the receptor for phage D4 attachment to mycobacterial cells.

2. A phenol-phthiocerol glycosides.

Mycosides lacking amino acids are typically derivatives of phenol-phthiocerol and have been isolated and characterized from *M. kansasii, M. bovis, M. marinum*, and *M. leprae*.

Table1 Classification of Selected Mycobacteria Pathogenic for human [36]

Groups	Organism
Non-Runyon group	*M. tuberculosis* *M. leprae* *M. africanum* *M. bovis* *M. bovis (BCG strains)*
Runyon group I (Slow-Growing Photochromogens)	*M. kansasii* *M. marinum* *M. simiae* *M. asiaticum*
Runyon group II (Slow-Growing Scotochromogens)	*M. szulgai* *M. scrofulaceum* *M. xenopi* *M. gordonae* *M. flavescens* *M. celatum*

Table1 Classification of Selected Mycobacteria Pathogenic for human (continued)

Groups	Organism
Runyon group III (Slow-Growing Nonchromogens)	*M. avium complex* *M. genavense* *M. haemophilum* *M. malmoense* *M. ulcerans* *M. gastri* *M. terrae* *M. triviale* *M. nonchromogenicum*
Runyon group IV (Rapid Growing)	*M. abscessus* *M. chelonae* *M. fortuitum* *M. mucogenicum* *M. phlei* *M. smegmatis* *M. vaccae* *M. shimoidei*

Species frequently involved in human

- ***M. tuberculosis* complex**

The term *tubercle bacillus* designates two species of the family Mycobacteriaceae, order Actinomycetales: *M. tuberculosis* and *M. bovis*. They different form many other mycobacterial species that share the staining characteristic referred to as acid fastness. Three other species- *M. microti*, a pathogen for rodents, and *M. africanum* and *M. canetti*, both rare causes of tuberculosis in Africa- are closely related and are the other members of the *M. tuberculosis* complex. The progenitor of these organisms likely arose from a soil bacterium, and the human bacillus may have arisen from *M. bovis* following the domestication of cattle. Disease caused by *M. bovis* is relatively rare, and the terms tubercle bacillus and *M. tuberculosis* are, practically speaking, synonymous.

Human are the only reservoir for *M. tuberculosis*, although many forming, nonmotile bacillus with a high cell wall content of high molecular weight lipids. Growth is slow, the generation time being 15 to 20 hours, compared with much less than 1 hour for most common bacterial pathogens and visible growth takes from 3 to 8

weeks on solid media. The organism tends to grow in parallel groups, producing the colonial characteristic of serpentine cording. The complete 4.4 Mb circular genome sequence of the H37Rv strain of the *M. tuberculosis* was recently reported. Initial analyses indicate that, in radial contrast to other bacteria, a very large portion of *M. tuberculosis* genes encode enzymes involved in lipogenesis and lipolysis. Knowing the genome sequence should accelerate progress in tuberculosis.

- ***M. avium*** complex

The bacteriological and clinical aspects of MAC infections have been reviewed by [60] and [61]. MAC organisms show characteristic heterogeneous colony morphology. Small translucent (smooth transparent (SMT)) colonies usually co-occur with glossy, whitish colonies (smooth domed (SMD)). There is even a third flat and dry morphology, which resembles *M. tuberculosis*. SMT are more frequently isolated from blood in AIDS patients [13, 62]. Isolates from patients with disseminated MAC often contain mixtures of colony morphologies [63-65]. SMT bacteria have greater potential for intracellular multiplication in macrophages, have greater virulence in animal models, and are more resistant to antibiotics than SMD bacteria.

Traditionally, MAC has consisted of 28 serotypes ('serovars') comprising *M. avium* and *M. intracellulare*. Serovars 1-6, 8-11, and 21 are *M. avium*, while serovars 7, 12-20, and 22-28 are *M. intracellulare*. Inclusion of *M. scrofulaceum* (serovars 41-43) in the group *M. avium- M. intracellulare- M. scrofulaceum* (MAIS complex) is no longer appropriate. *M. avium* and *M. intracellulare* are easily distinguishable with molecular methods [66]. Based on phenotypic and genetic characteristics, three subspecies of *M. avium* have been proposed: subsp. Avium, subsp. *Paratuberculosis*, and subsp. *Silvaticum* [67].

MAC has been isolated from bedding material, house dust, soil, plants, swimming pools, hospital water, and natural bodies of water [68-70]. The true prevalence of MAC infections is unknown because mycobacterioses are not reportable [17, 71]. Poultry, swine, and cattle may also be infected by MAC, which gets into the soil by fecal shedding from birds but not from cattle or swine [60]. Serovars similar to those from birds have been isolated from humans living in their close proximity, but animal-to-human transmission of infection has not been documented. The

predominant serovars in human and animal infections are different [72]. In spite of widespread environmental exposure to MAC in certain geographical regions, the incidence of clinical disease in immunocompetent hosts is extremely low. *M. avium* serovars account for more than 90 percent of typeable MAC that cause infection in AIDS, whereas in other patients almost half the isolates are *M. intracellulare* [73, 74]. In the USA, for unknown reasons, bacteremia in AIDS is associated with a limited number of *M. avium* serovars (serovars 1, 4 and 8).

- ***M. kansasii***

M. kansasii forms visible niacin-negative, photochromogenic colonies on LJ slants at 2-3 weeks. After 2 weeks in ambient light, the colonies turn bright yellow or orange. The 'yellow bacillus,' as it was originally called, must be distinguished from other photochromogenic species, such as *Mycobacterium simiae* and *Mycobacterium szulgai*, which also may cause pulmonary mycobacterioses. In sputum smears, *M. kansasii* may appear as distinctive large, cross-barred AFB [75]. Closely related to *M. gastri*, molecular studies have defined five genotypes of *M. kansasii*, all of which are able to cause human disease [76]. Subtyping *M. kansasii* may improve clinical management by distinguishing pathogemic from nonpathogenic subtypes, as demonstrated very recently in Switzerland [77]. The environmental reservoir of *M. kansasii* is unknown; it has occasionally been isolated from water sources but not from soil [75].

- ***M. scrofulaceum***

Derived from 'scrofula,' the historical term was used to describe mycobacterial infections of the cervical lymph glands. Until the 1980s, the organism was the most common cause of mycobacterial cervical lymphadenitis in children. Nowadays, it has been replaced primarily by MAC [78]. There are only a few reports of other human diseases, e.g. pulmonary disears, conjunctivitis, osteomyelitis, meningitis, granulomatous hepatitis, and disseminated disease.

- ***M. fortuitum***

M. fortuitum is mycobacteria in the rapidly growing mycobacteria (RGM) group. They were initially separated from the other nontuberculous mycobacteria by Runyon as members of his Group IV; as defined by him, these are 'Acid-fast bacilli which from small inocula mature at room temperature (25ºC) within one week. Unfortunately, not all isolates of all species of mycobacteria separate cleanly as either slow or rapid growers. For example, a significant percentage of *Mycobacterium marinum* and *Mycobacterium terrae* isolates-perhaps more than half of each-meet the technical definition of a RGM, although both species are traditionally grouped with the slow growers. It is also important to note that the definition of an RGM is not based on the time to detection of the organism on initial isolation; especially with modern liquid-based culture methods, some isolates of species generally considered to be slow growers, particularly when present in high concentration in a specimen, may occasionally be detected in less than 7 days. Conversely, some isolates of species that are generally considered to be RGMs may take more than 7 days to grow on initial isolation, especially if the culture is not incubated at the temperature optimum for the species in question. A further problem in the accurate initial categorization of an isolate is that different species of RGMs may be photochromogenic, scotochromogenic, or nonphotochromogenic (key features of the Runyon group I, II, and III, respectively, of slowly growing nontuberculous mycobacteria). Therefore, not only may some mycobacterial isolates that belong to species considered to be slow growers initially be thought to be RGMs, but also some isolates that belong to species that are considered RGMs may initially be thought to belong to one of the groups of slowly growing nontuberculous mycobacteria.

The disease

- **Tuberculosis**

M. tuberculosis is transmitted almost exclusively in cough spray from patients with open pulmonary TB and gains access to the body by inhalation of infective droplets usually less than 5 μm across. Patients with sputum that is positive on direct microscopical examination, and thus contains at least 5,000 bacilli in 1 ml, are the principal sources of infection [79]. Not all patients infected by the tubercle bacillus

develop overt disease. Only 10 percent of nonimmunocompromised infected people eventually develop active TB, 5 percent within the first 2 years following infection and 5 percent during their lifetime. The disease ratio in HIV-positive people is much higher, as indicated above. The interval between the initial infection and overt disease varies from a few weeks to several decades, and post-primary disease may be due to endogenous reactivation of the initial infection or to exogenous reinfection.

The initial lesion is usually in the lung, from which organisms reach other organs via the lymphatics and the bloodstream. Hematogenous spread occurs in primary TB with implantation of bacilli in many organs. In some people, particularly children under 3 years of age, these foci progress to serious, even fatal, disease principally involving the meninges, kidney, bones, and pleurae. Foci developing in the endothelium of major blood vessels may rupture and give rise to widespread small granulomata, a disease termed 'miliary TB' (Latin: milium, a millet seed). Disease occurring in a person never previously exposed to a tubercle bacillus is termed 'primary TB.' Infection results in a focus of disease (the Ghon focus) at the sites of implantation of the bacillus, usually the lung but occasionally other tissues. This focus, together with enlarged, infected regional lymph nodes, is termed the 'primary complex.'

The primary complex often resolves, but it may cause serious local complications. Most primary complexes resolve spontaneously, but a few tubercle bacilli may enter the poorly understood state of persistence of latency. Such infected but healthy people are usually, but not invariably, tuberculin-positive.

- Pathogenesis and immunology of TB

The clinical and histological features of TB are the result of the virulence of the tubercle bacillus and, more critically, the nature and effectiveness of the host's defense mechanisms. Protective immune reactions in TB are principally cell-mediated, relying on macrophage activation and granuloma formation. Another important defense mechanism is the recognition and destruction of exhausted macrophages and other cells in which tubercle bacilli are replicating. Thus, regulated and directed cell destruction is an inevitable and essential part of the protective immune response in

tuberculosis [80, 81]. The outcome of infection by the tubercle bacillus depends critically on whether the host responds with a protective or tissue-necrotizing reaction.

Tubercle bacilli entering the tissues are taken up by macrophages. Entry of *M. tuberculosis* into human monocytes appears to be by a phagocytic mechanism mediated by complement receptor and the macrophage mannose receptors [82, 83]. Recent results showed that heparin- or fibronectin-binding proteins, present on the bacterial surface, play a role to facilitate the binding to epithelial cells or macrophages [84, 85]. Once inside the macrophage, the intracellular mycobacteria employ a variety of survival strategies, which include: (1) prevention of an oxidative burst in phagocyte cells and inhibition of phagosome-lysosome fusion; (2) resistance to lysosomal enzymes and reactive oxygen intermediates (by means of cell-wall lipids, including peptidoglycolipids (mycosides) and LAM and secretion of superoxide dismutase; and (3) escape from the phagosome into the cytoplasm [6, 86, 87].

If the bacilli are not destroyed, they replicate and kill the cell. A local area of inflammation is thus established and more phagocytes are attracted to the site. Some bacilli are transported, probably within phagocytes, to the regional lymph nodes, where they are engulfed by antigen-presenting cells (APC). Other bacilli are transported further a field and may cause one of the extrapulmonary forms of primary disease such as tuberculous meningitis.

Epitopes from mycobacteria lying within phagosomes within the APC are presented on the cell surface by the major histocompatibility complex (MHC) class II (HLAD) molecules to CD4+ helper T-cells, which undergo activation and clonal proliferation. These T-cells produce a range of cytokines, including interferon-γ (IFN-γ), which activates macrophages. Some T helper cells, however, also produce factors that lead to tissue-destroying hypersensitivity, as described below.

If the tubercle bacilli proliferate within the APC and escape from the phagosomes, their epitopes are presented to CD8+ T-cells by MHC class I (HLA-A and B) molecules. The CD8+ lymphocyte population contains cytotoxic T-cells that are able to lyse any cell presenting antigen in this manner.

- **Diseases caused by nontuberculous mycobacteria**

NTM are frequently found in environmental habitats that may colonize and occasionally cause infection in humans and animals. Such infections are termed mycobacterioses. The increasing prevalence of immunocompromised hosts, particularly in relation to the AIDS pandemic NTM infections are becoming more prevalent [88]. The characteristics of these infections differ from those seen in immunocompetent human hosts, as do the organisms involved. Patients with depressed cellular immunity, such as those who have AIDS, lymphoproliferative disorders or transplants, and those on immunosuppressive therapy, are at particular risk.

AIDS patients differ from other immunocompromised patients in that high numbers of NTM can be recovered from blood. However, in these patients, NTM can also cause pulmonary infections [89]. Although NTM are recoverable from sputum or bronchoalveolar lavage fluid, true invasion of the lung is difficult to demonstrate and pulmonary symptoms are often minimal. The radiographic appearance of NTM in AIDS differs from that in immunocompetent hosts. The chest X-ray may be normal or show nonspecific mediastinal and/or hilar adenopathy or, rarely, patchy alveolar infiltrates. The frequent detection of NTM in blood cultures reflects the disseminated nature of infection and the high bacterial burden. The mechanism by which organisms in the blood become recoverable from respiratory secretions is unknown. The increased incidence of disseminated *M. avium* complex (MAC) infection in AIDS patients is attributable to two principal factors: (1) greater surveillance since MAC bacteria were recognized as a potentially treatable cause of morbidity and (2) the increased survival of patients with AIDS. Rarely, there may be a genetic basis for disseminated NTM infection.

In transplant patients [90], NTM infections generally occur late in the posttransplantation period (range 10 days to 269 months; mean 48 months). Most are chronic infections of soft tissues and joints (cutaneous lesions on the extremities, tenosynovitis, and arthritis) and osteomyelitis, often with multifocal involvement. Fever, leukocytosis, night sweats, weight loss, and lymphadenopathy are usually absent. Skin lesions are often painful erythematous subcutaneous nodules, which can progress to abscess formation. The most commonly involved joints include finger joints, wrists, elbows, ankles, and knees, and in these patients diagnosis is often

delayed by several months. Pulmonary involvement occurs in approximately a quarter of patients, with radiographic evidence of pulmonary nodules or infiltrates, and over half the patients have concomitant extrapulmonary involvement. Pulmonary involvement is most frequently with *M. kansasii*. Risk factors for mycobacterioses in solid-organ-transplant recipients are poorly defined. For heart recipients, they include a history of open heart surgery and immunosuppressive therapy. The environment is the likely source in patients with transplant-related immunosuppression and other immunodeficiencies.

Patients with the uncommon 'hairy cell' leukemia appear to have a predilection for mycobacterial infection (approximately 5 percent of patients), mainly with *M. kansasii* and MAC [91]. In contrast, infections by NTM in cystic fibrosis patients are common [92].

In the immunocompetent host, NTM can cause infections in cutaneous, deep soft tissues lymphatics, and other sites (e.g. skeletal, peritoneal catheter-related, ocular). These mycobacterioses are rare, indolent, and frequently misidentified. Active infections in immunocompetent hosts cause granulomatous inflammation of the lung.

However, very often, NTM lack clinical relevance and are, therefore, incorrectly labeled as colonizers or as the cause of infection. Discrimination between colonization and infection by NTM can be difficult. Some of the most important American Thoracic Society (ATS) criteria for the diagnosis of pulmonary mycobacterioses are as follows: cavitary or noncavitary infiltrates (e.g. nodular infiltrates or bronchiectasis) should be present on a chest X-ray. Two or more repiratory specimens (sputum or bronchial washings) should demonstrate AFB on smear examination and/or moderate to heavy growth (2+ to 4+) on culture.

To date, human-to-human and animal-to-human transmission of NTM has not been documented. Inhalation of contaminated aerosols is the presumed mode of inoculation of the lung. Geographical clustering of infections often matches the environmental distribution of NTM and supports the hypothesis that infections arise from environmental exposure. Host susceptibility is a factor in the development of infections, because in some areas exposure is universal. It is not known whether, as in the case of *M. tuberculosis*, latent infection is part of pathogenesis. Because of cross-

reactivity between shared antigens, species-specific skin test has not proved reliable to measure immune responses.

Susceptibility to antimicrobial agents

- ***M. tuberculosis* complex**

Diseases caused by *Mycobacterium* spp, are difficult to treat, and the search for effective drugs has been active, the most intensive search being for drugs offective against TB, the mycobacterial disease of greatest public health significance. Doub recounts some of the early events leading to the development of anti-TB drugs and concludes that 'of those that succeed (as anti TB drugs), it is a story of chemical intermediates for planned drugs. Synthetic drugs active against *M. tuberculosis* include PAS, PZA, INH, ethionamide, and EMB. Antibiotics are RMP, other rifamycins (e.g. rifabutin), SM, viomycin, kanamycin, capreomycin, and cycloserine. One of the newer active drugs is ofloxacin, a fluorinated carboxyquinolone, and linezolid, an oxazolidinone compound, has promising effects as well. The modes of action of the antimycobacterial drugs have been reviewed [93].

The reliability and standardization of methods for testing the susceptibility of *M. tuberculosis* isolates to anti-TB drugs were discussed by Canetti et al.[94], and the methods used routinely in most mycobacteriology laboratories have been reviewed. A key to producing reliable results is the use of standardized drug concentrations to determine if a strain is resistant. As defined by Canetti 'Resistance is clinically significant when at least 1 percent of the total bacterial population develops at the so called critical concentrations. The 'critical concentration' is the weakest concentration of drug that inhibits the growth of more than 95 percent of wild-type isolates of a given species using precisely defined experimental conditions. For *M. tuberculosis*, the critical concentration of INH in 7H10 agar medium is 0.2 µg/ml, and EMB is 5.0 µg/ml [95], while the critical concentrations in liquid media may be different (NCCLS 2003). Additional concentrations are often tested to provide information to physicians to assist them in designing a therapeutic regimen. However, the critical concentration is always included so that there is a historical basis to determine whether resistance increases in specific populations over time. Recognition of the emergence of

resistance and the need to insert new drugs into a treatment regimen are dependent on collection of accurate data.

- Drugs

1. Isoniazid (isonicotinic acid hydrazide) was first synthesized in the early part of the twentieth century, but its activity against tubercle bacilli was not discovered until mid-century. The drug has been the main stay of antituberculosis therapy for more than 40 years, but neither the target site in the mycobacterial cell nor the mode of action is well understood [93, 96]. The antibacterial spectrum of INH is limited largely to inhibition of strains of *M. tuberculosis*. The MIC for susceptible strains of *M. tuberculosis* is usually less than 0.05µg/ml, and other species in the *M. tuberculosis* complex are also highly susceptible. However, MAC strains and strains of other species are not susceptible to INH. Middlebrook (1954) found that there was a link between INH resistance and reduced catalase activity [97]. However, the exact relationship and molecular basis of INH resistance are still unknown. INH per se is not active and its activation is achieved by the catalase-peroxidase encoded by the *katG* gene. The KatG enzyme activates INH in a toxic metabolite that blocks the synthesis of mycolic acids. Numerous mutations, including point mutations and deletions, have been identified in the *katG* gene. Those located in part of the gene corresponding to the NH_2 domain of the protein, i.e. the active site of heme binding, are correlated with a loss of the KatG activity and high resistance to INH whereas those located in part of the gene corresponding to the COOH domain, where INH binds, still have some KatG activity and are correlated with a low INH resistance. The low INH resistance may also be related to surexpression of two enzymes involved in the synthesis of mycolic acids, InhA and the InhB complex, composed of AcpM and KasA proteins. It has been proposed that activated INH Binds to NADH, and that this complex once bound to InhA, and enoyl-acyl carriew protein reductase, inhibits mycolic acid synthesis. InhA surexpression correlated with mutations in the promoter region of *inhA* confers low resistance to INH and crossresistance to the second-line drug ethionamide. The *kasA* gene encodes an enoyl-acyl carrier protein synthase involved in mero-mycolic acid synthesis. Mutations in *kasA* have been found in low INH-resistant strains and also in sensitive strains. Mutations in INH resistant strains

are associated with mutations in *katG* or *inhA*, and the significance of mutations in *kasA* is still unclear. Another gene, *ahpC*, has been involved. The AhpC protein contributes to detoxification of organic peroxides into alcohols, according to a second pathway of defence against oxidative stress products. Mutations in the promother region of *ahpC* yields and overexpression of AhpC, which is compensates for the loss or diminished activity of KatG. Altogether, mutations in these genes may account for approximately 90 percent of INH resistance.

2. Rifampicin was introduced in 1968 for the treatment of TB and is a first-line drug along with INH, PZA, and EMB. It is a broad-spectrum antibiotic that is active against *M. leprae, M. kansasii, M. haemophilum, and M. marinum*, as well as bacteria in other genera. The mechanism of action is similar to that in *E. coli* and involves inhibition of DNA-dependent RNA polymerase [93, 98]. Studies showed that more than 96 percent of RMP resistance could be attributed to mutations within an 81 bp region of the *rpoB* gene [7]. The molecular mechanism of rifampin resistance in *M. leprae* is similar to that in *M. tuberculosis*.

3. Pyrazinamide is a synthetic derivative of nicotinamide that is rapidly bactericidal for reproducing cells of *M. tuberculosis*; the average MIC is 20µg/ml. The drug is inactive against nonreplicating tubercle bacilli and against all other species of mycobacteria, including *M. bovis*, MAC, and rapid growers. Optimal in vitro activity of PZA is observed at pH 5.5-6.0. Neither the mechanism of action nor the molecular basis for resistance is fully understood [93] although there is evidence that the enzyme that hydrolyzes PZA to pyrazinoic acid, pyrazinamidase, is essential to susceptibility to the drug [99]. Lack of pyrazinamidase activity and its correlation with PZA resistance has been associated with mutations in the *pncA* gene that encodes the enzyme [100]. Apparently, 72-97 percent of PZA resistance can be attributed to mutations in that gene.

4. Ethambutol is a synthetic compound introduced in 1961 for the treatment of TB. Preliminary studies were carried out with the racemic mixture of the compound, but only the D-isomer is effective, which is less toxic than the racemic mixture. MIC

against wild-type isolates of *M. tuberculosis* are 1-5 μg/ml, but the drug is rather inactive against other species. EMB inhibits cell-wall synthesis and is bacteriostatic. EMB resistance correlates with a specific mutation at codon 306 in the *embB* gene, which encodes arabinosyltransferase. Mutations in this codon were associated with MIC of 20-40 μg/ml for several EMB-resistant isolates of *M. tuberculosis* [22]. The mechanism of action probably involves inhibition of arabinogalactan synthesis [101] or synthesis of precursors of cell-wall components [93]. The drug has a synergistic effect in combination with ciprofloxacin, amikacin, and rifampicin against *M. malmoense* [102].

5. Streptomycin has been used extensively in the treatment of TB. Amikacin and kanamycin can be administered as well while gentamycin and tobramycin are inactive against mycobacteria at the usual concentrations attained in serum. SM is active in vitro against *M. tuberculosis*, *M. kansasii*, and *M. marinum*. The drugs must be given by injection and, over the years, SM in particular, lost popularity and became difficult to obtain. Ototoxicity is the major side effect associated with aminoglycosides. Concerning SM resistance of *M. tuberculosis*, about 66 percent of strains display changes in the *rpsL* or 16S rRNA gene, as is seen in other bacteria [93]. This observation implies that there is at least one additional mechanism conferring resistance.

- Drug regimens

Today's standard regimen (directly observed therapy, short-course (DOTS) according to the WHO consists of a combination of three or four drugs (INH, RMP, EMB, PZA) for 2 months, followed by a dual combination of INH and RMP for 4 months. For multidrug-resistant TB, additional drugs have to be adder and length of therapy adapted.

- **NTM**

Treatment of infections caused by NTM is less well established than for tuberculosis. Usually, a combination of several drugs is being administered for a long period of time (months up to 1-2 years, depending on the species and the extent of disease). All NTM have in common that they are inherently resistant to PZA.

- Pulmonary infections

With the advent of molecular testing in the laboratory, it is important to recognize the species, its potential to cause clinical disease, its susceptibility to antimicrobial drugs, and its response to drug/surgical therapy. In particular, with NTM present in respiratory specimens, it is not always easy to address these aspects, since not all individuals with sputum cultures positive for NTM require immediate treatment. In patients with severe lung problems or those with a single smear-negative, culture-positive sputum specimen, treatment should be delayed to determine whether the infection is invasive. The guidelines of the American Thoracic Society in 1997 can be used to determine whether the infection is active. The need for treatment should be assessed critically in patients with medication intolerance or allergy, elderly patients with indolent disease, and patients in whom previous treatment has failed. Overall, prediction of treatment outcome from *in vitro* susceptibility tests continues to be a problem in infections with NTM [103, 104].

1. MAC

The MAC cell wall is a barrier to many antimicrobial agents [105]. MAC isolates are generally resistant *in vitro* to most antituberculous drugs, and *in vitro* susceptibility correlates poorly with clinical response [106, 107]. In the past, long-term treatment failures had been commonplace and only 45-65 percent of patients responded long term to early multidrug treatment regiments [108], but surgery combined with medical therapy has been effective in highly selected cases [109].

With the availability of newer drugs, better outcomes can be expected. The ESM drugs (e.g. clarithromycin, azithromycin) are the first with strong *in vitro* activity against MAC. They are concentrated in lung tissue and macrophages, have a long half-life, have a postantibiotic effect, and are well tolerated orally. Availability of ESM

antibiotics has led to a re-evaluation of *in vitro* susceptibility testing to predict the likely clinical response. Resistance to ESM *in vitro* may develop during monotherapy and thus predict treatment failure [110].

Another advance is rifabutin, a derivative of rifamycin, which is more active *in-vitro* against MAC than rifampicin. Preliminary data suggest that clinical responses are improved only modestly when rifabutin is used in place of rifampicin. Side effects unique to rifabutin are also a drawback to its widespread clinical use. Drug combinations, such as ethambutol and rifampicin, are synergistic *in vitro* and effective *in vivo*, although MAC is resistant to these agents individually *in vitro*.

Usually, a regimen of an ESM, ethambutol, rifampicin, or rifabutin is recommende. In severe disease the addition of streptomycin for the first 2-4 months of therapy is recommended. Treatment should be continued for 18 months to 2 years or at least 12 months after the last positive sputum culture.

2. *M. kansasii*

This is susceptible to most antituberculous agents except PZA. Some strains show intermediate resistance to INH. A recommended regimen is INH, RMP, and EMB daily for 18 months. Strains isolated from patients treated with RMP are predictably susceptible to the drug at 1µg/ml. As with most other pulmonary mycobacterioses, large controlled clinical treatment trials have not been reported. Medication regimens that include RMP have relapse rates of less than 8 percent [23]. Resistance to RMP, which is caused by point mutations in the *rpoB* gene [111], predicts a poor outcome. RMP resistance has arisen as a result of inadequate initial therapy and has been a greater problem in HIV patients [112]. Newer agents such as the ESMs and fluoroquinolones are active against *M. kansasii in vitro* and may be useful to treat patients infected with RMP-resistant bacteria [113, 114]. A rifabutin-based regimen has been suggested for HIV-positive patients receiving antiretroviral therapy [115].

3. Other NTM

Treatment of pulmonary disease caused by other NTM remains controversial since it is uncertain whether *in vitro* testing predicts clinical response in the way it does for *M. tuberculosis* and *M. kansasii* and to a lesser extent for MAC. Agents to be primarily considered are clarithromycin, EMB, and RMP, while the utility for amikacin, INH, rifabutin, ciprofloxacin, and streptomycin are less certain. In a large randomized trial of treatments for pulmonary disease caused by MAC, *M. malmoense*, and *M. xenopi* in HIV-negative patients, the research committee of the British Thoracic Society in 2001 missed a correlation of the in vitro susceptibility results with the patients' reponse to chemotherap. In that study, treatment of *M. malmoense* with RMP and EMB for 2 years was preferable to RMP, EMB, and INH, Conversely, addition of INH reduced the failure of treatment/relapse rates for MAC and *M. xenopi*, but there were higher death rates overall. Lang-Lazdunski et al. pointed out that resection represented an important adjunct to chemotherapy for the treatment of *M. xenopi* pulmonary disease [72].

- Extrapulmonary infections

Most *M. marinum* isolates are predictably susceptible to RMP and EMB. Alternative agents include clarithromycin, amikacin, ciprofloxacin, tetracyclines, and sulfamethoxazole-trimethoprim. Even though treatment with adequate drugs is asdministered, surgical intervention (excision or debreidement) is often necessary. Wild-type strains of *M. haemophilum* appear to be susceptible to quinolones, rifamycins, clarithromycin, and azithromycin, and usually resistant to EMB, INH, and SM. *In vitro* susceptibility test results may be helpful for the treatment of other NTM such as *M. xenopi, M. szulgai*, and *M. malmoense* because of the lack of information about the susceptibility patterns of these organisms. However, reports indicate that the correlation between susceptibility test results and therapeutic success was inconsistent [116].

Prevention and control of human TB

The principles of TB surveillance and control have been reviewed in detail by Styblo (1986). As TB is almost always spread from person to person, chemotherapy, by rendering source cases uninfectious, is the main component of control programs. The impact of chemotherapy on the spread of infection depends on the effectiveness of the therapeutic regimens, the adhercence (compliance) of the patients, and the percentage of cases that are diagnosed. The impact of chemotherapy on disease control also depends on the interval between onset of infectivity and commencement of therapy. The epidemiological impact of chemotherapy is considerably reduced if source cases have infected all their household contacts by the time they commence therapy. Patients receiving effective chemotherapy are rapidly rendered noninfectious even though they continue to excrete cultivable tubercle bacilli for several weeks. For practical purposes, patients are usually regarded as being noninfectiuous from the time that chemotherapy is started [79], though this is a risky assumption in regions and communities where multidrug resistance is common.

Cases may be detected passively, i.e. by waiting for those with symptoms to attend health centers, or actively, by searching for suspects, usually defined as people with a cough for 1 month or more in duration.

Contact tracing is a key component of tuberculosis control and up to 10 percent of cases of TB may be found in this way. Household contacts of patients with smear-positive pulmonary disease should be screened, as should the contacts of patients with nonrespiratory smear-negative pulmonary disease, as they may prove to be the source cases.

Vaccination strategies

A living, attenuated vaccine was produced by Calmette and Guerin from a tubercle bacillus isolated from a case of bovine mastitis by passaging it 230 times over a period of 11 years, by which time extensive animal studies showed that it had lost its virulence. This vaccine, bacilli Calmette-Guerin (BCG), was given orally to neonates in 1921 but is now given by injection or multipoint inoculation.

The protective efficacy of BCG vaccine has been the subject of considerable controversy, as a number of major trials have shown its efficacy to vary from around

80 percent to none at all [117]. The various explanations of these discrepancies are outlined elsewhere. When given to an uninfected (tuberculin negative) child, BCG offers protection against the serious forms of primary infection, especially those due to dissemination of bacilli from the primary complex. It is, however, much less effective in preventing tubercle bacilli from persisting in the tissues and becoming reactivated later in life, which greatly limits its use in the control of TB.

BCG protects equally well against leprosy as against tuberculosis and protects children against cervical lymphadenitis caused by environmental mycobacteria [118]. Thus, there is little doubt that the mycobacterial antigens that induce protective immunity are mostly or entirely among those that are common to all species in the genus.

Laboratory Diagnosis

In recent years, there have been a growing number of new, rapid methods in clinical mycobacteriology, but remarkably, to date there is no single test for the detection of TB that can stand alone. Recent advances in this area include nonradiometric, fully automated systems for liquid culture and drug susceptibility testing, chemical methods such as HPLC analysis of mycolic acids [119, 120], and various molecular procedures for identification, as well as DNA fingerprinting techniques for molecular epidemiology.

In the model diagnostic mycobacteriology laboratory described by Salfinger and Pfyffer (1994) [121], specimen decontamination and concentration, staining with fluorochrome dye, inoculation of primary isolation media, and direct detection of *M. tuberculosis* complex by nucleic acids amplification-based assays are carried out on the day the specimen is received. Depending on growth, identification tests by HPLC, DNA probes, or gene sequencing can be carried out a few days after inoculation of liquid media. If not identified by molecular methods (e.g. by a line probe assay) biochemical tests require 7-21 days for identification within *M. tuberculosis* complex after the primary culture is grown fully. Drug susceptibility testing is being completed in 7-10 days using modern growth-based systems (compared with at least 21 days using the conventional agar proportion method).

- **Acid-fast smear microscopy**

Mycobacteria are acid-alcohol fast, i.e. once stained with a specific dye; the cells resist decolorization with acidified ethanol. This staining characteristic is shared only by species of closely related genera that have mycolic acids as a constituent of their cell walls, e.g. *Dietzia, Gordonia, Tsukamurella, Sermania, Nocardia, Rhodococcus*, and *Corynebacterium*. In addition, *Legionella micdadei*, as well as the oocysts of Cryptosporidium, Isospora, and Cyclospora, may exhibit various degrees of acid-fastness. The basis of the acid-fast staining reaction is not fully understood, but it appears to be related to the presence of mycolic acids in the cell wall and to the integrity and viability of the cell.

Microscopy provides a simple, sensitive, and rapid means of detecting open, infectious cases of pulmonary TB. Detection of acid-fast bacilli (AFB) by smear examination is, therefore, used in many developing countries as the only test to confirm the diagnosis of TB and to measure the relative infectiousness of a patient. To be detected microscopically, there must be between 5×10^3 and 5×10^4 AFB in 1 ml of sputum. While 10^6 AFB/ml of specimen usually results in a positive smear, only 60 percent of the smears are positive if 10^4 AFB/ml are present in European Society for Mycobacteriology in 1991. The overall sensitivity of the smear has been reported to range from 22 to 80 percent [122]. Normally, its predictive value for *M. tuberculosis* in expectorated sputum is over 90 percent. It is, however, not possible to distinguish members of the *M. tuberculosis* complex from other mycobacteria by their staining properties. Even though in liquid media *M. tuberculosis* shows serpentine cording, one has to be aware that cords are also seen with some NTM species [123].

Smears can be taken directly from sputum specimens. However, it is advisable to perform it from a homogenized, decontaminated, and concentrated specimen, i.e. after pretreatment to enhance sensitivity. Standard methods are the Ziehl-Neelsen acid-fast stain (hot staining procedure), Kinyoun acid-fast stain (cold staining procedure), or auramine & fluorescence acid-fast stain. In the classical Ziehl-Neelsen procedure, stain is prepared by dissolving 0.3 g basic fuchsin in 10 ml 90-95 percent ethanol or methylated spirits and mixing with 90 ml of phenol solution (5 g phenol crystals in 95 ml distilled water). A volume of approximately 0.01 ml of specimen is smeared over a 2 cm^2 area of the slide and heat-fixed. Approximately five drops of

fuchsin-phenol solution is added and the bottom of the slide heated with a Bunsen burner or electric heater until the stain begins to steam. After heating for 5 min, the smears are rinsed with tap water, and drained. Next, the smears are flooded with and acid-alcohol solution (3 ml concentrated hydrochloric acid plus 97 ml 90-95 percent ethanol or methylated spirits) for at least 2 min, rinsed in tap water, and drained. The smear is then counterstained with methylene blue for 1-2 min.

Fuchsin-stained smears should be examined with a light microscope by making three longitudinal sweeps of the stained area along the length of the slide. About 100 fields will be seen in a single sweep at a magnification of 1000-fold. Mycobacteria appear as red to pink rods on a blue background. Auramine-stained smears are examined with a fluorescence microscope at a lower power (250x). Therefore, only 30 fields need to be examined. A smear can be examined in approximately 1.5 min with fluorescence microscopy (30 fields) as opposed to approximately 15 min by light microscopy (300 fields). If fluorescent particles are seen, they must be observed with higher magnification to confirm the typical morphology of AFB. Ideally, all fluorochrome-positive smears should be confirmed by a carbol-fuchsin staining method. A smear is considered negative if no AFB were seen. One or two bacilli per 300 fields indicate that another specimen should be analyzed.

- **Culture**

Despite the advances in direct detection of *M. tuberculosis* complex in clinical specimens by molecular methods, culture is, at present, indispensable for a number of reasons. It (1) enhances diagnostic certainty, particularly when nucleic acid amplification (NAA)-based results are available; (2) provides biomass for further identification and antimicrobial susceptibility testing; and (3) detects NTM in addition to *M. tuberculosis* complex.

For detection of mycobacteria in clinical specimens, the current 'gold standard' consists of a combination of solid and liquid media. Solid and semisolid media have been known for a long time. Liquid media are more attractive as they offer significantly shorter turn around times for detection of mycobacteria. In detecting as few as 10^1-10^2 viable organisms/ml of specimen in the optimal case, culture is more

sensitive than smear. Also, it is the only reliable means to monitor effectiveness of therapy in TB patients [124].

- Solid media

Egg-based media such as Lowenstein-Jensen (LJ), Ogawa, or Stonebrink have the capability to bind and neutralize toxic compounds encountered in clinical specimens. Usually, they contain malachite green, a dye that inhibits growth of contamination organisms. Egg-based media have a long shelf life and support well growth of *M. tuberculosis* but need, on average, 18-24 days. Since they are nonsynthetic, the quality of the ingredients may vary considerably, which may affect the reproducibility of results. Also, when becoming contaminated, they may liquefy.

Agar-based media, e.g. Middlebrook 7H10 or 7H11, are chemically better defined and the growth of *M. tuberculosis* complex colonies is much easier observed. Negative aspects of agar-based media have limited shelf life and higher costs of preparation.

- Liquid media

A part from traditional broths (e.g. Middlebrook 7H9 or Dubos Tween Albumin) and the biphasic Septi-Chek System (Dickinson Microbiology Systems, Sparks, MD), the radiometric, semiautomated BACTEC 460TB System represented the most efficient and rapid technique to culture mycobacteria for more than two decades and is still used by many laboratories to date. By this method, ^{14}C-labeled palmitic acid as a carbon source in the medium is metabolized by microorganisms to $^{14}CO_2$, which is monitored by the instrument. The amount of $^{14}CO_2$ and the rate at which the gas is produced are directly proportional to the growth rate of the organisms. For *M. tuberculosis*, an average detection time of 8 days was found for smear-positive specimens, compared with 19 days on nonradioactive conventional solid medium [125]. For smear-negative specimens, an average recovery time of *M. tuberculosis* between 14 days in the BACTEC 460TB and 26 days on conventional medium has been reported [126]. Problems associated with the use of radioisotopes and the potential of needle punctures among technicians have implied, however, the search for nonradiolabeled and operationally safer alternatives.

Although utilizing different detection principles, the new culture concepts have in common that they are based on nonradiometric liquid media. Developments range from manual systems utilizing simple tubes (MB Redox, Heipha Diagnostica Biotest, Heidelberg, Germany; Mycobacteria Growth Indicator Tube (MGIT), Becton Dickinson) to fully automate systems (BACTEC MGIT 960, Becton Dickinson; MB Bac/T, Bio-Merieux, Marcy-L'Etoile, France; Esp Culture System II, Trek Diagnostic Systems, Westlake, OH).

The nonautomated MB Redox technique is based on a modified Kirchner medium that contains a colorless tetrazolium salt as a redox indicator, which is reduced to colored formazan by actively growing mycobacteria. AFB can then be detected visually as pink to purple pinhead-size particles. Another manual culture method is represented by the MGIT (Becton Dickinson Microbiology Systems), which contains a modified 7H9 broth in conjunction with a fluorescence-quenching-based oxygen sensor. Growth of mycobacteria or other microorganisms in the broth depletes the oxygen, and the indicator fluoresces brightly when tubes are illuminated with ultraviolet (UV) light at 365 nm.

The fully automated systems that allow continuous monitoring of mycobacterial cultures are (1) the BACTEC MGIT 960 based on the MGIT technology; (2) the MB-BacT utilizing a colorimetric carbon dioxide sensor in each bottle to detect growth of mycobacteria; and (3) the ESP Culture System II, which is based on the detection of pressure changes in the headspace above the broth medium resulting from gas production or consumption due to growth of microorganisms.

All these new systems have similar performance characteristics. In clinical evaluations, recovery rates and time to detection were similar to those of conventional solid media (MB Redox [127]; manual MGIT [127]; BACTECMGIT 960 [128]; MB/BacT [128, 129]; ESP Culture System II [130]). Fully automated systems are certainly less labor-intensive than conventional culture and address safety more appropriately. However, as a consequence of continuous monitoring, these systems are both instrument- and space-intensive. Also, some automated systems lack the versatility of the BACTEC 460TB System (e.g. no incubation temperature available other than 37ºC; no medium available for the inoculation of blood).

- Growth conditions

Inoculated Middlebrook agar media require a CO_2 atmosphere to ensure growth, but CO_2 is not essential for growth on egg-based media, although it stimulates early, more luxuriant growth. To ensure penetration of the gas into containers, tubes should be incubated in the slanted position with caps loose for at least 1-3 weeks before caps are tightened, and plates should be placed medium side down in CO_2-permeable plastic bags until the inoculums has been absorbed. The optimal temperature for recovery of most mycobacteria in the clinical laboratory is 35-37 ºC. *M. marinum*, *M. ulcerans*, and *M. haemophilum* require 25-33 ºC, while *M. avium* and *M. xenopi* should ideally be incubated at 40-42 ºC. The optimal pH for growth of mycobacteria is 6.0-6.5, but this may vary from species to species.

Many *Mycobacterium* spp. are obligate aerobes that will grow at or near the surface of semisolid medium [38]. Microaerophilic species such as *M. bovis* and some *M. africanum* strains grow as a narrow band 5-20 mm below the surface of the medium.

- **Identification and typing methods**

The traditional methods of identification of mycobacteria, based on morphological and biochemical properties, are well established, standardized, reproducible, and relatively inexpensive, but they are limited in scope to the high number of species currently described. Mycobacteria are usually preliminarily identified by traits such as growth rate and pigmentation according to Runyon's criteria. Additional biochemical tests help for further characterization but do not always allow an accurate characterization at the species level. The current identification scheme of mycobacteria mainly relies on molecular methods along with some key phenotypic tests.

- Phenotypic test

Biochemical tests

Determination of the growth rate remains a key test as mycobacteria are classified in rapidly and slowly growing species. Main tests also include determination of the preferential growth temperature, pigmentation, photoreactivity, and colony morphology. The most useful biochemical tests for the precise characterization of species within *M. tuberculosis* complex are the niacin accumulation test, two tests based on catalase, including a semiquantitative test at room temperature and thermotolerance, nitrate reductase, pyrazinamidase, and inhibition by thiophene-2-carboxylic acid hydrazide and p-nitrobenzoic acid. A limited list of other tests useful for other mycobacteria includes arylsulfatase, iron uptake, sodium chloride tolerance, tellurite reduction, tween 80 hydrolysis, and urease. Procedures and distinctive properties of most frequent mycobacteria in clinical practice have recently been revised.

Mycolic acid analysis

Mycolic acids are present in all mycobacteria. Their composition is constant for all strains of a given species and varies from species to species [55]. An HPLC method for analysis of mycolic acid esters, has been standardized and demonstrated to the rapid and reliable method for identification of many *Mycobacterium* species [26, 119]. In this procedure, mycolic acids are extracted from saponified mycobacteria, converted to p-bromophenacyl esters, and analyzed by HPLC. The mycolic acid esters are separated on a reversed-phase C18 column by a methanol-methylene chloride gradient elution and detected by UV or fluorescence detection spectrophotometry.

The standardized method recommends a visual comparison of a sample HPLC pattern to an atlas of reference strain patterns in combination with the use of peak height ratios. A fully integrated automated FL-HPLC system, SMIS (Sherlock Mycobacteria Identification System, MIDI, Inc., Newark, DE) was developed with a library containing entries for 26 mycobacterial species or groups. The SMIS has demonstrated an overall accuracy of 85-90.6 percent [120]. Although initial equipment costs are high the method represents an interesting diagnostic tool.

However, identification may be limited to assign a 'complex of species' and not individual species.

- Genotypic test

Reverse-Hybridization

In the InnoLiPA Multiplex Probe assay (INNO-LIPA Mycobacteria, Innogenetics, Ghent, Belgium), probes consist of the internal transcribed spacer (ITS) region of approximately 280 bp, which separates the 16S and 23S rDNAs. The first version of the kit included probes for the *Mycobacterium* genus, *M. tuberculosis* complex, and seven specific probes for NTM [131]. Several probes were included for *M. kansasii* and *M. chelonae*. Cross-reactions were expected to occur with *M. gastri* and *M. kansasii* as well as with *M. abscessus* and *M. chelonae*. A newly developed version includes additional probes and extends the identification to other species. Another Multiplex Probe assay is available from Hain Lifescience (Nehron, Germany). The kit is similar to the InnoLiPA, but probes consist of the specific alleles of the *gyrB* gene. The kit has no probe for the *Mycobacterium* genus but includes probes for *M. tuberculosis* complex and 14 other mycobacterial species.

Both assays run in 6 h and are more comfortably ensured with an automated machine, Auto-LiPA (Tecan, Switzerland), which performs washes and solution changes at the different steps of the procedure. According to various studies, specificity and sensitivity of the kits are high for most species but limited for the rapidly growing mycobacteria [5, 9, 132-134].

Real- time PCR technique

During the last 8-10 yr, Real-time PCR technology has been used for the following different purposes in mycobacterial research. Identification of mycobacteria by real-time PCR using reference strains and clinical mycobacteria isolates has been studies [8, 135]. Differentiation of MTB complex, *M. avium* and NTM has been done by using hybridization prode. Targeting the 16S rDNA, 3 different probes specific for mycobacteria, MTB complex and *M. avium* were constructed. The thermal melting temperature for the different mycobacteria was as follows: *M. tuberculosis* 64.35^{o}C, *M. kansasii* 58.91 ^{o}C, *M. avium* 57.82 ^{o}C, *M. intracellulare* 54.46 ^{o}C, *M. marinum*

58.91 ^{o}C, RPG mycobacteria 53.09 ^{o}C or 43.19 ^{o}C. This assay with melting curve analysis consistently accurately detected and differentiated *M. tuberculosis* from NTM [136]. For identification of *M. abscessus* (type I and type II) and *M. chelonae* using light cycler based analysis of *hsp65* gene has been published. Results from 36 clinical strains were compared with *hsp65* gene restriction analysis and biochemical profiles of bacilli. As all the three methods yielded identical resulta for each isolate, this procedure offer and results for each isolate, this procedure offers an alternative to previously established nucleic acid amplification based technique for the differentiation of *Mycobacterium* species. As also shown by several other studies [137] the identification of mycobacteria and differentiation between *M. tuberculosis* and NTM can be techniques and it provides an alternative method for differentiation between the closely related species like *M. abscessus* and *M. chelonae*.

PCR-Restriction Enzyme Analysis (PCR REA)

The polymorphism of conserved genes can be determined using a simple PCR amplification of the gene followed by restriction enzyme digestion. Telenti et al. (1993) developed a method for mycobacteria based on the *hsp65* gene encoding for the 65-kDa heat-shock protein and proposed an algorithm of species identification based on the restriction profiles generated by *Hae*III and *BstE*II. The method has been extensively applied and catalogs of profiles extended to various species [138-140]. However, the method is hampered by the polymorphism of the gene within individual species (i.e. several alleles) on the one hand and by the high number of mycobacterial species, which both contribute to ambiguous profiles and complex databases, on the other hand. A website is dedicated to polymerase chain reaction/restriction enzyme analysis (PRA) profiles.

Other genes have been studied following the same procedure, PCR amplification followed by restriction enzyme analysis. These include the *rpoB* gene that mediates rifampin resistance, the *dna*I gene, and the 16S-23S rRNA spacer gene. None have been studied as extensively as *hsp*65 [5, 141, 142].

DNA sequencing

Because of the slow growth of mycobacteria, DNA sequencing for species identification is very attractive. Identification of species-specific signatures within variable regions of highly conserved genes such as 16S rDNA (*rrs* gene) or *hsp65* allowed the design of PCR protocols using genus-specific primers followed by the direct sequencing of the complete or full PCR products [4, 143, 144]. Other targets may also be used such as the gene encoding the 32-kD protein [145], the *dna*J gene, the sod gene encoding the superoxide dismutase [146], the *gyrB* gene coding for the gyrase subunit B [147], the *rpo*B gene coding for the RNA polymerase [142], or the ITS 16S-23S sequence [28, 148]. Catalogs of sequences of mycobacterial species may be retrieved from databases [149].

Genes have different molecular clocks and the interspecies polymorphism varies from gene to gene. The molecular clock of the *rrs* gene (16S rRNA) is rather slow. In other words, species of recent divergence may have similar *rrs* gene sequences. As a matter of fact, *M. kansasii* and *M. gastri*, *M. ulcerans* and *M. marinum*, *M. shimoidei* and *M. triviale*, and *M. abscessus* and *M. chelonae* cannot be differentiated according to their *rrs* signatures [4]. Moreover, no single 16S rDNA interstrain nucleotide sequence difference value that unequivocally defined species boundaries has been established for the Mycobacterium genus [150]. For instance, the *rrs* gene shows a two-nucleotide difference only in the 1384-nucleotide sequence of the distinct species *M. szulgai* and *M. malmoense,* but one to seven different nucleotides in a 782-nucleotide segment of some *M. intracellulare* strains

Direct amplification tests

Because of the slow growth of the tubercle bacilli, great attempts were expected from direct amplification procedures to get a rapid, sensitive, and specific detection of *M. tuberculosis* complex directly from clinical specimens. Three commercial kits are currently available. Tests are directed against 16S rRNA (MTD, Gen-Probe Inc.) or against the 16S rRNA (*rrs* gene) [151]. Manufacturers recommend the tests to be applied to respiratory specimens only. However, with specific modifications, detection of *M. tuberculosis* has been reported in nonrespiratory specimens [152, 153]. Both commercial kits are rapid and can be performed in a single workingday.

Although in vitro sensitivity is very high (as few as one cell equivalent), sensitivity in clinical specimens turned out to be less accurate and mainly depends on the number of bacilli per milliliter present in the specimen. The sensitivity of specimens containing 100 or fewer cells of *M. tuberculosis* per milliliter has been reported to be between 65 and 85 percent, whereas the sensitivity with samples containing more than 1,000 cells per milliliter was almost 100 percent [154-156]. The specificity is excellent for both tests. However, a discrepant positive test result was obtained with *M. celatum* containing specimens with the MTD kit [157]. Since both tests rely on 16S rRNA or the *rrs* gene, it has to be stressed that detection corresponds to *M. tuberculosis* complex with on specific identification within the complex.

Principle of Multiplex PCR

Multiplex PCR is an amplification reaction in which two or more sets of primers specific for different targets are introduced in the same tube, allowing multiple target sequences to be amplified simultaneously. Primers used in multiplex reactions must be designed carefully to have similar annealing temperature and lack complementarily, in order to avoid dimerization. Extensive empirical testing is often needed. Co-amplification of multiple targets can be used for different purposes, detecting pathogens and identifying species of pathogen in single sample.