Nontuberculous *Mycobacteria*

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4.1 Introduction on the Genus *Mycobacterium*

After the first description of *Bacillus leprae* in 1875 by Hansen and the following discovery of *Mycobacterium tuberculosis* by Robert Koch in 1882, the genus finally emerged as a taxonomic group of pathogens named *Mycobacterium* as proposed by Lehmann and Neumann in 1896 [1]. The genus is composed of aerobic rod-shaped Gram-positive acid-fast microorganisms, most of them exhibiting facultative intracellular growth and having varied environmental reservoirs. Some *Mycobacterium* spp. also are associated with important well-known historical human diseases such as leprosy and tuberculosis, among others, while also being pathogenic for animals, some of them with zoonotic potential.

The use of the term "atypical acid-fast microorganisms" was introduced in 1935 to designate a mycobacterial isolate that caused human disease but could not be differentiated from *M. tuberculosis* by morphology, pigmentation, and virulence in animals [2]. Three years later, Costa Cruz isolated a fast-growing *Mycobacterium* from a human abscess that he named *M. fortuitum* [3]. A series of mycobacteria different from the *tuberculosis bacillus* started to be recognized as etiologic agents of human diseases, including *M. marinum* 1926 [4], *M. ulcerans* (1950) [5], *M.*

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intracellulare (initially named as *Nocardia intracellularis* in 1949 and later on renamed as *M. intracellulare* in 1965) [6], *M. kansasii* (1953) [7], and *M. scrofulaceum* (1956) [8], as main examples [9, 10].

It is known for decades that natural habitats of NTM are aquatic and soil environments [11]. The majority of *Mycobacterium* species have no impact on human health and occasionally, as opportunists, are responsible for human infections. However, some species are both environmental and pathogenic, while some are obligatory pathogenic. Contamination probably occurs through aerosolization or aspiration of water and/or soil particles and through exposure to traumatized skin and is generally not transmitted person to person. Therefore, it is important to identify the species that cause an infection in cases where the symptoms are sufficient to support sample collection [12–14].

4.1.1 The Taxonomy of Mycobacteria

Considering the present version of the List of Prokaryotic Names with Standing in Nomenclature (LPSN) database (available at http://www.bacterio.net/m/mycobacterium.html), a total of 192 validly published taxa are included in the genus *Mycobacterium*, including species and subspecies that are distributed in three major groups: (i) the *Mycobacterium tuberculosis* complex (MTBC), (ii) the distinct specie *Mycobacterium leprae*, and (iii) the nontuberculous mycobacteria (NTM), also called mycobacteria other than the MTBC. Traditionally, mycobacteria have been divided into rapidly (RGM) and slowly growing mycobacteria (SGM), the former needing less than 7 days for visible colony formation on solid culture media, the latter more than 7.

Through the years, the systematic taxonomy of this genus has evolved considerably based on grouping of phenotypic properties, analysis of chemotaxonomic characteristics, and sequence comparison of the 16S rRNA; of the 65-kDa heat-shock protein; of the genes *recA*, *rpoB*, *gyrA*, *gyrB*, *secA1*, *sodA*, *tuf*, and *smpB*; of the tmRNA; and of the 16S-23S rRNA intergenic spacer (ITS) region, performing a multilocus sequence analysis approach of concatenating several gene sequences, by interspecific DNA-DNA hybridization technique and/or, most recently, by genomic comparison.

Recent studies have suggested a new taxonomic classification and phylogenomic structure for mycobacteria based on datasets of genes/proteins from the genomes of different species. In 2018, Gupta et al. [15] suggested the redefinition of mycobacterial taxa based on amino acid insertions or deletions of fixed lengths within a specific position at a conserved region, named conserved signature indels (CSIs). These clade-specific marker gene sequences were proposed as a better definition of relationships among mycobacteria for determining the vertical inheritance and phylogenetic tree building as performed on datasets of concatenated protein sequences and proposed to relocate the mycobacterial taxa into five distinct genera: *Mycobacterium*, *Mycobacteroides*, *Mycolicibacillus*, *Mycolicibacter*, and *Mycolicibacterium*. However, Tortoli et al. [16] and most of the researchers in this study field preferred

Table 4.1 Nomenclature for the main *Mycobacterium* species as summarized by Tortoli et al. [16]

Classical nomenclature	Nomenclature adjustment proposed by Tortoli et al. [16][a]	Nomenclature according to Gupta et al. [15]
Mycobacterium abscessus subsp. *abscessus*		*Mycobacteroides abscessus* subsp. *abscessus*
Mycobacterium abscessus subsp. *bolletii*		*Mycobacteroides abscessus subsp. bolletii*
Mycobacterium abscessus subsp. *massiliense*		*Mycobacteroides abscessus subsp. massiliense*
Mycobacterium avium subsp. *avium*	*Mycobacterium avium* subsp. *avium var. avium*	
Mycobacterium avium subsp. *paratuberculosis*		
Mycobacterium avium subsp. *silvaticum*	*Mycobacterium avium* subsp. *avium var. silvaticum*	
Mycobacterium chelonae subsp. *chelonae*		*Mycobacteroides chelonae*
Mycobacterium chelonae subsp. *bovis*		
Mycobacterium chelonae subsp. *gwanakae*	*Mycobacterium chelonae* subsp. *bovis*	
Mycobacterium fortuitum subsp. *fortuitum*	*Mycobacterium fortuitum*	*Mycolicibacterium fortuitum*
Mycobacterium fortuitum subsp. *acetamidolyticum*	*Mycobacterium fortuitum*	*Mycolicibacterium fortuitum*
Mycobacterium gordonae		
Mycobacterium immunogenum		*Mycobacteroides immunogenum*
Mycobacterium intracellulare subsp. *intracellular*		
Mycobacterium intracellulare subsp. *chimaera*		*Mycobacterium chimaera*
Mycobacterium intracellulare subsp. *yongonense*	*Mycobacterium intracellulare* subsp. *chimaera*	
Mycobacterium kansasii		
Mycobacterium parafortuitum		*Mycolicibacterium parafortuitum*
Mycobacterium smegmatis		*Mycolicibacterium smegmatis*
Mycobacterium tuberculosis complex		*Mycobacterium tuberculosis*
Mycobacterium ulcerans		

[a]Empty field: nomenclature identical to the one of the first column

to use the classical nomenclature and reinforced the use of genome comparison for taxonomic classification.

By using the average nucleotide identity (ANI) and genome-to-genome distance (GGD) to analyze all the *Mycobacterium* taxa, Tortoli et al. [16] performed a detailed review and suggested specific adjustments for this genus. We detached the main species citing the classical and previously proposed nomina in Table 4.1.

4.1.2 *Mycobacterium tuberculosis* and *Mycobacterium leprae*

The two major human mycobacterioses are tuberculosis and leprosy. In 2018, ten million people fell ill of tuberculosis worldwide, killing 1.5 million of these, and 1 in 6 coinfected with HIV [17]. Although the disease is curable, a major problem is resistance to rifampicin, evolving often to multidrug and sometimes extreme drug-resistant disease, difficult to cure with long, toxic, and expensive treatment schemes and high mortality rates.

Almost 210,000 new cases of leprosy were reported in the same year, and just like TB, these are curable with a multidrug therapy and fortunately still presenting relatively low drug resistance levels [18]. However, relapse is quite common, and the World Health Organization recommends vigilance for drug resistance. The major problems regarding this disease are late diagnosis causing physical disability and stigma.

Tuberculosis is caused by organisms belonging to the MTBC that was recently redefined as a single species [19], disease being mostly caused by *M. tuberculosis var. tuberculosis* and *M. tuberculosis* var. *bovis*. Leprosy is caused mostly by *M. leprae* although a second species called *M. lepromatosis* and described mainly in Mexico is causing a particular clinical form of leprosy called Lucio syndrome [20, 21]. However, both disease characteristics and geographic distribution of the latter pathogen are under active study.

During the last two decades, basically since the availability of the complete genome sequences of *M. tuberculosis* [22] and *M. leprae* [23], a large number on studies on genetic variability between strains in either species have been described. Procedures for detection of strain variability have been used in studies on definition of species, phylogeny, evolution, strain virulence, transmissibility, molecular epidemiology, drug resistance, and host response, and these topics have been covered in several good reviews. Because another review on this is beyond the objective of this chapter, we refer to some recent papers and chapter, respectively, for MTBC [24–26] and for leprosy [27].

4.2 Clinical Significance of Nontuberculous Mycobacteria

4.2.1 Disease Caused by Infection with Rapidly Growing Mycobacteria

The clinically most prevalent RGM species are *M. abscessus*, *M. chelonae*, and *M. fortuitum*. While *M. abscessus* is mostly isolated from clinical respiratory specimens, *M. fortuitum* is recovered more frequently from non-respiratory specimens. The spectrum of diseases varies among the main species of the group [28–32]:

- *M. abscessus* – Pulmonary infections, primarily associated with bronchiectasis associated with cystic fibrosis or other comorbidities, skin and soft tissue infections after cosmetic procedures or surgeries, prosthetic device infection, tenosynovitis, and osteomyelitis
- *M. chelonae* – Surgical wound infections, abscesses, keratitis, catheter-related bacteremia, and hematogenously disseminated disease in immunosupressed individuals
- *M. fortuitum* – Skin and soft tissue infections (surgical or other traumatic lesions), chronic discharging sinuses, pulmonary infections among individuals harboring underlying pulmonary diseases, superficial lymphadenitis, prosthetic device infection, catheter-related sepsis, prosthetic valve endocarditis, and others

4.2.2 Disease Caused by Infection with Slowly Growing Mycobacteria

The major clinical syndromes associated with SGM include progressive pulmonary disease, skin and soft tissue infection due to direct inoculation, lymphadenitis, and disseminated disease in severely immunocompromised individuals by *M. avium* complex (MAC) and other NTM [31, 33]. The major clinical syndromes caused by specific species are:

- MAC – Lung diseases in HIV-negative patients, commonly associated with cystic fibrosis or middle-aged or elderly men, alcoholics, and/or smokers presenting or not underlying chronic obstructive pulmonary disease (COPD), mainly non-smoking women over 50. MAC also causes disseminated disease in severely immunocompromised patients (such as AIDS or other syndromes and upon use of immunosuppressive drugs), solitary pulmonary nodules, and hypersensitivity pneumonitis syndrome [34, 35].
- *M. avium* subsp. *paratuberculosis* (MAP) – One of the possible etiological agents of Crohn's disease (CD) due to the characteristic tuberculous-like gastroenteritis in humans and similarities to the clinical and histopathological findings to the Johne's disease in ruminants caused by MAP. Some studies have described the isolation of this pathogens from lymph nodes and blood of patients with CD [36].

- *M. kansasii* – Considered the second most common respiratory NTM and associated with pulmonary disease similar to tuberculosis in patients with COPD, malignancy, immunosuppressive drugs, pneumoconiosis, alcohol abuse, and/or HIV infection. This species has also been described causing disseminated disease, mainly in HIV-positive individuals [37].

Other human pathogenic SGM include *M. malmoense*, *M. marinum*, *M. simiae*, and *M. xenopi*, all associated with similar pathologies caused by other NTMs [38]. *Mycobacterium ulcerans* is particularly related to localized skin lesions progressing to extensive ulceration that may result in functional disabilities [39].

4.2.3 Considerations on Virulence and Drug Resistance

Because both virulence and drug resistance are important characteristics of NTM that can vary considerably both on a species and strain level, we mention these in this chapter as strain typing can be beneficial for the patient. Nonetheless, few data exist on direct correlation of these characteristics and characterization of NTM strains, and this in contrary to strains belonging to the MTBC (see part 1.2).

Virulence of NTM is related to their complex lipid-rich cell wall and cholesterol catabolism as a source of energy and material for the synthesis of the cell wall, proteins, and cell envelope lipoproteins responsible for bacterial adherence and their ability to form biofilms. Due to the hydrophobic nature of the cell wall, NTMs can adhere to a wide range of organic and inorganic materials, promoting as such colonization followed by either pseudo-infections or true infections. In the last few decades, there has been a report of an increase in outbreaks and diseases caused by NMT [40].

NTM are naturally resistant to a wide spectrum of antibiotics that include most TB drugs. The selective pressure imposed by other microorganisms in the soil and in the water, probably producing antimicrobials, may have led NMTs to develop innumerable resistance mechanisms to maintain their survival [41]. One of these is the thick hydrophobic double-membrane cell envelope of mycobacteria that also acts as a major permeability barrier. It was shown already in the 1990s that isolates of the then called *M. chelonae-M. abscessus* complex have a cell envelope about 10–20 times less permeable than that of *M. tuberculosis*. In addition, morphotypic antibiotic resistance, a phenomenon of varying degrees of drug resistance in *M. avium* which is associated with a reversible colony morphology switch (white/red on Congo red containing agar, transparent/opaque), is also attributed to changes in permeability owing to cell wall modifications [42]. Such morphologic changes might have a genetic basis and should therefore be traceable by genotyping.

Efflux pumps contribute to intrinsic drug resistance by preventing accumulation of antibiotics in the bacteria and have been mainly described for fluoroquinolones and macrolides [43]. The NTM species also induce the expression of certain genes resulting in the modification of the target binding site of the drug, the so-called inducible drug resistance, and in the case of macrolide resistance in *M. abscessus* which is mediated by the *erm(41)* gene, encoding a ribosomal methylase and

sequencing of this gene and *rrl* can predict susceptibility to clarithromycin in strains of the *M. abscessus* group [44], but this correlation does not seem to be absolute [45]. The use of strain typing for prediction of drug resistance in this group and more particular *M. abscessus* subsp. *massiliense* was demonstrated very recently by MLST and WGS [46]. The differentiation of the subspecies of the *M. abscessus* complex is indeed important because they differ in resistance to antibiotics and in treatment response.

Some genotyping tools allow simultaneous differentiation of NTM to the species and/or subspecies level and inform on drug susceptibility. GenoType NTM-DR (NTM-DR, Hain Lifescience, Nehren, Germany) line probe assay (LPA) is such a tool that enables identification of the MAC species (*M. avium*, *M. intracellulare*, and *M. chimera*), *M. chelonae*, and subspecies of the *M. abscessus* complex. The assay also allows for detection of antibiotic resistance to macrolides and aminoglycosides, including polymorphisms in the *erm (41)* gene.

4.3 Molecular Identification and Genotyping of Mycobacteria

Among the NTM species, only about one third is familiar to microbiologists and doctors, so their identification guides therapeutic treatment and provides clues regarding the source and route of exposure. Due to the presence of these mycobacteria in the environment, a laboratory control monitoring the growth of NMT is established following clinical and microbiological criteria known for decades. When dealing with sterile clinical specimens such as in pleural fluid, blood, cerebrospinal fluid, and tissues, among others, a single NTM is confirmative for infection, while for diagnosis of lung disease, positivity in two samples of spontaneous sputum or in one bronchoalveolar lavage sample is needed [47].

Traditional phenotypic identification procedures for NTM to the complex and sometimes species level are laborious and based on time-consuming biochemical and morphology-based tests, including their initial differentiation form the MTBC. For these tests, confluent growth is required, may take more than 20 days to achieve adequate growth, and has the limitation to be basically species-specific [48, 49]. Time for identification has much been reduced due to the development of molecular tools for NTM identification. Nevertheless, the combination of conventional and nucleic acid-based procedures is still used in many laboratories for precise diagnosis and eventual strain typing.

The molecular identification methods for diagnosis have expanded significantly, and among the most widely used are:

- The polymerase chain reaction restriction enzyme analysis of the *hsp65* gene (PRA-*hsp65* method) [50]
- Direct PCR (partial) gene sequencing with the principal target genes 16*S*, *hsp65*, and *rpoB* including single target or MLST analysis [51, 52]
- Commercial rapid test based on DNA-strip technologies: INNO-LiPA Mycobacteria v2 (Fujirebio, H.U. Group, Japan); Speed-oligo® (Vircell,

Granada, Spain); GenoType CMdirect VER 1.0 (Hain Lifescience GmbH, Nehren, Germany), detecting MTBC and more than 20 clinically relevant NTM from patient specimens; and GenoType *Mycobacterium* CM VER 2.0 (Hain Lifescience GmbH, Nehren, Germany), detecting MTBC and more than 20 clinically relevant NTM from cultures

Despite being useful for identification of the species level and thus for accurate diagnosis, (most of) these methods do not discriminate within the specie and subspecies levels, with an exception of sequencing.

Hence, molecular typing procedures that characterize below the species level have been developed almost simultaneously and almost exclusively based on nucleic acid analysis (Fig. 4.1). They have been used for improving the epidemiological vigilance of mycobacteriosis based on detection of strain variability, transmission, outbreak investigations, as well as differentiation of reinfection and persistence/resistance. Through genotyping, the general idea was created that infection with NTM normally occurs from environmental sources [53]. Nonetheless, patient-to-patient transmission has been demonstrated between cystic fibrosis (CF) patients [54], and more studies are needed to evaluate the extend of such transmission events. To illustrate the major typing procedures used for NTM and their main applications, we summarize literature according to publication data in Table 4.2.

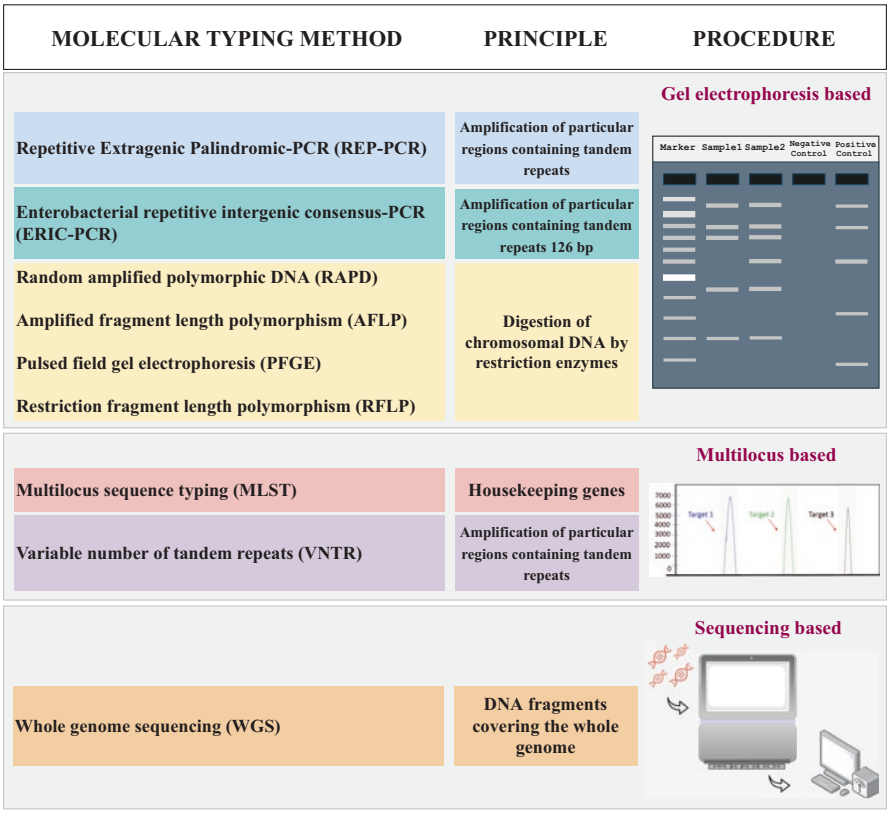

Fig. 4.1 The main molecular typing methods applied to Nontuberculous Mycobacteria

Table 4.2 Application and limitations of the main molecular genotyping methods applied to nontuberculous mycobacteria classified chronologically

Molecular typing method	Applications	Limitations
Repetitive extragenic palindromic-PCR (REP-PCR) [55]	Pseudo-outbreak [56], identification of source of infection [57], outbreak, and genetic diversity [58]	It is not an accurate tool for identifying organisms to the subspecies level. Low discriminatory power
Enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR) [99, 100]	Genetic diversity [53, 59], distinguish *M. paratuberculosis* from other mycobacteria (IS900/ERIC-PCR) [60], study of clonality [61], genetic diversity and infection control [62], outbreak [63–65]	It has necessarily a higher DNA quality. It has difficult reproducibility once it generates many bands, and thus, it is difficult to analyze
Random amplified polymorphic DNA (RAPD) [66]	Study of clonality [61, 67, 68], genotypic diversity and infection control [62, 69], outbreak [70], differentiation of infection and pseudo-infection/pseudoendemic [71], characterization of novel specie proposal [72], genetic diversity [73], identification of source of infection [74–77], clonality [78], strain discrimination [79], pseudo-outbreak [80, 81], outbreak [82]	Lacks inter-test and interlaboratory reproducibility; potential for misinterpretation is greater than that by PFGE. There is not a universal primer; we must test a set of primers. It generates many bands, and thus, it is difficult to analyze
Amplified fragment length polymorphism (AFLP) [83]	Identification of source of infection [84, 85], molecular epidemiology [86], genetic diversity [87, 88]	It is not an accurate tool for identifying organisms to the subspecies level. Low discriminatory power
Pulsed field gel electrophoresis (PFGE)	Outbreak [63–65, 89–94] and pseudo-outbreak [95, 96], molecular epidemiology [97], novel specie proposal [72], genetic diversity [58], identification of source of infection [98–104], study of clonality [59], differentiation of relapse from reinfection [105], specie differentiation [60, 106, 107]	PFGE depends on DNA quality, and the typing results can be influenced by a method of DNA isolation, electrophoresis/ running conditions [108]. Inability to type *M. abscessus* due to DNA degradation [107]. High cost of reagents compared to ERIC and RAPD [62]
Restriction fragment length polymorphism (RFLP) [109]	Genetic diversity [59, 108, 110], specie differentiation [106, 111], identification of source of infection [98, 112–115], specie identification and differentiation [116–120]	It is labor-intensive and requires a high level of operator skill
Multilocus sequence typing (MLST)	Specie identification [97, 110, 121, 122], molecular epidemiology [123], differentiation of infection from reinfection [124], phylogeny [97] and characterization of novel specie proposal (*Mycobacterium paraintracellulare* sp. *nov*. [125])	High cost of reagents compared to ERIC and RAPD [62]

(continued)

Table 4.2 (continued)

Molecular typing method	Applications	Limitations
Variable number of tandem repeats (VNTR) [126]	Influence of genotype [127], transmission [128, 129], genetic diversity [108, 130–134], phylogeny and association of genotypes to drug susceptibility [135–137], genotypes associated with clinical aspects [138], phylogeny [139, 140], identification of coinfection, source of infection [141, 142]	The genetic diversity can be influenced by homoplasy [143]
Whole-genome sequencing (WGS)	Transmission assessment [144], novel mutation proposal [46], strain discrimination [145], identification of source of infection [146, 147], taxonomy/phylogeny [148–151].	Higher cost compared to others

Because methods are based on different procedures that might include enzymatic digestion, PCR amplification, agarose gel analysis, sequence or fragment analysis, and fragment size estimation or counting, among others, their applicability depends on the diagnostic or typing purpose, and choice is therefore based on a combination of characteristics such as simplicity and speed of execution, cost, and differentiating power. However, one of the most important characteristics of a genotyping technique for strain differentiation is the discriminatory power, and in the case of several NTM species, PFGE presents the highest value and could in some way be considered as the reference technique [152].

Alternative fragment analysis-based procedures such as REP, AFLP, RAPD, and ERIC-PCR might be easier to perform but have the limitation that patterns and interlaboratorial comparison can be more complex. Moreover, simple variations on the DNA extraction protocol can have serious impact on the result.

One interesting application of such procedures was a study on *M. fortuitum* isolates from mammaplasty patients belonging to ITS genotype V that had indistinguishable RAPD-PCR and ERIC-PCR patterns, confirming that infections at other hospitals were caused by different *M. fortuitum* genotypes and that there was no clonal dissemination between hospitals [65]. Another study using the same tools demonstrated that ERIC-PCR has the potential to be used as a screening tool and useful for rapid epidemiological typing tools for *M. fortuitum* infections [62].

When compared to ERIC, both PFGE and RFLP demonstrated a higher resolution [60]; however, ERIC is still valid as a complementary or alternative tool for outbreak investigation, especially when working with *M. abscessus*. Compared to RAPD, however, ERC demonstrates either a higher [62, 64, 65] or a similar discriminatory power [61].

In other studies, PFGE showed similar results as REP-PCR for *M. abscessus* typing [153, 154]. Combined with VNTR typing, PFGE demonstrated a nice tool for discrimination within *M. kansasii* [108], a species that was described as being

composed of seven subtypes [155]. Recently, six of these subspecies have been elevated to a species rank and named *M. kansasii* (former type I), *M. persicum* (II), *M. pseudokansasii* (III), *M. ostraviense* (IV), *M. innocens* (V), and *M. attenuatum* (VI) [150, 156, 157]. Even so, this organism is still presenting considerable variability as presented by division of *M. kansasii* (type I) into two *hsp*65 subtypes as observed also by the overall genome organization [158]. This was confirmed in a later study adding more genomes [159], so WGS seems WGS a promising tool for future strain typing studies.

Despite being time-consuming, labor-intensive, and resource- and expertise-demanding, turning PFGE difficult to perform on a large-scale basis, it is still considered by many as the pillar method for molecular typing of NTM because of its high discriminatory power [93]. However, for some species, particular caution needs to be taken such as the case for *M. abscessus* that may present DNA degradation [152]. The subjectivity part of comparison of PFGE restriction patterns can reduce guideline focused on interpretation and using rigid algorithms but not totally eliminated [160].

The MLST technique has shown the highest sensitivity and specificity for identification to the species level of NTM [51], including discrimination of *M. abscessus* from other NTM species [97, 121]. But for typing of isolates of this species, again, PFGE was superior [161]. In another study, as expected, WGS showed a clearly higher discriminatory power in comparison with VNTR and therefore in practice the only molecular tool suitable to effectively discriminate isolates of *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *bolletii*, with clonal groups with different drug resistance patterns and suggesting transmission between patients [145]. Interestingly, a recent study compared a large amount of clinical strains completing a total of 175 NTM species by comparing whole-genome data and developing a new MLST algorithm based on 184 genes [122]. Their MLST-based identification showed higher accuracy than conventional MLST, and besides the potential to rapidly detect pathogens, the higher amount of data might, future wise, allow the use of this combined MLST-WGS approach for strain typing.

A sometimes very severe infection of subcutaneous tissue is observed during Buruli ulcer (BU), a neglected tropical skin disease caused by *M. ulcerans* [162], and molecular tools have contributed considerably to understanding the transmission and disease reservoirs [129, 151, 163]. Among these, VNTR has demonstrated a large genetic diversity [128] also adequate for phylogenetic assessment [132] of this species. Recently, the application of WGS through a phylogeographic analysis revealed a predominant sublineage of *M. ulcerans* that arose in Central Africa and proliferated in its different regions of endemicity during the Age of Discovery [151].

A recent excellent review by Shin et al. [164] focuses on genotyping of MAC/MAP and demonstrated that these species are mostly isolated from environmental sources such as in water and soil, therefore being the ecological niche for *M. avium* and *M. intracellulare*. Despite *M. avium* being excreted from infected animals and contaminates the environment, there seems to be no evidence for similar environmental contamination by *M. intracellulare*. Typing methods for strains from this complex can improve our understanding of estimating the infection pathway among

animals, humans, and the environment and evaluation of the treatment outcomes and the pattern of recurrence of MAC infection. The transmission of MAC species is not yet clearly defined, and together with the complex drug susceptibility pattern, more reliable and feasible genotyping methods of MAC are urgently needed,

The RGM *M. chelonae*, besides causing infections as related above, is commonly associated with skin and soft tissue infections and postsurgical infections after implants, transplants, and injections such as sclerotherapy and mesotherapy [165, 166]. Detection of source of infection is possible by molecular epidemiology studies on [56] and outbreaks by PFGE [90, 99] and/or ERIC-PCR [64]. Although considered a single species with *M. abscessus* until 1992, when *M. chelonae* was elevated to the species status, they share partial 16S rRNA signatures and are therefore still called the *M. chelonae-M. abscessus* group [167]. Among other phenotypical and molecular tools, RFLP was used to propose a division of this group [168] separating these species [111]. Genotyping by MLST to what were apparently particular stains of *M. chelonae* [169, 170] or of the *M. chelonae-M. abscessus* group [171] has also led to the identification of new (sub)species of these organisms.

Among the molecular tools, the only non-nucleic acid-based identification and typing technique for *Mycobacterium* isolates that we cover here is that based on matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS), a technique that during the last decade has turned into a timely and cost-effective identification procedure in routine microbiology laboratories [172]. In brief, a small amount of bacterial mass from a log phase culture is collected, heat inactivated, and treated with ethanol and the dried cell pellet vortexed or sonicated with beads in acetonitrile and formic acid before covering the dried extract with a special matrix. Identification is obtained both at the genus and species level between a range of 80% and 98% depending on the study [173]. The method has some limitations that have been only partly resolved. One is the impossibility to identify subspecies within the so-called *Mycobacterium* complexes that is still not possible for the MTBC. In the case of the *M. abscessus* complex, an algorithm for differentiation of the three subspecies was described [174], while the same was obtained by the use of principal component analysis [175]. Interesting also is that the formerly single species *M. kansasii* composed of seven genotypes resulted in reproducible and unique MALDI-TOF spectra that differentiated six of these [176], now separate species [150, 156, 157]. Another example of the promising evolution of this identification technique is the recently described algorithm for the differentiation of *M. intracellulare* from *M. chimaera* [177].

Two commercially systems for MALDI-TOF, each with their own *Mycobacterium* reference library, that of Bruker Biotyper with Mycobacterial Library v5.0.0 (164 species) and bioMérieux VITEK MS with v3.0 database, were recently compared and yielded similar results, although some problems were encountered in both systems for differentiation within complexes [178]. Because of the increasing number of *Mycobacterium* species and redefinition of their taxonomy, the constant need of updating of such databases to maintain accuracy of the identification is obvious [168]. Such databases have been constructed for MALDI-TOF users and can be accessed at https://microbenet.cdc.gov.

To our opinion, combined MALDI-TOF and genotyping analysis might be useful future wise, but the recent tentative to use the former technique alone for strain typing or lineage definition within the MTBC seems nothing but what the author's nicely called "a dream for the moment" [179].

4.4 Summary

Mycobacterium is a genus of *Actinobacteria* that are acid-fast bacilli closely related to *Corynebacteria*, *Rhodococcus*, and *Nocardia*. The genus now contains almost 200 recognized species with pure pathogenic species with best known examples *Mycobacterium tuberculosis* and *Mycobacterium leprae* and many environmental species that are sometimes also opportunistic pathogens. Mainly due to the evolution of genotyping techniques, many new species have been described during the last (two) decades, and many are to follow. Besides recognition of species, identification to the subspecies or strain level can teach us about disease transmission and bacterial population genetics and speeds up diagnosis, prediction of drug susceptibility, and evolution of disease and can therefore improve treatment. This chapter concentrates on current knowledge of strain typing of the main clinically important mycobacteria.

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