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Chapter 33

The Genus Mycobacterium

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Introduction

The genus *Mycobacterium* consists of more than 190 species and belongs to the family of *Mycobacteriaceae*, order Corynebacteriales, phylum/class Actinobacteria, and kingdom Bacteria (http://www.dsmz.de/bacterial-diversity/prokaryotic-nomenclature-up-to-date).[1] Mycobacteria are nonmotile, rod-shaped bacilli, characterized by an extremely lipid-rich cell wall comprising mycolic acids. These long-chain (C-60 to C-90) fatty acids make all mycobacterial species "acid-fast" as they resist acid-alcohol decolorization following staining with phenicated dyes like fuchsine, enabling their microscopic detection. Mycobacteria should not be confused with members of the closely related genera *Nocardia, Corynebacterium*, or *Rhodococcus*, which have shorter-chain (C-22 to C-64) mycolic acids and can be partially acid-fast.[1]

Based on phenotypic characters, the genus was proposed in 1896 by Lehmann and Neuman.[2] With the advent of DNA-based analysis, mycobacterial species were initially differentiated at <70% similarity by DNA-DNA hybridization,[3] and later by <98.8% 16SrRNA Sanger sequence similarity[4] with or without

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inclusion of additional housekeeping genes like *hsp65* or *rpoB*.[5] These phenotypic and genotypic analyses are all in agreement with the initially described genus *Mycobacterium,* phylogenetically distinguishing five major groups.

Recently, the taxonomy of the genus *Mycobacterium* has been challenged based on a core protein analysis—identifying insertions/deletions of amino acids, or proteins exclusively found in evolutionarily-related groups of species—suggesting to split the genus in five monophyletic groups designated as the "Tuberculosis-Simiae," "Terrae," "Triviale," "Fortuitum-Vaccae," and "Abscessus-Chelonae" clades.[6] The five proposed genera exactly overlap the major clades of the classical taxonomy. Gupta et al. propose to keep the genus "*Mycobacterium"* for species belonging to the "Tuberculosis-Simiae" clade and to introduce four novel genera hosting the nontuberculous mycobacteria (NTM) of the above mentioned clades in "*Mycolicibacter*," "*Mycolicibacillus*," "*Mycolicibacterium*," and "*Mycobacteroides*," respectively. This reclassification has been debated and questioned for reasons of methodology and clinical relevance.[7,8] Tortoli and colleagues argue:

> NTM may be diverse in their genetics and biology, but they produce remarkably similar disease manifestations in distinct populations at risk. This uniformity and the lessons to be learned from it are important for both clinicians as well as the affected patients.

The newly proposed reallocation risks to cause confusion among clinicians and clinical microbiologists, while not being beneficial for the patient. Also, the novel genus "*Mycobacterium*" is said to include all of the major human pathogens[6] but does not comprise clinically important species like *M. abscessus*.[9] Regarding the methodological approach for this reclassification, while Tortoli and colleagues identified minor technical errors (mislabeling for some of the sequences used),[10] Meehan and colleagues questioned the correctness of the analysis in a more fundamental way.[8] Analyzing the percentage of conserved proteins (POCP) revealed that all 145 tested species in the original *Mycobacterium* genus fell within the 50% boundary of all others, while all 223 tested non-*Mycobacterium* species from the *Corynebacteriaceae* family had <50% POCP to all *Mycobacterium* species, strongly supporting retention of the classical genus *Mycobacterium*.[8] Given the equal taxonomic validity of both classification systems, the newly proposed genus and species names can be used as synonyms.[7] For sake of clarity in this handbook on clinical practice, we will stick to the classical genus "*Mycobacterium*" and related species names in this chapter.

With their generation time varying from 2 to 48 hours, mycobacterial species can be classified as slow growers—requiring more than 7 days to form visible colonies on solid medium—and rapid growers exhibiting visible growth in less than 7 days. This growth-rate-based separation is supported by phylogenetic trees constructed from 16S rRNA or whole genome sequences.[11] Combining growth rate with ability to produce pigmentation and clinical significance, Runyon classified members of the *Mycobacterium* genus into five groups,[12,13] a classification that is barely used nowadays.

According to their infectiousness, mycobacteria are classified in three categories: (1) strict pathogens, with members of the *M. tuberculosis* complex (MTBc) and *M. leprae* having the biggest public health impact; (2) opportunistic pathogens like *M. avium*, that are found in the environment and may cause disease in persons with predisposing conditions or compromised immunity; and (3) saprophytes like *M. gordonae*, that are ubiquitous and can be found in environmental sources, yet rarely or never cause infection and are considered overall nonpathogenic. Most (opportunistic) pathogenic mycobacteria are slow growers.

Clinical Importance of Mycobacteria

The clinical importance of mycobacteria cannot be overemphasized, especially for members of the MTBc, *M. leprae*, *M. ulcerans*, and some opportunistic NTM in specific vulnerable populations.

Mycobacterium tuberculosis Complex (MTBC)

Tuberculosis (TB) is caused by members of the *M. tuberculosis* complex (MTBC) comprising *M. tuberculosis sensu stricto*, *M. africanum*, *M. bovis*, *M. canettii, M. caprae, M. microti*, *M. mungi, M. pinnipedii*, *M. orygis*, and *M. surricatae*.[14] While *M. tuberculosis* and *M. africanum* are human-adapted species, the other species prefer (specific) animal hosts with possible zoonotic spill over to humans, mostly from *M. bovis* via cattle.[14] An attenuated strain of *M. bovis*, *M. bovis* BCG (Bacille Calmette-Guérin), is used as a vaccine in many high-burden countries to protect against acquiring TB, though its efficacy is questionable.[15]

The two human-adapted species comprise eight phylogenetic lineages (L1–L8),[16] with L1–L4 and L7–L8 being *M. tuberculosis sensu stricto*, and L5 and L6 *M. africanum*. As for their geographic repartition, L2 and L4 are the most widespread, while L1 and L3 are intermediately distributed, L5 and L6 are restricted to West Africa, and L7 is found only in Ethiopia.[17] *M. africanum* is responsible for up to 40% of TB in West Africa.[18]

Worldwide, TB is among the top 10 causes of death, and the first cause of death by a single infectious agent (above HIV/AIDS).[19] It was estimated that in 2018, worldwide, 10 million people developed TB, of whom 1.2 million died. Two-thirds of cases occurred in eight high-burden countries, with India (27%) and China (9%) ranking first. Only 6% of global cases were in the World Health Organization (WHO) European Region (3%) and the WHO Region of the Americas (3%). In terms of TB incidence, Africa ranked first with 231 per 100,000 population, which is almost twofold the worldwide incidence (132 per 100,000 population).[19] Likewise, the highest TB mortality rate was reported from Africa, where the TB epidemic is fueled by the HIV/AIDS epidemic. People living with HIV are 20–30 times more likely to develop active TB disease than people without HIV. Following huge TB control efforts, increased accessibility to antiretroviral treatment and availability of new anti-TB drugs, the worldwide TB incidence rate is falling at about 2% per year, with the fastest regional declines between 2013 and 2017 in the WHO European (on average, 5% per year) and African (3.8% per year) regions.[19] Nevertheless, TB continues to be a major public health problem in many countries.

Of added importance is the number of MTBC strains that are found resistant to one or more of the antitubercular agents.

TB disease is always treated with a combination therapy. The preferred regimen for treating adults with TB consists of an intensive phase of 2 months of isoniazid (INH), rifampin (RIF), pyrazinamide (PZA), and ethambutol (EMB) followed by a continuation phase of 4 months of INH and RIF. RIF, a strong bactericidal and sterilizing drug, is considered the core drug for standard TB treatment.[20] Patients infected with MTBC strains resistant to (at least) RIF or RIF and INH—defined as multidrug resistant (MDR)—should be treated with alternative drugs in so-called MDR-TB regimens. While these drugs are commonly known as "second-line" drugs, recent guidelines from the WHO classify these drugs in group A, B, and C.[21] Individualized MDR-TB regimens using three drugs from group A, complemented by one or two drugs from group B, and one or more from C, have been proposed for a duration of 18 months or more.[22] Alternatively, a standardized short MDR-TB regimen (9–12 months) has been shown to be noninferior compared to the long regimen,[22] and was successfully implemented under programmatic conditions in multiple African and Asian countries.[23,24] The short regimen includes a fourth-generation fluoroquinolone (gatifloxacin, moxifloxacin, or levofloxacin) as core drug.[20] Recently, the use of injectable aminoglycosides (amikacin or kanamycin) or polypeptides (capreomycin) has been discouraged, due to the irreversible ototoxicity.[21]

An MDR patient may further develop resistance to other drugs during the course of treatment. MDR combined with resistance to a fluoroquinolone and an injectable aminoglycoside or polypeptide is defined as extreme drug resistance (XDR). XDR-TB treatment regimens require further inclusion of less efficient, more toxic group C drugs[21] or new drugs like bedaquiline and delamanid. XDR-TB treatment is difficult and costly.

MDR- and XDR-TB are a major concern worldwide, with cases reported in all countries. According to the WHO, in 2018, globally 3.4% of new cases and 18% of previously treated TB cases had RIF-resistant/MDR disease, with three countries accounting for half of the world's cases of RIF-resistant/MDR-TB: India (27%), China (14%), and the Russian Federation (9%).[19] Among MDR cases 6.2% were estimated to be XDR, numbers that were slightly lower compared to 2018 data.[19]

Non-tuberculous Mycobacteria (NTM)

Mycobacterial species different from MTBC are grouped as NTM, also referred to as atypical or anonymous mycobacteria, or mycobacteria other than tuberculosis (MOTT).

Humans can acquire NTM infection from various sources in the environment such as natural and engineered water systems, mist, aerosols, dust, and soil,[25] or from contact with plants, birds, fish, and other animals.[26,27] In humans, NTM can be found as colonizers without clinical relevance—with chronic lung disease as predisposing factor—or they can be seen as mostly intracellular pathogens. Human-to-human transmission of NTM was not known to be a common route of transmission, till recent evidence documented human-to-human transmission in cystic fibrosis patients.[28–30]

While the frequency of clinical isolation of NTM is increasing in many countries with decreasing MTBC prevalence,[27,31–33] and even in countries endemic for TB,[34,35] their clinical relevance remains often unclear.

The diagnosis of NTM disease remains a clinical dilemma for clinicians as NTM exist in the environment, and mere isolation from an unsterile site could be a reflection of environmental contamination. Correctly identifying and determining the clinical relevance are however paramount, as the time-consuming and complicated treatment of NTM disease will vary depending on the species. NTM infections most commonly manifest as pulmonary disease (especially in adults), followed by skin and soft tissue infections, lymphadenitis (especially in children), or disseminated disease in severely compromised patients.[36]

General criteria for the diagnosis of pulmonary NTM disease as established by the American Thoracic Society (ATS) may guide on clinical decision-making.[37] One of the criteria states that two positive cultures should be obtained from subsequent sputum specimens OR a single positive culture from a bronchial aspirate. Nevertheless, single-sputum NTM isolation can provide evidence of true NTM-lung-disease, taking into account patient's characteristics like bronchiectasis, younger age, and more severe radiographic pulmonary lesions.[38]

It is to be noted that only around 20–25% of patients with pulmonary NTM isolates meet the ATS criteria,[32,35,39,40] which can increase up to 60% among patients at higher risk like cystic fibrosis patients.[41] Of note, a considerable proportion of patients with NTM pulmonary disease in Europe have no detected underlying lung disease or immunodeficiency.[42] In the United States, the annual rates of pulmonary NTM disease range from 1.4 to 13.9 per 100,000 inhabitants,[43] while in Europe rates seem to be lower ranging from 0.2 to 2.9 per 100,000 population.[42]

The probability of clinical relevance varies across species. NTM commonly found in patients with pulmonary symptoms are *M. avium, M. intracellulare*, *M. kansasii*, *M. xenopi*, *M. abscessus*, *M. fortuitum*, and *M. scrofulaceum*.[32,36,40,44] *M. abscessus* is increasingly seen as pulmonary pathogen.[39,40] While the less common *M. genavense* has a very high likelihood of being clinically relevant in pulmonary disease, *M. avium* and *M. kansasii* have a moderate odds, and *M. gordonae* is almost never involved in disease though frequently being isolated.[32,40] The frequency of isolated NTM species may vary across geographical regions and settings.[45] Also, the relative frequency of NTM species may change over time, as has been noticed in Europe with shifts of *M. kansasii* and *M. malmoense* frequency.[42]

Regarding extra-pulmonary NTM disease, the majority of lymphadenitis cases are seen in young children, and most often caused by *M. avium, M. scrofulaceum, M. simiae, M. haemophilum, M. malmoense*, or *M. marinum*.[32,46-48]

NTM species most commonly reported from skin and soft tissue infections are *M. fortuitum, M. abscessus, M. chelonae* among the rapidly growing mycobacteria, and *M. marinum, M. ulcerans, M. chimaera,* and *M. haemophilum* as slowly growing species.[27] Using inadequately disinfected medical equipment has been associated with outbreaks of NTM skin and soft tissue infections. Because of their lipophilic character, environmental mycobacteria are frequently found in biofilms, making them even more resistant to decontamination with standard antiseptics and biocides, including chlorhexidine and glutaraldehyde.[42,49] Recently, some compounds have been described to be active against NTM in biofilms.[50,51] Potable water systems have been proposed as an advantageous pre-infection niche for bacteria—including NTM-colonizing lungs of CF patients.[52] In a recent

comparison of Norwegian chlorinated and non-chlorinated drinking water distribution systems, it was found that residual chloramine may increase mycobacterial biomass, while it may also decrease mycobacterial diversity.[53] As a result, mycobacterial species have been associated with both pseudo-outbreaks and real outbreaks of nosocomial infections following surgery or cosmetic procedures. In the United States, cosmetic surgery tourism constitutes an increased risk for NTM soft skin tissue disease.[54,55] Molecular typing of the isolated strains during an outbreak with *M. abscessus* after breast augmentation surgery in Venezuela undoubtedly identified tap water as the source of infection.[56] Also from nonhospital/cosmetic settings, outbreaks have been reported, such as *M. marinum* infections following fish handling[57] or use of premixed ink as the common source of infection with *M. chelonae*.[58]

The NTM commonly found as causative agent in patients with disseminated disease are *M. avium, M. intracellulare*, *M. kansasii, M. haemophilum,* and *M. chelonae*.[36,59]

M. gordonae and *M. engbaekii* are among the NTM most frequently found as contaminants with no or very rare clinical implication.

Trying to ascertain the prevalence of NTM disease may be complicated by several factors. As opposed to TB, NTM disease is not notifiable to public health authorities at a national or an international level, with a few exceptions such as Oregon State (the U.S.A). Therefore, underreporting might occur, which can prohibit collection of systematic data and potentially lead to an underestimation of NTM disease. Conversely, overdiagnosis may occur when patients are diagnosed with (and treated for) nonpathogenic mycobacteria, and in those cases in which the clinical relevance of the potential pathogenic mycobacterium isolated has been misjudged. Finally, care must be taken with regard to the trend analysis of certain species, as the degree of taxonomical diversification is much higher in contemporary studies using molecular identification techniques, than it is in older studies based on phenotypic characteristics.[42]

Clinically Significant Species of Slowly Growing NTM

The following are some of the most important slow-growing NTM species that have been associated with clinical disease. An overview of the clinical relevance for additional slowly growing NTM species can be consulted at https://bccm.belspo.be/about-us/bccm-itm.

Mycobacterium leprae

This mycobacterium is an obligatory intracellular pathogen and the causative agent of leprosy or Hansen's disease. The disease mainly affects the skin, the peripheral nerves, mucosa of the upper respiratory tract, and the eyes. It can range from mild and self-limiting (tuberculoid type) to severely debilitating (lepromatous type). Leprosy is also classified as paucibacillary (PB) or multibacillary (MB), based on the number of skin lesions and the presence of nerve involvement. It is an ancient disease with cases depicted around 600 BC. There has always been a large social stigma associated with leprosy, which resulted in victims being segregated to leper colonies.

In humans, *M. leprae* is a very slow-growing organism with a long incubation period, it can take from 6 months to 20 years for an infected individual to develop symptoms after exposure (WHO fact sheet). *M. leprae* does not grow on laboratory media, likely due to the loss of important genes, with a reduced genome size of 3.3 Mbp compared to the 4.1 Mbp of MTBC.[60]

In its efforts to eliminate leprosy, the WHO recently introduced the concept of "one report for leprosy" by launching an online data collection tool to facilitate the gathering of epidemiological and programmatic indicators from member states. In 2018, there were 208,619 new leprosy cases registered globally, according to official figures from 159 countries from the six WHO Regions (https://www.who.int/news-room/fact-sheets/detail/leprosy). The majority of these cases were found in nine countries in Asia, Africa, and Latin America. In the United States, leprosy is seen in about 200 new cases every year, with nine-banded armadillos being an important reservoir.[61]

Current diagnosis of leprosy relies often solely on clinical findings, without microbiological confirmation. Microscopy for detection of acid-fast bacilli can confirm the diagnosis of more advanced leprosy, but it is less effective in the early stages or in paucibacillary cases. Nucleic acid-based detection techniques for *M. leprae* are becoming a standard of care for supporting the diagnosis of leprosy, with the repetitive sequence RLEP as a promising target for qPCR, allowing the detection of approximately one *M. leprae* genome in a sample (https://internationaltextbookofleprosy.org/chapter/pathogen-detection). No commercial molecular tests for *M. leprae* detection are available.

Clinical diagnosis of leprosy is based on the presence of at least one of three cardinal signs: (1) definite loss of sensation in a pale (hypopigmented) or reddish skin patch; (2) thickened or enlarged peripheral nerve with loss of sensation and/or weakness of the muscles supplied by that nerve; or (3) presence of acid-fast bacilli.[62]

Leprosy is now totally treatable with a free-of-cost multidrug therapy including dapsone, RIF, and clofazimine.

Mycobacterium ulcerans

This species is a close relative to *M. marinum,* but differs in its ability to produce a mycolactone toxin. It is the causative agent of Buruli ulcer (BU), a disease which occurs in at least 33 countries with mostly tropical and subtropical or temperate climates. Most cases are reported in West and Central Africa, while Australia remains an important non-African endemic country. In 2018, 2,713 suspected BU cases were reported from 14 countries, confirming the recent increase after years of decline from 2010 (5,000 cases) to 2016 (1,961 cases). The reasons for the initial decline and recent increase remain unclear https://www.who.int/news-room/fact-sheets/detail/buruli-ulcer-(mycobacterium-ulcerans-infection).

The disease begins as a painless nodule, but if left untreated, can progress to a debilitating skin ulcer or osteomyelitis, and can lead to permanent disfigurement and long-term disability.[63] It is the mycolactone that causes subcutaneous tissue destruction and local immunosuppression, aggravating the symptoms. Most of the lesions appear on the limbs, especially on the lower

extremities.[63] *M. ulcerans* is an environmental bacterium, and the exact means of transmission remain unknown, but most infections appear to be found adjacent to bodies of (stagnant) water. There is some indication that mosquitoes may be responsible for transmission of the disease in Australia,[64,65] although evidence of *M. ulcerans* positive mosquitoes remains limited.[66] In Africa, where BU is most prevalent, no environmental source has been identified so far, with one exception of a *M. ulcerans* isolate grown from a water strider in Benin.[67]

Although the disease can often be diagnosed on clinical presentation, this organism can grow on standard media suitable for mycobacterial growth, with a growth temperature optimum of 30°C. The most sensitive diagnosis for BU, however, is done by PCR targeting the insertion sequence IS*2404*.

Buruli ulcer is treatable with combination therapy using RIF and clarithromycin for 8 weeks. Surgical debridement is often required as well, especially for larger ulcers or advanced disease.[63]

Mycobacterium avium Complex (MAC)

Originally, *M. avium* and *M. intracellulare* were the only two species belonging to the *Mycobacterium avium* complex (MAC).[37] Sequencing studies of the 16S rRNA gene and 16S–23S internal transcribed spacer (ITS) confirmed the MAC as a diverse, yet relatively clearly defined and clustered group among the slowly growing NTM, comprising the species *M. avium, M. intracellulare** (including *M. chimaera*, M. yongonense*, M. paraintracellulare**), *M. colombiense, M. arosiense, M. vulneris, M. bouchedurhonense, M. timonense, M. marseillense*, and *M. lepraemurium*.[68–70]

M. intracellulare cannot be easily differentiated from *M. avium* through traditional physical and biochemical tests;[37] it requires genetic analysis.

Members of the MAC are ubiquitous in soil and many natural and man-made water systems.[25,71,72] Although usually not considered pathogenic in the general community, these organisms are the most commonly isolated causative agents of NTM-mediated lung disease with TB-like symptoms,[37,70] and they may cause lymphadenitis in children and/or other extrapulmonary and disseminated infections in immunocompromised pati ents.[37,68,73] Pulmonary disease attributed to the MAC is mainly caused by *M. avium* and *M. intracellulare*. Other MAC species have been increasingly reported as potential causative agents of NTM lung infections, but their full spectrum of diseases is not yet entirely known.[68] One member of the MAC, namely *M. lepraemurium*, is known as the etiologic agent of feline and murine leprosy.[74,75]

Infections are most probably acquired by inhalation or ingestion of the MAC species. Although frequently isolated from respiratory specimens, isolation of MAC does not necessarily imply clinical relevance in pulmonary disease.[37]

To date, there is still an ongoing debate on whether or not specific species should be assigned within the complex or if the MAC should be considered as a single entity. Moreover, the American Thoracic Society and Infectious Diseases Society of America (ATS/IDSA) stated that although the differentiation between *M. avium* and *M. intracellulare* may be important epidemiologically for research purposes and in the future for therapeutic use, the distinction is not yet clinically significant enough to routinely identify MAC isolates to the species level.[37] Also, disease caused by all members of the MAC is treated in a similar manner using a regimen specific to the MAC.[68]

Treatment regimens for MAC infections include combination therapy, typically with a macrolide (clarithromycin or azithromycin) as core drug, with RIF (or another companion drug), EMB and occasionally amikacin in cases of disseminated disease when a more aggressive therapy is required.[37,76] ATS recommends clarithromycin susceptibility testing for new, previously untreated MAC isolates.[37]

Mycobacterium avium

M. avium was originally divided into three subspecies corresponding to pathogenicity and host-range characteristics: *M. avium avium*, *M. avium paratuberculosis*, and *M. avium silvaticum*.[77,78] To elucidate the epidemiology of *M. avium*-associated disease, Mijs et al. clustered the isolates originating from humans and pigs together as a suggested fourth subspecies called *M. avium hominissuis*,[79] the most abundant etiological agent of *M. avium*-related disease in humans.[80,81] Additionally, Mijs and colleagues stated that the designation *M. avium avium* should be reserved for bird-type strains because of its association with avian TB.[79] *M. avium paratuberculosis* on the other hand is the causative agent of Johne's disease resulting in chronic granulomatous enteritis of ruminant livestock and wildlife and may have a similar—yet not proven—role in Crohn's diseases in humans.[82-84] Finally, *M. avium silvaticum* is highly related to *M. avium avium* and has been isolated primarily from wood pigeons wherein it can cause TB-like lesions.[78]

Tortoli and colleagues suggest combining these *M. avium* subspecies within a single taxon, and to regard them as synonyms based on their very high average nucleotide identity (ANI) and genome-to-genome distances (GG) values well exceeding the subspecies thresholds.[85] In consideration of their adaptation to specific hosts, the previously named subspecies should be reclassified as variants.[85] Subspecies status of *M. avium paratuberculosis* is proposed to be maintained due to the significant differences in clinical presentation between this subspecies and other variants of the MAC.[77,85]

This would result in *M. avium variant avium*, *M. avium variant hominissuis*, *M. avium variant silvaticum*, and *M. avium paratuberculosis*

M. avium grows at 30–37°C on media suited for mycobacterial growth. Primary isolation of both *M. avium paratuberculosis* and *M. silvaticum* requires adding mycobactin to the culture medium.[86] Besides, *M. silvaticum* is unable to grow on egg media and needs the stimulation of growth by pyruvate and at pH 5.5, gradually losing this phenotype upon subculture.[78]

Mycobacterium intracellulare and Closely Related Species

This mycobacterium is mainly a respiratory pathogen in humans, responsible for most MAC lung diseases in the United States, while an association with disseminated disease is less common.[35,77]

M. chimaera became well known for its association with contaminated heater-cooler devices used in open heart surgery during a worldwide outbreak.[87,88]

M. yongonense and *M. chimaera* were recently reclassified as subspecies of *M. intracellulare*, resulting in the designation of three new taxa: *M. intracellulare intracellulare*, *M. intracellulare yongonense*,[89] and *M. intracellulare chimaera*.[69] In 2019, *M. intracellulare chimaera* has even been proposed as synonym of *M. intracellulare yongonense*.[85]

Likewise, *M. paraintracellulare*[90] was proposed to represent a fourth subspecies or even a synonym of *M. intracellulare* rather than a separate species based on its average nucleotide identity (ANI) value being well within intraspecies values.[68,69] Also, the need to distinguish *M. paraintracellulare* from *M. intracellulare* is questioned as there is no reason of clinical relevance to do so.[68]

This would result in a new taxonomy to recognize only a single species, *M. intracellulare*, with two subspecies: *M. intracellulare intracellulare* and *M. intracellulare chimaera*.[85]

Mycobacterium haemophilum

This organism was first isolated in 1978 from a patient with Hodgkin's disease, and it was shown to cause skin lesions and disseminated infections in patients with AIDS in the early- to mid-1990s. Indeed, *M. haemophilum* has been described in immunosuppressed patients, including those with lymphoma, those who undergo bone marrow or cardiac transplantation, and patients receiving steroid therapy.[91] It has also been described as a cause of lymphadenitis in immunocompetent children.[92]

The growth requirement of these organisms is rather unique among mycobacteria as they require hemin or ferric ammonium citrate for growth, at an optimal temperature of 30°C.[93] This probably accounts for the relative infrequency of its isolation, especially in laboratories solely relying on culture at 37°C. It is advisable that all patients presenting with a skin lesion have their specimen cultured onto medium containing a source of hemin with incubation at 30°C.

There is no standard therapy for infections with *M. haemophilum* but regimens including clarithromycin, doxycycline, ciprofloxacin, amikacin, INH, and RIF have led to resolution of the disease,[94] while surgical intervention may be favored in case of cervicofacial lymphadenitis.[95]

Mycobacterium kansasii

This mycobacterium causes a disease that resembles pulmonary TB. The patients present with cough, fever, and night sweats as is seen in TB.[96] Typically, the patient is an older male with a prior history of TB, chronic obstructive pulmonary disease or other chronic lung diseases. Cavitary disease may be seen on x-ray. Extra-pulmonary disease caused by *M. kansasii* has also been described occasionally.[59,97] The odds of clinical significance for a positive *M. kansasii* culture is relatively high.[32] In a Korean study, 52% of all patients with *M. kansasii* respiratory isolates exhibited clinically significant disease.[98]

In the past, seven subtypes of *M. kansasii* have been reported based on target sequences such as *hsp65*. Recent WGS analyses support a reclassification of this polyphyletic group in separate species rather than subtypes,[99,100] with clearer separation of their clinical relevance (Table 33.1).

M. kansasii has similar growth conditions as *M. tuberculosis*. Standard treatment involves the use of first-line anti-TB drugs RIF, INH, and RMB, while RIF-resistant *M. kansasii* treatment can be based on drug-susceptibility testing data.[29]

TABLE 33.1

Reclassification Species Within the *Mycobacterium kansasii* Group

(New) Species Name[99,100]	Former *M. kansasii* Subtype	Pathogenicity	Isolation from Clinical Specimens
M. kansasii	1	Most pathogenic	Frequent
M. persicum	2	Pathogenic	Limited data
M. pseudokansasii	3	Rarely pathogenic	Frequent
M. species[a]	4		
M. innocens	5	Rarely pathogenic	Limited data
M. attenuatum	6	Nonpathogenic	Very rarely
No suggestion[b]	7		

[a] To be defined as soon as a Type strain is available; [b]Lack of Type strain and full genome.

Mycobacterium malmoense

This mycobacterium is rarely seen in the United States but is recognized as causing pulmonary disease in Northern Europe, with a frequency exceeding that of *M. kansasii*-isolation in some UK settings. In the United Kingdom, *M. malmoense* isolates had a moderate chance of being clinically significance,[40] while in the Netherlands *M. malmoense* was found to be clinically relevant at much higher (80% of cases) levels. These usually cause pulmonary disease with serious morbidity.[101] The typical patient is a male over his 50s with prior or underlying lung disease.[101,102] There have also been reports of—mostly pediatric—lymphadenitis caused by *M. malmoense*.[101,103]

M. malmoense has no specific growth requirements—even though growth on solid medium can remain very slight, with good growth at 37°C.[104]

Therapy includes the use of RIF and EMB, supplemented with a macrolide,[29] although some studies showed no additional benefit of adjunctive clarithromycin.[101,105]

Mycobacterium xenopi

Like most of the other slowly growing opportunistic pathogenic NTM, this species causes pulmonary disease, with various presentations (cavitary, nodular, or diffuse infiltrate).[106] *M. xenopi* disease has become prominent in Northern European and American countries with a low TB prevalence, yet is rarely or not seen in TB high prevalent countries (China,[107] Tanzania,[34] and Mozambique[35]). Nevertheless, *M. xenopi* is a fairly ubiquitous environmental bacterium found mostly in water, including the water supplies of hospitals. The typical patient is an older male with a prior history of TB or other lung disease. The patient often presents with cavitary lung disease. The probability of being clinically relevant was found to be moderate 40–60%.[3239,40]

This organism is easily grown in culture but prefers an elevated growth temperature (42°C) and forms typical "bird's nest" colonies when grown on solid media.[108]

M. xenopi disease is hard to treat with around 30% success rate.[109] Variable success has been reported using a four-drug

regimen comprising RIF, EMB, a macrolide (clarithromycin or azithromycin), and a fluoroquinolone (cipro- or moxifloxacin) or INH.[29,106] For severe disease an injectable aminoglycoside (amikacin or streptomycin) can be added.[29]

Mycobacterium celatum

This mycobacterium was first described in the early 1990s as a causative agent of disseminated disease in AIDS patients with a very low CD4 count.[110] Subsequently, *M. celatum* has been identified as a cause of pulmonary disease in immunocompetent individuals as well, with disease resembling that of MTBC and other NTM species.[111] *M. celatum* isolates can be easily misidentified as *M. xenopi*, sharing many biochemical/phenotypic characteristics including a typical fatty acid pattern.[112] *M. celatum* can be differentiated from *M. xenopi* by its poor growth at 45°C, and production of larger colonies on 7H10 agar.[112] Using the AccuProbe assay—which detects MTBC-specific nucleic acids—*M. celatum* isolates can be falsely positive.[113]

There is no standard therapy for infections with *M. celatum*. Isolates of *M. celatum* are generally resistant to RIF. Successful treatment has been achieved by combining clarithromycin, EMB, and ciprofloxacin.[114]

Mycobacterium marinum

Mycobacterium marinum was first observed in 1926 causing infections in saltwater fish in the Philadelphia aquarium.[115] The first reported cases in humans were from an epidemic in Sweden in 1954. These patients, who all swam in the same swimming pool, developed lesions, which began as papules at the site of infection and mostly became ulcerated and/or exudative. *M. marinum* disease, often referred to as "swimming pool granuloma," has also been associated with fish tank ownership.[116] The infection usually occurs on the upper extremities, including the hands, where the organism gains entrance through scratches and abrasions, and generally remains localized at the site of infection.

M. marinum can be cultured on standard media used for mycobacterial growth, but with incubation at 30°C, the optimum growth temperature for this species.

Therapy with regimens containing EMB, RIF, clarithromycin, amikacin, or doxycycline is usually effective. In some instances, surgical intervention is required.[117]

Clinically Significant Species of Rapidly Growing NTM

At present, rapidly growing mycobacteria comprise approximately 50% of all recognized and validated species belonging to the genus *Mycobacterium*.[118,119] Although ubiquitous in environmental reservoirs, some rapidly growing NTM are frequently isolated in healthcare facilities and have emerged as important human pathogens.[120] They are commonly found in abscesses formed after an unintentional inoculation or puncture wound, through contamination of surgical wounds or via traumatic injuries.[27] *M. abscessus* is increasingly seen as pulmonary pathogen.[39,40] *M. abscessus*, *M. chelonae*, and *M. fortuitum* together embody more than 80% of clinical isolates of rapidly growing NTM.[119,121]

First-line TB drugs are ineffective against NTM disease due to rapid growers.

An overview of the clinical relevance for additional rapidly growing NTM species can be consulted at https://bccm.belspo.be/about-us/bccm-itm.

Mycobacterium abscessus

This organism was first identified in 1952 by Moore and Frerichs, who isolated it from a patient's knee abscess.[122] Species name designation was based on the mycobacterium's ability to cause human skin and soft tissue infection, producing deep subcutaneous abscesses.[123]

Over time, *M. abscessus*' taxonomy, particularly the identification of species and subspecies, has faced many challenges resulting in a cumbersome cascade of nomenclature changes. *M. abscessus* and *M. chelonae* were originally considered to be the same species as they present almost identical biochemical features.[10,121,123] After first being designated subspecies status in 1972,[124] Kusunoki and Ezaki re-elevated *M. abscessus* to species status in 1992 based on genomic DNA hybridization studies.[125] As a result, *M. chelonae* subspecies *chelonae* was also reinstated again to its former name *M. chelonae*. After *M. abscessus* was recognized as a distinct species, the mycobacterium has been further categorized into three distinct subspecies: *M. abscessus abscessus*, *M. abscessus bolletii*, and *M. abscessus massiliense*.[126-128]

Precise identification to subspecies level of the disease-causing strain in *M. abscessus* pulmonary infections is essential for diagnosis and management as the subspecies differ in clinical disease presentation as well as in susceptibility to drugs and in optimal therapeutic regimens.[129] *M. abscessus abscessus* and *M. abscessus bolletii* have an *erm* (erythromycin ribosomal methylase) gene that confers inducible macrolide resistance, whereas most *M. abscessus massiliense* strains have a dysfunctional *erm* gene, resulting in macrolide susceptibility.[130] Consequently, better treatment outcomes have been reported in patients with *M. abscessus massiliense* pulmonary infection than those with *M. abscessus abscessus* disease.[131]

M. abscessus abscessus is the most common pathogen of the three *M. abscessus* subspecies,[132] and typically it is associated with a wide range of skin and soft tissue infections in human hosts,[37,123,133] while being increasingly the cause of pulmonary infections in patients with underlying structural lung disease such as cystic fibrosis and bronchiectasis.[134]

Skin and soft tissue infections often develop after the use of equipment or injected substances contaminated with these rapid growing mycobacteria during medical, cosmetic, and plastic surgery (such as liposuction,[135] mammaplasty,[56] and mesotherapy[136]), pedicures,[137] and after trauma.[138,139] Also pseudoinfections due to contaminated bronchoscopes and endoscopes have been ascribed to *M. abscessus*[140] and *M. abscessus bolletii*.[141]

M. abscessus is a nonchromogenic rapidly growing NTM characterized by its inability to grow at 42°C.

Current guidelines recommend an initial treatment for 2–4 months consisting of two parenteral intravenous agents (such as amikacin, cefoxitin, imipenem, and tigecycline) and a macrolide antibiotic if the species is macrolide susceptible.[37,133] If not, the macrolide should be replaced by another agent based

on *in vitro* drug susceptibility testing data. Secondarily, an additional inhaled/oral antibiotic (fluoroquinolone, linezolid, or clofazimine) can be added during the continuation phase.[133]

Mycobacterium chelonae

As stated before, *M. chelonae* is closely related to *M. abscessus* but lacks the *erm* gene that confers macrolide resistance in *M. abscessus*.[142] *M. chelonae* is also highly resistant to cefoxitin, while *M. abscessus* is intermediately susceptible. Additionally, *M. chelonae* is only a rare cause of pulmonary infections and instead has a greater preference for skin and soft tissue infections.[27,143] Furthermore, *M. chelonae* has been associated with catheter-related, disseminated and bone infections, mainly in immunocompromised patients with risk factors such as organ transplantation, renal failure, surgery, long-term corticosteroid therapy, or chemotherapy.[144,145] Infections of *M. chelonae* can also be linked to ophthalmic surgery such as LASIK (laser in situ keratomileusis)[146] and cosmetic procedures (inoculations, plastic surgery, mesotherapy).[147]

This species of rapidly growing NTM is abundant in man-made environments, such as tap water, but has also been retrieved from natural environments, such as lakes, freshwater rivers, and seas.[148] Given its ubiquitous presence in the environment, exposure to *M. chelonae* is common and is often seen in clinical labs, contaminating solutions, medical equipment, and surgical wounds.

M. chelonae grows optimally at 30–32°C.

Treatment regimens of *M. chelonae* infections may include tobramycin, clarithromycin, linezolid, imipenem, or clofazimine. To avoid the development of macrolide resistance, clarithromycin will usually be combined with a second agent.[37]

Mycobacterium fortuitum Group

In 1938, da Costa Cruz (1938) proposed the name *M. fortuitum* for a strain isolated from a patient with a postinjection skin abscess.[121] More than 30 years later, Stanford and Gunthorpe showed that the isolate was identical to a formerly recognized species named *M. ranae*, isolated from a frog by Küster in 1905.[149,150] In 1972, the request of Runyon to maintain the species designation *M. fortuitum* was accepted, which to date still stands in nomenclature as such.[121]

The *M. fortuitum* group comprises the species *M. fortuitum*, *M. peregrinum*, *M. senegalense*, *M. alvei*, *M. houstonense*, *M. setense*, *M. neworleansense*, *M. boenickei*, *M. septicum*, *M. brisbanense,* and *M. porcinum*,[151,152] and its species are commonly found in drinking water, water distribution systems, and in a variety of soil worldwide.[148]

In a series of cases of extrapulmonary disease caused by the *M. fortuitum* group, Wallace et al. [153] reported that almost 80% of the infections were due to the single species *M. fortuitum*.

M. fortuitum has also been associated with a number of nosocomial pseudo-outbreaks,[154,155] and it has been linked to surgical site infections, skin lesions, postinjection abscesses, otitis media, and catheter-related infections.[156–160] Whirlpool pedicure footbaths in nail salons have also been identified as a potential source of *M. fortuitum*-associated furunculosis.[118,161]

M. fortuitum grows under the same conditions as MTBC.

Treatment of *M. fortuitum* infections consists of minimal 4 months of therapy with at least two agents with *in vitro* activity against the clinical isolate.[76] This regimen may include a combination of amikacin, fluoroquinolones, some tetracyclines, cefoxitin, imipenem, or sulfonamides.[153,162,163] In some cases where drug therapy is difficult, surgical intervention is required.[118]

Diagnosis

Clinical suspicion of mycobacterial disease (including x-ray or other imaging techniques) can be confirmed bacteriologically.

For decades, smear microscopy to detect acid-fast bacilli (AFB) and isolation of mycobacteria by culture were the most important diagnostic tools for mycobacterial disease, especially in low-resource settings where microscopy often was the only available and affordable method. Microscopy however lacks sensitivity (for pulmonary TB overall around 60% and only 22–43% in HIV-positive individuals; 10–60% for clinically significant NTM disease),[40] while culture is significantly more sensitive (80–95%) but can take weeks for *M. tuberculosis* complex and slowly growing NTM to become positive. With the advent of nucleic-acid amplification techniques in the 1990s, rapid, specific, and sensitive diagnosis became conceivable, but it was mostly restricted to well-equipped laboratories in high-income countries. Only with the introduction and roll out of the cartridge-based GeneXpert MTB/RIF assay (Cepheid, U.S.A), molecular diagnosis for TB—with simultaneous detection of rifampicin resistance—has become widely available and increasingly is the first test of choice in TB high prevalent settings.[164,165,166] TB-LAMP, another relatively easy isothermal DNA amplification assay to detect MTBC, has been endorsed in 2016,[167] but it has been rolled out in only a limited number of settings.[168,169]

The antigen-detection-based lateral flow-lipoarabinomannan (LF-LAM) assay has been recommended to assist in TB diagnosis in specific populations, namely adults, adolescents, and children living with HIV with signs and symptoms of TB and CD4 cell count under 200 in inpatient settings as well as in adults, adolescents, and children living with HIV with signs and symptoms of TB irrespective of the CD4 cell count in outpatient settings.[170,171] LAM, a lipopolysaccharide, is present in mycobacterial cell walls and is released from metabolically active or degenerating bacterial cells. An LF-LAM test is to be done manually on a urine sample.[170]

Regarding serodiagnostics, the WHO strongly recommended that commercially available assays should not be used for the diagnosis of pulmonary or extra-pulmonary TB.[172]

Despite progress in molecular diagnosis, culture remains important for subsequent phenotypic drug-susceptibility testing (especially for new drugs like bedaquiline), monitoring of patients during treatment, and as a gold standard test for most mycobacterial diseases. Once a culture becomes positive and is confirmed to yield AFB, the isolated bacteria can be identified by phenotypic or genotypic means prior to phenotypic drug-susceptibility testing.

Of note, two test systems are available for the identification of latent TB infection: the tuberculosis skin test (TST) and interferon-gamma assays (IGRAs). The TST consists of an intradermal injection of purified protein derivatives (PPD, tuberculin),

with a read-out by qualified staff after 48–72 hours. This assay, albeit cheap and not requiring any laboratory facility, is not specific because of cross-reaction due to prior BCG vaccination or exposure to NTM.[173] Efforts are ongoing to validate new skin tests using more specific RD1-antigens, not shared by *M. bovis* BCG, showing similar sensitivity.[174,175] IGRAs are *in vitro* blood tests to measure cell-mediated immune response, and make use of the same RD1-antigens.[173] Both test systems have limited value to predict progress to active TB.

Specimen Selection

Respiratory specimens constitute the most common type of specimen submitted for the culture and isolation of mycobacteria, with only 10–20% of clinical specimens originating from nonpulmonary sites.[176] For respiratory infections, sputum is to be obtained by preference early in the morning. The patient should be instructed to rinse his/her mouth with bottle water (tap water is not recommended), and cough up expectorate into a sterile sputum collection device specifically designed for mycobacterial culture. Multiple early morning sputum samples improve the chances to grow mycobacteria, especially NTM. If the patient can't produce sputum, which is often the case for children and very sick persons, a sputum induction can be performed. In this instance, the patient is either placed inside a special chamber or a hood is placed over the patient's head, and nebulized saline is introduced, causing the patient to cough up sputum.

In children or in presumptive patients having difficulty expectorating, specimens can also be obtained by the use of a bronchoscope, yielding a bronchial wash, bronchial alveolar lavage, or biopsy.[177] Bronchoscopy can assist in specifically sampling the affected area of the lung identified by x-ray. Alternatively, gastric lavage is used in patients who are not able to expectorate sputum and, hence, swallow it. Gastric lavage specimens need to be processed within 4 hours after collection.[178] Gastric lavage had higher positivity rates compared to induced sputum for TB diagnosis in children and adults.[177,179] Data on the isolation of MTBC and especially NTM from gastric lavage are scarce.[179] Recently, examination of stool by GeneXpert MTB/RIF (Cepheid, U.S.A) yielded good results and was superior compared to gastric lavage in individuals unable to produce sputum.[180] This application has however not yet been recommended by WHO.

For extrapulmonary disease, biopsies, or body fluids can be used for diagnosis. Biopsy specimens can be obtained from any site suspected of being infected with mycobacteria. Biopsies of an organ can be sampled either by ultrasound guidance or through general surgery. Skin lesions can be sampled by punch biopsy, scraping the margin of the lesion, or swabbing the ulcerated region. Drying of swabs should be avoided. It is rarely productive to collect samples from the necrotic center of the lesions. Body fluids can be obtained through aseptic fine needle aspiration. Urine samples are rarely positive for growth of mycobacteria. If indicated, early morning midstream urine should be collected. For the LF-LAM assay, any urine sample may do. Blood is not commonly used for detection of mycobacteria except for disseminated disease, especially in AIDS patients with low CD4 counts. Blood can be inoculated directly into special blood culture bottles of some of the automated mycobacterial culture systems.

Specimen Storage and Transport

Although mycobacteria can be recovered several days after sample collection, non-delayed processing will increase chances of successful isolation. Long delays in processing the sample increase the risk of overgrowth by microorganisms from the common mircroflora or environmental contamination. This risk can be reduced by ensuring refrigerated storage and transport (2–8°C), or in case of expected long delays from remote places (>7 days) and/or in the absence of an ensured cold-chain, transport medium can be added. To this end, a commercial medium like OMNIgene.SPUTUM (DNA Genotek, Canada) or the noncommercial cetylpyridinium chloride (CPC) can be added, allowing storage and transport at ambient temperature for up to 8 days or 28 days, respectively, prior to culture. Both reagents are also suited to conduct subsequent molecular analysis.[181] If only molecular analysis are to be done, addition of ethanol (final concentration ≥50%) allows for long-term storage (years) and shipping at ambient temperature.[181]

Isolation of Mycobacteria from Clinical Specimens (Culture)

Specimen Processing

Sputa and other specimens collected from the respiratory tract are "contaminated" with normal microflora. In addition, sputum is a viscous nonhomogenous specimen. Hence the need to process these specimens with a mucolytic agent and a decontaminating agent prior to inoculation on culture medium. If not, the normal flora will overgrow the more slowly growing species of mycobacteria, making their isolation from clinical samples impossible.

Several mucolytic and bactericidal/fungicidal reagents are used for this purpose.[178] The most commonly used are 4% NaOH or equal quantities of 4% NaOH solution and sodium citrate plus N-acetyl L-cysteine (NALC) solution. The 4% NaOH helps in liquefying the specimen as well as killing the normal flora of bacteria/fungi. NALC at concentrations of 0.5–2.0%, when combined with NaOH, facilitates decontamination by further digesting mucopurulent specimens, which allows the NaOH to penetrate; sodium citrate aids in the liquification by binding heavy metals, thus stabilizing NALC and allowing it to work properly. NALC can be used with reduced concentrations of NaOH (final concentration of 1% in sputum). For isolation of mycobacteria from specimens that are likely contaminated with Gram-negative organisms such as *Pseudomonas aeruginosa*, as observed in patients of cystic fibrosis and bronchiectasis, a combination of Nalc-NaOH processing with 5% oxalic acid treatment may be more successful.[178]

The optimal volume of sputum to be processed is 5–10 mL. An equal volume of the digesting/decontaminating solution is added to the specimen, vortexed, and allowed to stand for 15–20 minutes at room temperature. Then, the alkali are either neutralized by adding an equal volume of phosphate buffered saline (PBS; pH 6.8, 0.067 M) or HCl buffer (1N) till neutral pH as indicated by the phenol red indicator, or "washed" by adding an excess

(usually till 45 mL total volume) of PBS buffer.[178] PBS lowers the specific gravity of the specimen and gently neutralizes the specimen. The amount of NaOH used, the time allowed for decontamination, and the addition of neutralizing or washing buffer to the digested/decontaminated specimen are critical because both the common microflora and mycobacteria are affected by the exposure to the high pH of sodium hydroxide.

The specimen is then centrifuged at 3,000 × g for 15–20 minutes to concentrate the bacilli at the bottom.[182] The obtained sediment can be used to inoculate appropriate culture media, and—in case not yet done directly from the nonprocessed specimen—to prepare a smear for microscopic examination, or to perform a DNA extraction and subsequent molecular analysis.

Urine and other large volume samples should be concentrated (15 minutes at 3,000 × g), and nonsterile biopsies minced (sterile mortar and pestle) prior to processing. Specimens aseptically collected from normally sterile body sites do not need to be processed before inoculation into the appropriate media.

Smear Preparation

Smears can be prepared directly from clinical specimens (direct) or from the sediment of processed specimens in case culture is done as well (indirect).

Staining and Reading of Smears

Since the detection of TB bacilli by Robert Koch, the most commonly used dye for staining mycobacteria is basic fuchsine. This dye can stain all microorganisms, but only few will retain the dye in the cell wall after discoloration with an acid-alcohol solution. The composition of the mycobacterial cell wall (mycolic acids) of these organisms renders them resistant to discoloration of the primary dye, hence the term "acid-fastness."

The classic method, the Ziehl-Neelsen stain, uses carbolfuchsin (0.3%) as the primary dye. In this method, the stain is heated until steam arises (three times at intervals of 3–5 minutes) to allow optimal penetration of the cell wall. The modified or cold Kinyoun stain also uses carbolfuchsin as the primary dye but without heating and a contact time of 20–25 minutes. In both methods, acid-fast organisms will appear red against the background stain, which is usually methylene blue. Reading of the slides requires objective 100× and the use immersion oil.

Alternatively, fluorescent stains are used that consist of the fluorochrome stain auramine, alone or in combination with another fluorochrome, rhodamine. These stained smears must be read using a fluorescent microscope, where mycobacteria will fluoresce a bright yellow to orange. Fluorescence microscopy allows reading at a lower magnification (objective 25× or 40×), speeding up analysis. However, care must be taken to distinguish fluorescing artifacts from true organisms. The introduction of LED-based microscopes has enabled the roll out of fluorescence microscopy, even to remote places. In 2011, the WHO recommended that conventional fluorescence microscopy be replaced by LED microscopy, and that LED microscopy be phased in as an alternative for conventional Ziehl-Neelsen light microscopy.[183]

A minimum of 5,000–10,000 bacilli per mL specimen is needed to be observable in microscopy.

Isolation of Mycobacteria by Culture

For an optimal diagnosis of mycobacterial infection, their isolation from clinical specimens is considered as a gold standard. Digested, decontaminated, and concentrated specimen is inoculated on culture medium and incubated at 30°C and/or 37°C for 6–8 weeks to allow growth of mycobacteria. As a rule, media inoculated with specimens obtained from skin lesions should always be incubated at the regular 37 ± 1°C as well as at the lower 30 ± 1°C temperature. Once growth of AFB shows up and is confirmed, it is reported as positive. Traditionally one or more pieces of a solid medium or one solid and one liquid medium are inoculated.

Solid media used for mycobacterial culture can be divided into two main categories: egg-based and agar-based media. The egg-based media consist of (part of the) eggs, salts, potato flour, amino acids, glycerol, and malachite green as an inhibitor of bacterial growth from the common microflora persisting prior specimen processing. The media are solidified by heating the mixture to a temperature of 85°C for approximately 45 minutes in a process called inspissation. This process also sterilizes the medium. The most common form of egg-based medium used in the United States and many other countries is Löwenstein-Jensen (LJ) medium. Modified egg-based media may be more suited for the isolation of specific species. For example, *M. bovis* (BCG) grows better on medium without glycerol and supplemented with pyruvate (Coletsos or Stonebrink medium), while *M. haemophilum* needs addition of ferric-ammonium citrate for its growth. Agar-based media commonly employed are Middlebrook 7H10 and 7H11, both containing defined salt concentrations, growth factors such as oleic acid, albumin, catalase, glycerol, and a low concentration of malachite green. Medium 7H11 differs from 7H10 by the addition of pancreatic digest of casein to facilitate the growth of fastidious MTBC cultures.[184] Recently a new selective agar-based medium has been developed ("rapid growing mycobacteria" RGM), which enabled sensitive (98%) detection of NTM from CF patient samples.[185] Prolonged incubation up to 26 days further increased growth of MAC, with higher recovery compared to the classical medium for isolation of mycobacteria.[186,187]

Solid media can be prepared in house or purchased as ready-to-use from commercial companies. They may be supplemented with antibiotic mixtures to further reduce culture contamination rates. As, the optimal growth temperature for most NTM ranges between 28°C and 37°C, it is important to incubate at least one tube at 37°C and one at 30°C, especially in case of biopsies or pus from skin lesions.

In resource-poor settings, specimens can be cultured using only LJ medium, but for optimal isolation of mycobacteria from clinical specimens, both liquid and solid media should be employed to decrease the time to positivity and to increase the probability of a positive result (>10%).[188] The number of positive cultures for liquid is significantly higher than for any solid medium. Several automated systems using liquid medium for isolation of mycobacteria have been developed and are now used routinely in many laboratories, with the BACTEC 460 (BD Diagnostic Systems, U.S.) being the first introduced in the 1980s. This semiautomated system utilized a modified Middlebrook

7H9 broth containing a radiolabeled substrate that would be hydrolyzed by microorganisms growing in the medium, releasing radiolabeled carbon dioxide that was measured as an indicator of growth. The BACTEC 460 system has been discontinued due to concerns of radioactivity and its disposal and has been replaced by the nonradiometric BACTEC MGIT 960 system (BD Diagnostic Systems, Sparks, MD). This system builds on the principle of oxygen consumption by growing organisms. Oxygen present in the medium quenches the fluorescent indicator embedded in silicone at the bottom of the tube. When the oxygen is consumed by growing bacilli, the tubes will begin to fluoresce in the presence of UV light. Automated reading each hour, will flag these tubes as positive. The instrument provides the time to positivity and a growth-unit value (GU). Prior to specimen inoculation, a growth supplement including an antibiotic mixture (PANTA) must be added. MGIT 960 can be used for all types of specimens except for blood. Blood must be inoculated into a different type of bottle, the MYCO/F Lytic F, and incubated in a different instrument, the BACTEC 9240.

Bacterial contamination, which survives decontamination and grows in the presence of added antibiotics, can lead to a false-positive result. Unlike solid medium, liquid medium does not allow for the observation of colony characteristics, colony counts or mixed cultures. Any positive culture must therefore be confirmed by acid-fast staining, and, if possible, by purity check on a blood agar.

Scores of publications have verified MGIT 960 superior performance over solid media.

A couple of other commercial automated liquid systems have been introduced, such as VersaTREK Myco System (Trek Diagnostic Systems, U.S.A) and BacT/Alert (Biomérieux, France), but these systems are less commonly used.

Identification of Mycobacteria

It is imperative that MTBC can be immediately distinguished from NTM.

Upon isolation, initial mycobacterial species identification may rely on the observed growth rate (rapid versus slow), pigmentation, and colony appearance, the latter mainly observable from solid medium. They are described as either buff (white- to cream-colored colony) or a chromogen (yellow to orange pigmentation). The chromogens are classified as scoto- or photochromogenic, with scotochromogens being pigmented regardless of growing in the light or dark, whereas photochromogens will only develop pigment if exposed to light.[183] Most mycobacteria can be speciated by their biochemical profile,[183] which can be very tedious, requiring up to several weeks, and may not always allow for reliable (sub) species differentiation. Susceptibility to paranitrobenzoic acid (PNB; 500 µg/mL) has for long been a marker to differentiate MTBC from PNB-resistant NTM.[189] In most laboratories, however, molecular assays have displaced biochemical tests.

To rapidly differentiate MTBC from NTM growth, a lateral flow immunochromatographic assay detecting the presence of the MTBc-associated MPT 64 antigen has been developed. Three commercial tests are available: Capilia TB NEO (Tauns, Japan), BD MGIT™ TBc ID test (Becton Dickinson, U.S.), and SD Bioline TB Ag MPT64 Rapid (Standard Diagnostics, South Korea). In 15 minutes, the tests differentiate MTBC species from NTM in a positive culture. In a multicenter study, all three tests showed 100% sensitivity for detection of MTB from MGIT on the day of positivity. While the BD assay is not indicated for use with solid media cultures, the sensitivity of Capilia and SD Bioline assays was also 100% when applied on isolates grown on LJ cultures.[190] The SD Bioline assay may yield a weak false-positive result with *M. gastri*.[190] In a comparative study in Uganda, the SD Bioline assay performed equally well as the PNB assay.[191] Of note, not all MTBC lineages may be detected with a similar sensitivity, as evidenced by false-negative reactions for *M. africanum* in Benin and the Gambia.[192,193]

In case no MTBC is detected by an MPT64-based assay or an MTBC-specific molecular assay, most laboratories rely now on molecular testing for further NTM speciation. In house PCR- and sequencing-based identification of conserved genes is the reference method for the identification of mycobacteria.[5] This technology commonly targets a number of housekeeping genes, such as the ones encoding the 16S rRNA (*rrs*), the 65-kDa heat shock protein (*hsp65*), the RNA polymerase β-subunit (*rpoB*), bus is mostly restricted to reference laboratories. The vast majority of clinical laboratories will make use of commercially available assays.[5] While these can reliably differentiate MTBC from other mycobacterial species with very high sensitivity and specificity, they can identify only a limited number of NTM species. A comprehensive overview of various (molecular) assays for NTM species identification, discussing advantages, and shortcomings is provided by Misch and colleagues.[27] Some examples are discussed next.

The FDA-approved AccuProbe test (Gen-Probe, U.S.A) has single-stranded DNA probes that hybridize with ribosomal RNA from several mycobacterial species. It has probes specific for MTBC, *M. avium* complex, *M. kansasii*, and *M. gordonae*. The test can be used directly from positive cultures, within clinically relevant time frames (24–48 hours), with a sensitivity and specificity comparable to the lateral flow assay, yet requiring more complex manipulations.

Numerous assays based on the reversed hybridization line probe technology have been developed for the identification of mycobacteria: the InnoLiPA Mycobacteria (Innogenetics, Belgium) identifying 13 NTM species, and various GenoType assays from Hain Lifescience (Germany). The GenoType *Mycobacterium* CM and GenoType Cm*direct* both differentiate >20 common NTM species and MTBc, with the former requiring a positive culture, while the latter can also be used directly on decontaminated sputum. The GenoType NTM-DR allows for the same species identification as "CM," but in addition detects resistance mutations associated with clarithromycin and amikacin. For further identification of 19 less common NTM species, the GenoType *Mycobacterium* AS can be used on positive cultures.

In addition, molecular tests used for direct detection of MTBC in clinical specimens can also be used for identification of MTBC in grown cultures.

The cumbersome analysis of mycolic acid profiles using high performance (or pressure) liquid chromatography (HPLC), is nowadays rarely used in routine practice. The commercialized Sherlock Midi system (Midi Labs, Newark, DE), contains a database to which the profile of the unknown organism can be matched to speciate the isolate.

In clinical practice, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry has shown value in replacing the more costly and time-consuming 16S rRNA gene PCR and sequencing-based approach as the primary method for identification of microorganisms.[194] In a recent multicenter study, two commercial databases (Bruker Biotyper with Mycobacterial Library v5.0.0 and bioMérieux VITEK MS with v3.0 database) showed comparable performance, with 92% and 95% of NTM strains identified at least to the complex/group level, and 62% and 57% to the highest taxonomic level.[195] Differentiation between members of *M. abscessus, M. fortuitum, M. mucogenicum, M. avium*, and *M. terrae* complexes/groups was problematic for both systems, as was identification of *M. chelonae* for the Bruker system.[195] Similarly, a seeded-culture study that incorporated clinically prevalent respiratory microbiota, achieved 99% correct identification of mycobacteria, indicating that residual patient microbiota in a positive liquid culture was reduced by the Vitek MS.[196] In another study, the MAC subtyper module using the MALDI Biotyper algorithm, allowed for correct identification of 100% of the *M. intracellulare* and 82% of the *M. chimaera,* two closely related species.[197] Forero-Morales and colleagues have described a novel mycobacterial inactivation and protein extraction protocol (MIPE), which provides reliable MALDI-TOF results from solid and liquid media, while ensuring laboratory safety.[198] Finally, MALDI-TOF MS is intended for the identification of pure cultures; mixed *Mycobacterium* cultures present a challenge, as both species may not be distinguishable, especially in case of closely related taxa.[199]

Finally, the next-generation sequencing based Deeplex Myc-TB assay (Genoscreen, Lille, France), can detect 156 mycobacterial species either directly from smear-positive clinical specimens or from positive cultures, using nucleotide identity at the *hsp65* gene.[200]

Direct Testing of Specimens for *Mycobacterium Tuberculosis* Using Molecular Methods

Molecular diagnostics can be used to test directly on clinical specimens. Several commercial assays for detection of MTBC are being used since the 1990s (Table 33.2), such as the isothermal MTD amplification test by Gen-Probe (San Diego, U.S.A), the ProbeTec ET *Mycobacterium tuberculosis* complex Direct Test (DTB) (Becton-Dickinson), or the COBAS Taq-Man MTB Test (Roche Molecular Systems, Indianapolis, U.S.A). These assays were approved for AFB smear-positive respiratory specimens, yet require large instruments, are expensive or can be cumbersome.

Among the most common newer commercial molecular tests are the GenoType MTBC (Hain Lifesciences, GmbH, Nehren, Germany) and the less commonly used INNO-LiPA Mycobacteria (LiPA Innogenetics, Ghent, Belgium). These tests detect MTBc in a clinical specimen. The sensitivity and specificity are good in smear-positive sputum specimens but low in smear-negative specimens.

The most recently introduced is the Gene Xpert MTB/RIF (Cepheid, California, U.S.A) automated test. It is the most simple and rapid test with results available within 2-hours' time. Specimen is mixed with a reagent in a measured quantity and is placed in the instrument. All the necessary molecular procedures are automatically carried out inside the instrument, and results for the presence or absence of the MTBc and presence of rifampicin resistance is displayed within two hours. Sensitivity and specificity for smear-positive is very high, but for smear-negative specimens is rather low. This system has been tried in the peripheral sites with encouraging results.[40] Detection from smear-negative and culture-positive specimens needs improvement while detection of rifampicin resistance at a low level may be a concern.[41]

Other less commonly used commercial CE-labeled real-time PCR systems are available:

- The artus *Mycobacterium tuberculosis* RG PCR (artus MTB) kit (QIAGEN, Germany) has also been approved by the FDA and detects MTBc and/or MAC.

TABLE 33.2

Overview of FDA-Approved Nucleic-Acid Amplification Techniques (NAAT) for the Detection and Identification of Mycobacteria

Target	Assay	Company
Mycobacterium tuberculosis	Xpert MTB/RIF Assay	Cepheid
	BDProbetec ET Mycobacterium tuberculosis complex culture identification kit	Becton, Dickinson, & Co.
	Amplified Mycobacterium tuberculosis Direct Test	Gen-Probe, Inc.
	Amplicor Mycobacterium tuberculosis test	Roche Molecular Systems, Inc.
	SNAP M. tuberculosis complex	Syngene, Inc.
	Accuprobe Mycobacterium tuberculosis complex Test	Gen-Probe, Inc.
	Rapid Diagnostic System for Mycobacterium tuberculosis	Gen-Probe, Inc.
	Rapid Identification Test for Mycobacterium tuberculosis complex	Gen-Probe, Inc.
Mycobacterium species	Accuprobe *Mycobacterium avium* complex culture	Gen-Probe, Inc.
	Accuprobe Mycobacterium kansasii Identification Test	Gen-Probe, Inc.
	SNAP *Mycobacterium avium* complex	Syngene, Inc.
	Accuprobe Mycobacterium intracellulare Culture Identification Test	Gen-Probe, Inc.
	Accuprobe Mycobacterium gordonae culture identification Test	Gen-Probe, Inc.
	Rapid Diagnostic System for Mycobacterium gordonae	Gen-Probe, Inc.
	Rapid Diagnostic System for Mycobacteria	Gen-Probe, Inc.
	Rapid Identification Test for *Mycobacterium avium*	Gen-Probe, Inc.
	Gen-Probe Mycobacterium Rapid Confirmation System	Gen-Probe, Inc.

Source: https://www.fda.gov/medical-devices/vitro-diagnostics/nucleic-acid-based-tests (update September 8, 2020).

- The Geno-Sen's MTC/MOTT Real-Time PCR (Corbett Research, Australia) detects the genus *Mycobacterium* and MTBC.
- The Abbott RealTime MTB (Abbott Diagnostics, U.S.A) detects MTBC, while the variant Abbott RealTime MTB RIF/INH allows for simultaneous detection of resistance to rifampicin and/or isoniazid.
- The Speed-oligo Direct *Mycobacterium tuberculosis* assay (Vircell, Spain) includes an automated or manual post-PCR strip hybridization (dipstick format).
- The Truenat MTB Plus (MolBio, India) allows for detection of MTBC, while the Truenat MTB-RIF Dx subsequently detects rifampicin resistance in *M. tuberculosis* in Truenat MTB/MTB Plus positive specimens.

More sophisticated micro-array or lab-on chip-based CE-approved systems comprise:

- The CapitalBio Mycobacteria Real-Time PCR Detection Kit (CapitalBio Corporation, China) detects and differentiates MTBC from NTM, while the CapitalBio M. tuberculosis Drug Resistance Array Kit in addition detects 14 of the most frequent mutations associated with resistance to isoniazid and rifampicin.
- The VereMTB™ Detection Kit (Veredus Laboratories, Singapore) simultaneously detects MTBC, resistance to rifampicin and isoniazid, as well as 14 NTM species.

A non-CE-IVD approved test includes the REBA Myco ID (YD Diagnostics, South Korea).

Drug-susceptibility Testing of Mycobacteria Species

Details of drugs effective against clinically important mycobacteria and procedures for drug-susceptibility testing (DST) can be found in the Clinical Laboratory Standard Institutes (CLSI) guidelines.[201]

Susceptibility testing of MTBc isolates is advisable even in newly diagnosed infections, as an individual may have been infected with a resistant strain (primary resistance),[202] and drug resistance rates vary widely across global regions and settings. The WHO therefore recommends "universal DST," i.e., the search and identification of RR-TB among new TB patients, wherever this is logistically feasible.[203] Universal DST in all presumptive TB cases in the GeneXpert-based algorithm in South Africa resulted in a higher overall proportion of MDR-TB cases being diagnosed.[164]

Regarding susceptibility testing of NTM, the U.S. CLSI provides clear guidance on when and how to test and how to interpret minimal inhibitory concentrations (MICs),[201] while the British Thoracic Society recommends to follow CLSI, admitting that evidence is poor and DST should only be carried out on isolates where there is clinical suspicion of disease.[29] In the absence of clear *in vitro/in vivo* associations and clear-cut test concentrations for most NTM and drugs, the British guidelines distilled some evidence statements that could guide patient management (Table 33.3).

Overall, there are two major approaches for testing drug resistance, using culture-based or phenotypic and molecular-based or genotypic methods, with NTM-DST being mostly limited to phenotypic DST, with preference for MIC determination and reporting.

Phenotypic Susceptibility Testing

Culture-based or phenotypic DST can be performed on solid or in liquid medium, the latter having the preference for MTBC in terms of speed, and for NTM in terms of reliability. TB-DST should always be carried out in a confined laboratory following at least the safety precautions recommended by WHO.[204]

Classically, TB drug susceptibility testing is performed on the same solid media as used for primary isolation, such as LJ or Middlebrook 7H10 and 7H11 agar medium. The proportion method is the most applied, establishing the proportion of bacilli growing on culture-containing medium as compared to drug-free slants, considering an isolate as clinically resistant if >1% of the bacterial population resists the drug. To this end, an appropriate bacterial suspension (e.g., 10^{-3} dilution MacFarland #1) of the isolated culture is inoculated on the drug-containing slants, while a 1:100 dilution of the same suspension is inoculated on ad drug-free control. Solid media for DST can be obtained commercially or prepared in house. The drugs are added to the medium prior to coagulation. With exception of pyrazinamide (PZA) all TB drugs can be tested on solid medium, with clearly defined critical concentrations, specific for the drug and medium used.[205] Of note, LJ medium should not be used for bedaquiline susceptibility testing, because of its high protein-binding capacity. Solid medium DST, while being reliable, requires at least 3–4 weeks of incubation, with preferably a second reading at 6 weeks,[205] which is not in keeping with current U.S. Centers for Disease Control recommendations.[202]

TABLE 33.3

Evidence-Based Impact of Drug Resistance on NTM Treatment Outcome

Species	Disease Presentation	*In Vitro* Resistance[a] to	Impact	Evidence Level[b]
MAC	Not specified	Macrolides & amikacin	Treatment failure	2++
M. kansasii	Not specified	Rifampicin	Treatment failure	2++
M. abscessus	Pulmonary	Macrolides	Treatment failure	2++
M. abscessus	Extrapulmonary	Cefoxitin, amikacin and co-trimoxazole	Treatment failure	2++

Source: Adapted from Ref. [28].

[a] determined by broth dilution.

[b] 2++ = High-quality. Systematic reviews of case–control or cohort studies, or high quality case–control or cohort studies with a very low risk of confounding, bias or chance, and a high probability that the relationship is causal.

The use of liquid medium for detection and drug susceptibility testing has been recommended by the U.S. Centers for Disease Control and WHO,[202,204] MGIT 960 is the most widespread used liquid culture system for susceptibility testing of isolated MTBC against first- and second-line drugs, as well as new drugs like bedaquiline[208] and delamanid.[209,210] The system also relies on the proportion method principle, and results can be reported as early as 5–7 days after primary isolation. Details of the MGIT DST procedure are covered in the MGIT Manual published by FIND.[211] First-line and some of the second-line drugs are available in ready-to-use lyophilized form, while others need to be purchased and dissolved/diluted in house. Although recommended by WHO and CDC, MGIT960 testing risks to miss some cases of rifampicin resistance.[212,213]

PZA testing requires a low pH (pH 5.0) which can be achieved using adapted MGIT medium (BACTEC™ MGIT™ 960 PZA Kit), the only recommended phenotypic assay, despite previous association with a high rate of false-resistance results.[201,207] Several studies have shown good agreement of MGIT-PZA testing with *pncA* sequencing, if done meticulously; careful inoculum preparation is essential for correctly performing PZA testing reliably.[214]

Regarding NTM susceptibility testing, a variety of methods have been proposed, including broth dilution tests, the E-test, and proportional- and absolute-concentration methods.[42] E-tests suffer from high intra- and interlaboratory variability, and the proportional method on solid media or in MGIT has its breakpoints not yet defined for NTM. Hence the preference for the broth microdilution method.[201]

Molecular-Based Drug-susceptibility Testing

In the last decade, several genotypic DST procedures have been introduced, detecting mutations in one or more specific genes associated with resistance to (a) particular (class of) drugs. Genotypic testing is significantly faster than phenotypic testing. Among the first-line drugs, molecular testing overall has an excellent sensitivity for rifampicin and a good sensitivity for isoniazid, while both having excellent specificity. Sensitivity and specificity for ethambutol and PZA are debatable. Regarding second-line drugs, both for injectables and fluoroquinolones a relatively high sensitivity is observed. Details of the likelihood of association with drug resistance can be found on the Relational Sequencing TB Knowledgebase (ReSeqTB) platform (https://platform.reseqtb.org/).[215]

The FDA-approved GeneXpert MTB/RIF is the most widely used assay, with a pooled sensitivity and specificity of 96.8% and 98.4%, respectively, to correctly identify rifampicin-resistance in clinical specimens.[216] False-resistant results have been associated with a low bacillary load,[217,218,219] or the presence of synonymous mutations not associated with resistance.[220] Therefore, it is recommended to repeat testing in case of a low pretest probability for rifampicin resistance or a very low bacillary load. The new version Xpert Ultra may be more specific as it includes melting temperatures to identify the mutation type.[221] Further roll out of this version will provide data on its performance as an initial diagnostic test in field settings.

Results for the clinical evaluation of the chip-based real-time micro-PCR Truenat MTB-RIF Dx (MolBio, India) are expected by 2020.

While not being FDA approved, the WHO has endorsed the GenoType MTBDR*plus* (Hain Lifesciences, Germany) and the Genoscholar NTM-MDR TB (Nipro, Japan) line probe assays for detection of resistance to rifampicin and isoniazid in cultures and smear-positive samples.[222] Likewise, the GenoType MTBDR*sl* (Hain Lifesciences, Germany) can detect resistance to fluoroquinolones and second-line injectables.[223] The only assay for rapid detection of PZA resistance (Genoscholar PZA-TB II) has not been endorsed yet.[224]

Finally, next-generation sequencing-based Deeplex Myc-TB assay (Genoscreen, Lille, France) can predict resistance to 13 anti-TB drugs/drug classes, directly from smear-positive sputum or from culture isolates.[225] This assay has not yet been CE or FDA approved. Likewise, whole genome sequencing can predict TB drug resistance with a good sensitivity and specificity for most drugs,[226] albeit with yet unclear genotypic-phenotypic associations for new drugs like bedaquiline and delamanid.[227,228]

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