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# Species identification of non-tuberculous mycobacteria.

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**Key words**: NTM; mycobacteria; species; identification; methods.

**Abstract.** Non-tuberculous mycobacteria (NTM) prevalence has risen during recent decades. NTM species identification is crucial for treatment and epidemiologic control since, as opportunistic pathogens, they become sources of infections. Classical biochemical tests and phenotypic methods are not accurate and are time consuming. Other accurate and reliable species identification methods are available. Most are DNA-based from simple PCR-based to those more complex and high-tech based. Not all laboratories can afford technology transfer from simple to more complex and expensive methods, especially in low-income countries. The price has dropped for some high-tech methods, hence we may think about its possible widespread use, even though some limitations exist regarding data analysis and skill acquisitions. The identification of NTM has been problematic due to their evolving taxonomy, limitations of current phenotypic methods, and absence of a universal gene target for reliable speciation. Accurate NTM identification is a challenging task in diagnostic laboratories due to the absence of a universal robust method and to the difficulty to distinguish between closely related species. This work reviews the most commonly available methods for NTM species identification nowadays.

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## Identificación de micobacterias no tuberculosas a nivel de especie.

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**Palabras clave:** MNT; micobacterias; especies; identificación; métodos.

**Resumen.** La prevalencia de las micobacterias no tuberculosas (MNT) se ha incrementado durante las recientes décadas. La identificación de las MNT a nivel de especies es crucial tanto para el tratamiento como para el control epidemiológico, debido a que son patógenos oportunistas y se vuelven fuentes de infección. Las pruebas bioquímicas clásicas y los métodos fenotípicos no son precisos y consumen un tiempo considerable para obtener resultados. Existen métodos precisos y confiables, la mayoría moleculares, desde aquellos basados en PCR hasta otros más complejos de alta tecnología. No todos los laboratorios pueden costear una transferencia de tecnología de métodos simples a más complejos y costosos, sobre todo en países con bajos recursos. El precio ha caído para algunos métodos tecnológicamente avanzados, por lo que se pudiera pensar en su amplio uso, aunque todavía existen limitantes en relación al análisis de datos y la adquisición de destrezas. La identificación de MNT ha sido problemática debido a: una taxonomía en constante evolución, limitaciones de los métodos fenotípicos actuales y la ausencia de un marcador genético universal para una especiación confiable. La identificación precisa de MNT es una tarea desafiante en los laboratorios que hacen diagnóstico debido a la ausencia de un método robusto universal y a la dificultad de distinguir entre especies estrechamente relacionadas. Este trabajo revisa los métodos más comunes disponibles para la identificación de las especies de MNT hasta el presente.

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### INTRODUCTION

Two ancient pathogens, responsible for causing Tuberculosis (TB) and Leprosy, lie inside the genus *Mycobacterium*. Although these diseases are still major public health concerns, their incidence, mortality and prevalence have been reduced over the past decade (1,2). Our research have been focused on Molecular epidemiology and population structure of *Mycobacterium tuberculosis* complex (MTBC) strains before (3-12), however nontuberculous mycobacteria (NTM) are gaining attention.

In the early 1900s, there were approximately 100 TB cases for each NTM case. At that time, a NTM isolated from sputum cultures was considered to be a contaminant (13). For a long time NTM were not the focus of interest because of difficult diagnosis, due to culturing problems and the underestimation of NTM prevalence (14). It has been known for more than 64 years that NTM (or mycobacteria other than tuberculosis, MOTT) are able to cause disease in humans (15). Although NTM were previously believed to be low pathogenic (16), some species are producing a variety of infections in humans, especially when associated with particular risk factors like immunosuppression (17).

In recent decades, NTM infections have risen steadily regarding high TB rates in de-

veloping countries. In Taiwan, they went from 2.7 to 10.2 per 100.000 patients. The increase of all NTM diseases was predominately found in pulmonary diseases (18). Fifty years ago, a case of pulmonary NTM disease (PNTMD) was described for older male patients with predisposing conditions such as emphysema; but in almost 80% of recent reports middle age or older females are described with the same condition. Increased cigarette smoking could explain this, but many females with PNTMD have never smoked. Bronchiectasis has been found in most female patients, a condition associated with previous history of lung infection. Interestingly, a gender shift has been produced (13).

In North America, the rates of NTM infection have been reported to be between 1 to 15/100.000 and for NTM disease are from 0.2 to 2/100.000 inhabitants. These rates have increased with coincident decrease of TB rates. Similar rates have been observed in European countries except the Czech Republic, and mining industry could be the reason for this. Regarding NTM prevalence, a marked geographic variability is shown in many of these studies. Rates from Japan and Australia are quite similar to those found in North America and Europe, with significant increases over time. The exception is South Africa, where NTM infection rates are three times greater than in any other part of the world (19). Changes in clinician awareness, increased field investigations or improved laboratory methods that led to the isolation and identification of more NTM could have played a role in this trend. However, a true increase could be related to the host, the pathogen, or both; thus explaining the significant high rates.

Potential pathogen changes include increased virulence (13). The most prominent pathogenic NTM species include the *M. avium* complex (MAC), *M. kansasii* and *M. abscessus* that are associated with PNTMD (20). The latter is the most pathogenic and chemotherapy-resistant rapid growing mycobacteria (RGM). It accounts for 80% of lung disease caused by RGM and is the second most common RGM species present in extrapulmonary disease (following *M. fortuitum*) (21). Other NTM causing diseases in humans are: *M. ulcerans*, *M. marinum*, *M. scrofulaceum M. abscessus*, *M. fortuitum*, and *M. chelonae*. *M. ulcerans* is responsible for an ulcerative skin infection known as Buruli ulcer, this disease has become the third most common mycobacterial disease worldwide after TB. *M. marinum* is responsible for fish tank or swimming pool granulomas. *M. scrofulaceum* is involved in children cervical lymphadenitis. *M. abscessus*, *M. fortuitum*, and *M. chelonae* are responsible for soft tissue and skeletal infections (14). All these findings point out that some species of NTM have become emerging pathogens of human populations, especially in low income countries.

Those NTM causing pulmonary disease vary according to the geographic region. In most regions, *Mycobacterium avium* complex (MAC) is the predominant pathogen. *M. kansasii* is more common in middle USA and England while *M. xenopi* it is in northern USA, Canada, UK and in some regions of Europe. *M. malmoense* disease is more common in UK and in northern Europe but uncommon in the USA. For *M. simiae* disease, it has been found that is more common in arid regions of southwestern USA, Cuba and Israel. Thus, it is possible that regional variations in environmental conditions, may favor differences in predominant NTM populations on water and soil reservoirs to which susceptible patients are exposed (13).

To date more than 160 NTM species have so far been identified (22), but most of them are still poorly characterized. They are a heterogeneous group ubiquitously distributed in diverse environments (water, sewage, soil, air, dust, food, animals and humans), like normal inhabitants of these settings; they act as opportunistic pathogens of humans and animals, causing mycobacterioses of skin, soft tissues and lungs. Some NTM, such *M. avium-intracellulare*, *M. kansasii*,

and *M. abscessus* can cause disease in immunocompetent hosts (24). NTM are also responsible for disseminated disease (25) and outbreaks (26, 27) previously attributed to *M. tuberculosis*.

Some laboratories discriminate between *M. tuberculosis* and NTM, but they do not attempt to identify NTM to the species level. Without NTM's species identification there will be a lack of knowledge about its distribution and epidemiology. Therefore, reliable and sensitive species identifications by laboratories are normally required. Treatment options differ according to the species isolated, so it is important to assess their clinical significance (28) and epidemiological trends through a correct species identification. The treatment for NTM disease involves multiple drugs, is lengthy and varies by species; therefore, is challenging. In general, the therapeutic regimen selection to treat NTM disease has been based on NTM species identification rather than upon results of *in vitro* drug susceptibility testing. The main reason behind this, is that the clinical response does not correlate well with the *in vitro* susceptibility to the many drugs used to treat the NTM disease (23).

Moreover, it is possible to isolate different NTM species from the same patient during observation or treatment (29). Griffith *et al.* (30), observed the coexistence of *M. avium-intracellulare* with other NTM in 8% of 154 patients with lung disease caused by RGM. More recently, two NTM species were simultaneously isolated from different sputum samples collected from one patient on the same day (29). In addition, they detected multiple NTM species in 25% of 133 patients. Among these, two NTM species were isolated from 29 patients and three species were isolated from 4 patients. In 23 patients the isolated NTM changed to a different species, most frequently from *M. avium* to *M. abscessus* (6 patients) followed by changes from *M. avium* to *M. intracellulare* (three patients) and from *M. abscessus* to *M. avium* (three patients). Alternating isolation of two or three NTM was also observed in 9 patients, such as *M. abscessus* to *M. avium* and then to *M. abscessus*. Only one patient was under immunosuppressant therapy, but other clinical findings were present in the lungs of some of the patients. Because of the above mentioned, the identification of NTM species is important.

**Taxonomy**

Classification and identification of organisms has always been a priority among the early scientists to understand their environment. Zoologists and botanists have plenty of morphological traits to identify animals and plants. However, morphological characters for identifying bacteria are few and limiting. Also, little variations are present in those traits (31).

Runyon classification is known since the first reports of NTM (32). This classification, that remains popular, deals mainly with the growth rate (slow and fast) and pigmentation abilities of these mycobacteria recognizing four groups. Groups I, II and III are for slowly growing mycobacteria (SGM) that requires a similar time to grow in culture as *M. tuberculosis* while group IV are for rapid growers, less than 7 days. SGM are further differentiated according to their ability to produce yellow pigment. Runyon I (Photochromogens): produce a yellow-orange pigment when exposed to light (*M. kansasii*, *M. marinum*). Runyon II (Scotochromogens): produce a yellow-orange pigment in light or in the dark (*M. scrofulaceum*, *M. gordonae*, *M. szulgai*). Runyon III (Nonchromogenic): This group include mycobacteria that do not produce pigment (*M. avium-intracellulare*, *M. xenopi*, *M. terrae*). Runyon IV: This group include mycobacteria that do not produce pigment (*M. fortuitum*, *M. peregrinum*, *M. abscessus*, *M. chelonae*). This classification has become less relevant in recent years due to advances in mycobacteriology and to the fact that has no added value for clinicians (33).

NTM identification by traditional methods such as culture and biochemical analy-

sis is not very sensitive or specific (34). Errors in species identification underestimate the diversity within the genus *Mycobacterium* (35). Molecular methods such as polymerase chain reaction and sequencing of specific genes have recently offered the best approach to the NTM species identification (36). Several complexes have been described for NTM consisting of genetically closely-related microorganisms. *M. avium* complex (MAC) is composed of a heterogeneous group of NTM with varying epidemiologic implications comprising *M. intracellulare*, *M. avium* (*M. avium subsp. avium*, *M. avium subsp. hominissuis* (this designation has yet to be formally validated) *M. avium subsp. silvaticum*, and *M. avium subsp. paratuberculosis*) (37). *M. avium subsp. hominissuis* has been described to reflect the distinction of human and porcine isolates from bird-type strains (38). Over the years, there have been species that typically have similar characteristics to MAC organisms but lack features that typify them as MAC species. *M. chimaera* and *M. colombiense* are MAC-like organisms. *M. lepraemurium* refers to the agent of rodent leprosy, which is considered unculturable and can be identified reliably only by sequencing methods. The association with MAC stems from serological groupings and because the organism is characterized by only two single nucleotide polymorphisms (SNPs) in the 16S rRNA gene from that of *M. avium*, but has a highly divergent *hsp65* sequence. Although it is highly related to MAC organisms typically is not considered to be part of them. When faced with such organisms the clinical or reference laboratory may best report a MAC-like organism (37).

The taxonomy and epidemiology of MAC strains are being re-evaluated using different approaches from genetic and more recently genomic analysis (39). The taxonomic validation of *M. avium subsp. silvaticum* remains uncertain by Turenne et al. (39), since they presented molecular data strongly suggesting that *M. avium subsp. silvaticum* strains are simply *M. avium subsp. avium*. Recently, it was proposed *M. paraintracellulare* as a novel MAC species. Strains were isolated from Korean patients with pulmonary infections. Sequences of the 16S rRNA gene and internal transcribed spacer 1 (ITS1) were identical to those of *M. intracellulare* ATCC 13950T, but multilocus sequence typing (MLST) analysis targeting five housekeeping genes (*hsp65*, *rpoB*, *argG*, *gnd* and *pgm*) revealed the phylogenetic separation of these strains from *M. intracellulare* 13950T (40).

The *M. abscessus* complex (MabC) comprises a group of rapidly growing mycobacteria (RGM) representing the most common etiological agents of lung disease, *M. abscessus* subsp. *abscessus* (MAA), *M. abscessus* subsp. *massiliense* (MAM), and *M. abscessus* subsp. *bolletii* (MAB). MabC are resistant to many antibiotics *in vitro* leading to difficulty in making treatment decisions. *M. abscessus* has emerged as major respiratory pathogen in individuals with Cystic fibrosis (CF) and can result in an accelerated pulmonary decline (41). The prevalence of *M. abscessus* infections in patients with CF is increasing reaching around 3 and 10% in North America and Europe respectively (42). The reasons for this could rely on (i) exposure increases through more permissive temperatures of home water heaters (43), (ii) contact with aerosols from contaminated showerheads (44), (iii) the establishment of permissive lung niches through increased antibiotic usage (45), (iv) impairment of host autophagy inhibition by chronic azithromycin therapy, and (v) spread of NTM through person to person transmission (46,47).

*M. terrae* complex (MTC) was created in the 1970s to gather *M. nonchromogenicum* (described in 1965) (48), *M. terrae* (described in 1950) (49), and *M. triviale* species (described in 1970) (50) for which differentiation was not possible at that time by the available biochemical and cultural methods. These species share important cultural features including intermediate growth rate (from 5 to 15 days are required for the development of clearly visible colonies from

diluted inocula on solid media) and lack of pigmentation. In the early 1990s, the presence of a two-nucleotide insertion in helix 18 of the 16S rRNA gene (a unique genetic signature) (35) compared with other SGM, confirmed the consistence of the MTC. This signature still remains the most reliable marker for the attribution of mycobacteria to the MTC. *M. triviale* showed a 14 nucleotides (nt) shorter helix 18, as also seen in rapid growers, demonstrating the unrelatedness of this species to the complex (51).

*M. terrae* was initially considered as nonpathogenic because when inoculated cutaneously into guinea pigs it caused no ulceration or regional node involvement. Later, these organisms were occasionally identified in the setting of clinical diseases involving joints, tendons, lungs, gastrointestinal tract and genitourinary tract (52). More recently, *M. terrae* and *M. nonchromogenicum* were implicated in pulmonary infections (53,54).

A gap of more than 20 years separates the recognition of the original members of the MTC from the description of a novel species related to this group, *Mycobacterium hiberniae* (55). This novel *Mycobacterium* is characterized by a unique phenotypic feature, pink pigmented colonies, but the major role in its differentiation is played by genetic analysis that was beginning to emerge at that time. The boom years of the MTC start however in 2006 with the description of three novel species *Mycobacterium kumamotonense* (56), *Mycobacterium senuense* (57) and *Mycobacterium arupense* (56). Identification at species level within the members of the MTC still remains problematic as well as biochemical and cultural tests, the analysis of cell wall lipids is poorly discriminative. More recently, DNA probes specific for the species of the MTC have not been introduced by any of the commercial hybridization kits; probably as a consequence of the limited interest aroused by organisms that have been grossly labelled as nonpathogenic. Moreover, MTC identification using DNA sequencing remains elusive as hundreds of sequences related to their members are stored in public domain databases with unreliable species allocations (51).

Over the years, the MTC has expanded including other species: *M. paraterrae* (58), *M. sp. sinense* JDM601 (59), *M. engbaekii, M. longobardum* and *M. heraklionense* (51). Most MTC members are environmental bacteria of little clinical significance but some have been isolated from animal hosts (60) and human patients (52). In addition to the chronic and debilitating infections reported in humans by these strains, multiple antibiotic resistance has contributed to the therapeutic problems posed by these infections (61).

The *M. fortuitum* complex (MFC) causes a wide range of infections involving various wounds, catheters, lungs and others. The MFC has 3 biovars: *M. fortuitum* biovar *fortuitum*, *M. fortuitum* biovar *peregrinum* and the third biovariant (62). Schinsky *et al.* (63), divided the third biovariant into 5 species: *M. porcinum*, *M. boenickei*, *M. houstonense*, *M. neworleansense* and *M. brisbanense*. The first 4 species diverged by only 0 to 2 nt according to 16S rRNA genes representing the *M. porcinum* group, but *M. brisbanense* differs by 37 and 39 nt from *M. porcinum* and *M. fortuitum* respectively; thus it should be separated from the MFC despite their biochemical similarities. In addition, other species also belong to this group: *M. mageritense* (64), *M. senegalense*, *M. septicum*, *M. farcinogenes*, and *M. conceptionense* sp. nov. (65). Lamy *et al.* (66), reported a new species of the *M. fortuitum* group, *M. setense* sp. nov.

The *Mycobacterium marinum* complex (MMC) is a group of mycobacteria that include *M. marinum* and *M. ulcerans*. *M. marinum* is the cause of chronic systemic infections in fish and cold-blooded animals (67); it occasionally causes superficial and self-limiting granulomatous skin lesions in humans involving the hands, forearms, elbows, and knees (68). The cutaneous infection known as Buruli ulcer in humans is a necrotizing skin disease produced by *M.*

*ulcerans* (MU). MU is thought to have recently evolved from *M. marinum* progenitor by plasmid acquisition and reductive evolution (69,70). MU produces mycolatone, an unusual macrolide with cytotoxic and immunosuppressive properties responsible for the massive tissue destruction seen in Buruli ulcer. Besides, MU harbors a 174 kb plasmid bearing a cluster of genes encoding a giant polyketide synthases (PKSs) and polyketide-modifying enzymes sufficient for the mycolactone synthesis (69).

Other members of the MMC are *M. shottsii* and *M. pseudoshottsii* (71), they are mycolactone-producing mycobacteria (MPM) but they are not associated with Buruli ulcer. In 2001, *M. liflandii* appeared in laboratory colonies of *Xenopus tropicalis* in USA (72), it appeared later in colonies of *Xenopus* laevis in the same lab. The taxonomy of *M. liflandii* is uncertain, although the evidence suggests to be placed as a variant of *M. ulcerans* or *M. marinum*. *M. liflandii* appears to be a *M. marinum* like organism with a MU plasmid (73). Others propose *M. liflandii* as an MU ecotype, hence the name *M. ulcerans* evovar Liflandii (74).

The *M. simiae* complex is comprised of several phylogenetically related species, is the largest group of mycobacteria. A 12 nt deletion in the 16S rRNA gene, starting at position 459 in *E. coli*, characterizes the *M. simiae* group (75). Species belonging to this group are: *M. simiae, M. intermedium, M. interjectum, M. genavense, M. triplex, M. lentiflavum, M. heidelbergense, M. kubicae, M. palustre, M. montefiorense, M. parmense, M. sherrisii, M. saskatchewanense, M. parascrofulaceum, M. florentinum, M. stomatepiae, M. europaeum, M. paraense,* and *M. ahvazicum* (76).

The *M. mucogenicum* group comprises: *M. mucogenicum, M. aubagnense*, and *M. phocaicum* (78). Initially, these organisms were named *M. chelonae-like*. Later, it was proposed to call them *M. mucogenicum* reflecting their highly mucoid character (79).

Finally, *M. smegmatis* group contains: *M. smegmatis* sensu strictu, *M. goodie* and *M. wolinskyi* (80). Although *M. smegmatis* is a biosafety 1 level organism, members of this group have seldom been associated with human infection, including orthopedic device infection and bacteremia (81,82).

The taxonomy of the genus *Mycobacterium* seemed to be far from being fully elucidated and the reporting of unusual strains provides the best background for the recognition of new species (34). Hence accurate species identification relying on a systematic approach will then be mandatory for the establishment of a correct *Mycobacterium* taxonomy.

Recently, Gupta *et al.* (83), built robust phylogenomic trees based on the genomic sequences of 80% of all known Mycobacteria. These trees were based on core proteins from *Mycobacterium* species and some from the Actinobacteria phylum. This work proposes a new taxonomy for Mycobacteria based on 5 genera, each represented by a well-defined and supported clade: *Mycobacteroides gen. nov* (*Abscessus- Chelonae* clade), *Mycolicibacterium gen. nov*. (*Fortuitum- Vaccae* clade), *Mycolocibacter gen. nov*. (*Terrae clade*), *Mycolicibacillus gen. nov*. (*Triviale* clade), and the emended genus *Mycobacterium* (*Tuberculosis- Simiae*). This work constitutes the most comprehensive phylogenomic proposal for Mycobacteria at present.

## METHODS

**High performance liquid Chromatography (HPLC).** Tests for NTM identification were not developed for the majority of NTMs since they were not considered a serious threat to public health. Fatty acids are abundant in the phospholipid bilayer of bacterial membranes. They are found in unique combinations in several bacteria making the widely used fatty acid profiling a useful identification tool (84).

High-pressure liquid chromatography (HPLC), developed in the mid to late 1970, use the pressure produced by pumps to force a sample suspended in a liquid to pass through a solid stationary phase enclosed in a metal column in such a way that the sample will rapidly fractionate into their components. These separated components will be distinguished by a detector and depicted as peaks by a recorder. Peaks are defined by specific elution, emergence, or retention times. In 1985, scientists at the Centers for Disease Control and Prevention (CDC) proposed the use of HPLC as an aid in mycobacterial classification. Four years later it was incorporated into the regimen of tests at the CDC Mycobacteriology Reference Laboratory and in 1990 was offered as a standard test for the identification of Mycobacterial species. An isolate submitted on culture could be analyzed on hours by HPLC instead than weeks with traditional biochemical routine methods. HPLC is considered a sophisticated procedure compared to other laboratory methods and a dedicated highly trained operator is required (85). Instrumentation is costly compared to other traditional methods. Laboratories that detect acid fast bacilli only by smear may not have the capacity to develop proficiency with the HPLC method. However, many high- throughput laboratories incorporated HPLC into their methodologies (86). A significant challenge for laboratory personnel lies in developing expertise in visual interpretation of chromatographic patterns.

Complex high- molecular- weight β-hydroxy fatty acids with a long alkyl chain at the α position are features of mycolic acids from mycobacterial species. Butler *et al.* (87), developed an HPLC procedure for mycobacteria identification extracting mycolic acids from saponified mycobacteria cells and examined them as p-bromophenacyl esters by HPLC. Standard HPLC patterns were developed from culture collections and other well characterized isolates. Standardization by subtraction of the peaks absolute retention times (RT) from the high-molecular-weight standards produced relative RTs that were reliable for selecting comparable peaks. Visual observations of the heights of comparable peaks with the same relative RT were used to calculate the peak height ratios used in the empirical design of a flowchart decision scheme. No discrimination between MTBC strains was achieved except for *M. bovis* BCG.

Current methods for the interpretation of HPLC- generated chromatographic data do require some expertise. Each chromatogram must be visually evaluated and often hand calculations and flow charts are necessary to validate the identifications for many mycobacterial species, hence most of the time the process becomes tedious and time-consuming for laboratories. Glickman *et al.* (88), provided means of evaluating chromatographic data generated by HPLC for NTM but later it was found that mycolic acids were not completely chemically resolved when subjected only to reverse-phase partition chromatography. Therefore, some previous close relationships were not supported. RT increased when carbon chain length increased, revealing extensive overlapping of chemical fractions (85). Mycolic acids patterns are sometimes indistinguishable for closely related species (89,90).

**16S rRNA sequencing**. The development of molecular tools for the identification of novel or previously disregarded mycobacterial species helped to produce the available growing amount of information. The classification of organisms, based on similarities in their morphological, developmental and nutritional characteristics do not necessarily correlate well with natural (or evolutionary) relationships. 16S rRNA gen has proven to be a valuable tool due to its high information content, conservative nature, and universal distribution; having a broader coverage of known bacteria (91,31).

Bacteria were primarily grouped based on their morphological characteristics (shape, presence or absence of flagella), substrate utilization, Gram staining, and

growth pattern. Woese *et al.* (92,93), provided new standards to identify bacteria through phylogenetic relationships by comparing a stable part of their genetic code. 16S rRNA has been the marker most commonly used for taxonomic purposes since it has shown sufficient variation to distinguish between taxa (93), enhancing our understating of prokaryotic diversity and phylogeny, including that of non-culturable organisms. 16S rRNA analysis usually requires PCR amplification and to sequence the PCR product. We can identify the sequence when it matches those present in DNA databases. Since 16S rRNA has been widely adopted, databases are flooded with this sequence. If a 16S rRNA sequence does not match any known bacterial species it is believed to be a new one. Culture-independent studies can be conducted using genomes from mixed microbial communities in case of metagenomics hence there is no need of pure cultures. The 16S rRNA gene has hypervariable regions (HV1-HV9) flanked by conserved regions. These HV regions are an indication of divergence over evolutionary time (94). Conserved regions are used as targets for primer designing. When identifying species, there is no use to analyze the whole 16S rRNA gene as variation in HV regions is correlated with taxa identity. Sequencing artifacts and bacterial purity are problems to be faced when using 16S rRNA leading to incorrect identification arguing for the use of alternative methods to confirm findings (31).

In prokaryotes, this gene comes into several copies in a single genome. Around 15% of bacterial genomes contain only a single 16S rRNA copy and half of the genomes currently analyzed harbor five or more copies (95). These intragenomic copies can differ in sequence leading to identification of multiple ribotypes for a single organism (96,97). These copy numbers have shown narrow and wide variation within the tested bacteria genera (98,99). As more sequence information becomes available, it is evident that the resolution power of 16S rRNA is too low to allow the differentiation of closely related species (100,101,97). Fox *et al.* (100), noted that the use of 16S rRNA gene sequences for species identification is most useful in distinguishing relationships between genera and will resolve the identification of some species but not the more recently diverged ones. Although the 16S rRNA marker had the above limitations still was used until now.

**16S-23S Internal transcribed regions (ITS)**. Few studies have focused on the internal transcribed spacer (ITS) region for NTM identification (102,103). A multiplex PCR for NTM locally recovered was developed by Ngan *et al.* (104). The forward primer was common to all NTM species, and the reverse targeted several conserved regions based on the 16S–23S ITS region. This region is 270 to 360 bp long within species. They obtained species-specific fragments of different sizes and found *M. abscessus*, *M. fortuitum* complex, and *M. chelonae* in their Hospital. Mwikuma *et al.* (105), targeted the same region by PCR and found *M. intracellulare* followed by *M. lentiflavum* and *M. avium*. Other organisms different than NTMs were identified with the same procedure: *Rodococcus equi*, *Nocardia carnea*, *Tsukamura pulmonis,* and *Paenibacillus* species. These species are known to cause pulmonary disease as MTBC species do.

Park *et al.* (106), identified genotypes in *M. intracellulare* related strains using the ITS region in conjunction with other markers (*hsp65* and 16S rRNA). These results showed the high homogeneity of Korean *M. intracellulare* strains. This region has also been used to establish an association between patients with MAC strains and pulmonary tuberculosis (107). Since the accurate identification of slow-growing NTM of clinical significance remains problematic, Subedi *et al*. (108), proposed an ITS-based method using a Sequencer-Based Capillary Gel Electrophoresis (SCGE) system to identify these strains. These results showed that it was more discriminatory than HPLC.

The ITS region is less highly conserved than the 16S rRNA, thus it has a higher rate of polymorphism. Therefore, the presence of intraspecies variability may result in confusion (109).

**Matrix- assisted laser desorption/ ionization time of flight mass spectrometer (MALDI-TOF)**. The most used mass spectrometer with MALDI is time of flight mass (TOF). First studies regarding the MALDI-TOF technique for bacteria identification were conducted towards the end of 1990s when it was made available as a research tool, but it was only in 2008 that it was formally commercialized for private and public laboratory use. The delay was mainly due to the lack of robust information tools and efficient databases. MALDI- TOF measures peptides and other compounds in the presence of salts. Bacterial cultures are spotted on the MALDI-TOF plate placed on the TOF chamber. Each sample is spotted at least twice to ensure reproducibility. A control specimen of known identity is placed for correct identification. Samples are allowed to air-dry at room temperature, inserted into the mass spectrometer and subjected to MALDI-TOF analysis (31). Different experimental factors like sample preparation, cell lysis method, matrix solutions and organic solvents may affect the quality and reproducibility of bacterial MALDI-TOF MS fingerprints. This argues in favor to use alternative methods to guarantee correct identification (110). There has been limited work thus far on the use of this technique for mycobacteria identification (111,112). Organism's identification relies on unique spectral fingerprints produced by extracted proteins. A specific protein extraction protocol was designed for mycobacteria by Saleeb *et al.* (113), since protocols previously employed did not yield adequate spectra. With minor exceptions, identifications showed to be accurate to the species level at least for tested species.

Recently, Cao *et al.* (114), showed results for two MALDI-TOF systems (bioMérieuxVitek MS and Bruker MALDI Biotyper) for identification of mycobacteria. They found that application of MALDI-TOF, in clinical pathogenic mycobacteria identification, is less satisfactory to date and an increasing need for improvement is important; especially at the species level. An updated library is a must.

***hsp65*-based Polymerase chain reaction restriction analysis (*hsp65*-PRA)**. In 1992, Plikaytis *et al.* (115), introduced the PRA method based on the polymorphism present in the gene encoding for the 65-kDa heat shock protein (*hsp65*). This method was only for SGM without any identification algorithm. In 1993, Telenti *et al*. developed a method for rapid NTM identification by PRA (116). Due to the conserved nature of this gene, primers common to all mycobacteria were used. Later, digestion with two enzymes, *Bst*EII and *Hae*III, allow the differentiation. An algorithm was developed based on digestion fragments migration patterns. Reference strains which cannot be differentiated by 16S rRNA sequencing exhibited distinct PRA patterns like *M. kansasii* and *M. gastri*. In contrast to other mycobacteria, *M. gordonae* generated multiple PRA patterns which are consistent with previously reported microheterogeneity patterns within the rRNA-encoding region (117). They referred to those isolates whose patterns were not present in the reference algorithm (like *M. terrae* and others) as environmental mycobacteria or *Mycobacterium spp.* The MFC was separated to subspecies level and the *M. avium-M. intracellulare* complex to *M. avium* and *M. intracellulare*. In 1997, Taylor *et al.* (118), added five additional PRA patterns (4 new subtypes of species already described and one additional specie). Devallois et al. (119), proposed a new algorithm for the Telenti *et al. hsp65*-PRA method (116). They focused on the analysis of mycobacteria frequently found in drinking water, which are also resistant to commonly used drugs. These authors introduced 11 additional pat-

terns (5 additional species and 6 subtypes of species already described). Because some patterns may be close enough (5 bp difference), it may be difficult to differentiate between such isolates without appropriate software.

In 2001, Brunello *et al.* (120), modified the PRA method and included 22 species into an algorithm from a 10% polyacrylamide gel electrophoresis (PAGE) of restriction digests to improve the resolution of low-molecular-weight fragments and to extend the identification capacity of the method. More precise estimates of the real size of restriction fragments were obtained and deduced from sequence analysis allowing identification of PRA patterns shorter than 60 bp. Most of the additional species are not frequently isolated from humans. We learned from intraspecies variability studies that only some subtypes of *M kansasii* strains are associated with human infections (121). Later, Kim *et al*. (122), developed a *hsp65*- PRA method targeting a 644 bp DNA fragment compared to the previous 441 bp of Telenti *et al.* (116). This method has a higher resolution than other PRA methods being able to identify: *M. tuberculosis*, *M. avium* and *M. intracellulare* to the species or strain level using only a single enzyme digestion *Ava*II. For this purpose, an algorithm was developed. There is a PRA site database (http://app.chuv.ch/prasite) with 135 restriction patterns described for 174 mycobacterial species (123).

**RNA polymerase beta (β) subunit (*rpoB*) sequencing**. Approaches that sequence important genomic regions associated with drug resistance and or pathogenicity islands can simultaneously identify species and provide insight into the organism´s population structure (124). The *rpoB* gene coding for the β-subunit of the RNA polymerase is one of the very critical housekeeping genes closely related to cell viability, which explains why it was chosen as a drug target (Rifampicin). It is reasonable to assume that its genetic structure is highly conserved within the same species (125). Telenti *et al*. (116), characterized first some mutations within an *rpoB* region (411 bp long) of *M. tuberculosis*, no polymorphism other than those conferring drug resistance were observed. Ninety to ninety-five percent of mutations found to be associated with Rifampicin resistance fall within an 81 bp region (referred as the Rifampicin-resistance-determining region, RRDR) (126). A larger segment (705 bp) allowed simultaneous species identification and detection of rifampicin-resistance mutations (124). Because the *rpoB* region is present in NTM and due to some emerged problems regarding 16S rRNA NTM identification, a *rpoB* oligonucleotide array and conventional dideoxynucleotide sequencing were used to analyze sequence diversity within NTM species by Gingeras *et al.* (124). Only 5 SGM and 5 RGM were taken into account.

The *rpoB* gene is highly conserved in eubacteria (127). Kim *et al.* (128), worked within a region of 342 bp from the *rpoB* gene. They were able to differentiate pathogenic *M. kansasii* from non-pathogenic *M. gastri* that have been shown to be identical by 16S rRNA- based analysis despite major clinical differences and phenotypic traits. They also showed that they could differentiate between 44 mycobacterial species by direct sequencing. Troesch *et al.* (129), worked on a GeneChip technology that was used for monitoring gene expression and screening of mutations and polymorphism in genes; but this time focusing on Mycobacteria to interrogate the sequence of 16S rRNA and *rpoB* loci. Unique hybridization patterns allowed both identifications of species and rifampin-resistant alleles.

Lee *et al.* (130), developed a new *rpoB*-based PRA method showing more advantages than others. Most of the species had unique PRA profiles, unlike other PRA profiles that required computer-assisted analysis and interpretation. The restriction analysis of the 360 bp fragment by *Msp*I gave highly effective results for species differentiation. Only

few species required further digestion with *Hae*III, *Sau3*AI or *Hinc*II. For some species like *M. gordonae*, *M. kansasii*, *M. fortuitum* and *M. celatum* the discrimination reached the subtype level. A similar idea was developed by Kim *et al.* (131).

de Zwaan *et al.* (132), showed that *rpoB* gene sequencing is a more discriminative identification technique than the combination of reverse line-blot (INNO-LiPA Mycobacteria v2- Innogenetics, Ghent, Belgium) and 16S rRNA sequencing, it could introduce a major improvement in clinical care and epidemiology of NTM disease. Several other studies based on *rpoB* that included direct sequencing, PRA or hybridization methods support NTM species identification (78,133,134,123).

**Commercial Strip-based Assays**. Commercial hybridization-based methods (strip assays) are also available like Accu-Probe (16S rRNA) (non-amplification-based) (Genprobe, San Diego, Calif.)., INNO-LiPA (16S-23S) (Innogenetics, Ghent, Belgium) or GenoType Mycobacteria assay (23S rDNA) (Hain Diagnostika, Nehren, Germany) (135,136). These methods are being widely used for routine diagnostic in some laboratories. Sometimes INNO-LiPA identifications require complementary tests for some RGM due to their great heterogeneity, especially MFC and *M. chelonae*. Therefore, it only provides a few advantages over conventional methods (137). de Zwaan *et al*. (132), showed that *rpoB* gene sequencing proved to be more discriminative than INNO-LiPA identifying 15 more known species (*Mycobacterium vulneris, M. colombiense, M. mantenii, M. timonense, M. yongonense, M. heidelbergense, M. tilburgii, M. conceptionense, M. porcinum, M. houstonense, M. septicum, M. peregrinum, M. alvei, M. setense*, and *M. novocastrense*). A greater diversity was observed resulting in a more precise NTM taxonomy.

INNO- LiPA misidentifications have already been reported for *M. heidelbergense*, *M. mantenii*, and *M. parascrofulaceum*. *M. houstonense* was not identified as a MFC member (*M. fortuitum-peregrinum*) by INNO-LiPA as it was by 16S rRNA gene and *rpoB* gene sequencing. Most clinically relevant species have *rpoB* gene sequences of type strains available through public databases; thus, *rpoB* sequencing could be an appropriate first-line identification method for NTM isolated from human samples (132).

The GenoType Mycobacterium assay enables rapid identification of a broad range of potentially clinically significant Mycobacterium species, but some species require further testing to differentiate or confirm ambiguous results (136). Sarkola *et al.* (138), made a prospective evaluation of the GenoType assay for routine identification of mycobacteria. Even though INNO-LiPA and GenoType were rapid, sensitive and cost-effective they preferred the latter because its wider strain selection, less stringent reaction conditions and slightly better performance. They obtained 19 discordant results of which two reacted both *M. avium* and *M. intracellulare* despite the presence of specific probes on the strip. However, these same species were all correctly identified in a previous study (139). Therefore, intraspecies variations can occur in the 23S rDNA gene and perhaps be a reflection of their geographical origin. 12 samples that were negative for Accu-probe were identified as MTBC by the GenoType assay. These samples were later confirmed to be MTBC strains by 16S rDNA sequencing. The growth produced in automated culture systems may not be sufficient to allow sample identification by Accu-probe. *M. mucogenicum* gave a positive result with the *M. fortuitum* probe; the former belongs to the same complex suggesting an identical nucleotide sequence in the targeted region (138).

Turenne *et al.* (37), have shown that the recently described *M. palustre* and *M saskatchewanense* species gave positive reactions with the MAC Accu-Probe test, therefore they can be confused with MAC strains but they are otherwise genetically distant from MAC.

The number of NTM to be identified was recently expanded and ameliorated by Genotype kits. GenoType CM is capable to identify 24 NTM, while GenoType AS can identify other 19 NTM. Both kits were tested using samples recovered from a multicenter study of 14 participating countries over a period of 20 years. The CM kit correctly identified 96.,4% of the strains while the AS did it with an additionally 0.9%. The prevalence and frequency of NTM species, isolated from different countries, may influence the clinical performance of these tests; hence, before the test implementation as a routine application NTM diversity should be estimated. Extensive expertise is not required to interpret results coming from GenoType strips, as it is for a biochemical test or DNA sequencing (140).

**Multigene approaches**. Some studies have focused on a multiphasic approach to identify NTM mainly involved in outbreaks (90) or for phylogenetic purposes (141). Cooksey *et al*. (90), observed extensive conservation throughout the 16S rRNA gene among RGM outbreak associated strains. Therefore, multiple gene analysis is needed to confirm identification. The use of several genes, in a concatenated fashion, enabled robust phylogenetic distinction of NTM (77,142,141) allowing conclusions like *M. avium subsp. paratuberculosis* and *M. avium subsp. avium/M. avium subsp. silvaticum* evolved independently from *M. avium subsp. hominissuis*, which represents a diverse group. Thanks to this approach it was revealed horizontal genetic exchange (HGE) between homologous genes involving housekeeping genes from *M. abscessus* group strains. In such a way, certain *M. abscessus* strains have a composite genetic structure as a result of HGE between MabC members questioning the distinction between *M. abscessus*, *M. massiliense*, and *M. bolletii* within the *M. abscessus* group as truly distinct species (143).

Devulder *et al*. (142), tested separately the congruence of four genes (16S rRNA, *hsp65*, *rpoB* and *sodA*), normally used in a multigene NTM approach. *M. doricum* did not fit within the SGM (forming colonies in about 2 weeks) in any of the single-gene phylogenies; instead, it was close to RGM. Inversely, even though *M. holsaticum* is a RGM (forms visible colonies within 1 week), in the 16S rRNA phylogeny-based tree it was close to the SGM. For all the tested genes, the separation between RGM and SGM is clear even though not supported by a high bootstrap value. Therefore, it seems to be linked to a whole evolutionary process. The observed single gene congruence allowed the concatenation of these genes to increase the precision of phylogenetic relationships by using variable regions from a great number of sites. The generated supertree could differentiate each species as a separate entity, and it agreed with preexisting phylogenetic relationships. Some gene discrimination power loss, present in some species, would be compensated by other genes. Hence, when bootstrap values increase the phylogenetic relevance improves. The discriminatory power of a gene is related to its ability to discriminate between closely related species (142). Multigene or concatenated approaches came from the search for phylogenetic DNA markers rRNA-independent (144) to improve species identification resolution power. A species is a category that circumscribes a genomically coherent group of individual isolates/strains sharing a high degree of similarity in many independent features, comparatively tested under highly standardized conditions (145). DNA- DNA similarity (based on hybridization, DDH) and the ΔTm (whenever determinable), remain the acknowledged standard for species delineation (144). Anyway, researchers are encouraged to develop new methods to supplement or supplant the DNA- DNA re-association methods.

Mignard and Flandrois (146), performed a multilocus study (*hsp65*, *rpoB,* 16S rRNA, *smpB*, *sodA*, tmRNA and *tuf*) calculating evolution rates (ER) for each gene. They found that rapidly evolving genes (like *smpB* or *sodA*) were buffered by slowly evolv-

ing genes (like 16S rRNA and/or tmRNA). They also showed that pathogenic and SGM (like *M. kansasii*, MTBC, *M. leprae*, and *M. haemophilum*) were close clustered. Even though gene concatenation is the commonest method used in phylogenetic reconstruction it does not consider gene´s ER. The studied genes had different ER, also SGM and RGM has contrasting ERs. They suggested that the used phylogenetic approach should consider this variability.

Some authors have questioned the current taxonomic classification of *M. abscessus*, *M. bolletii*, and *M. massiliense* based on partial *rpoB* sequence. Macheras et al. (147), decided to study a large collection of these strains by partial *rpoB* sequencing and MLSA. Eight housekeeping genes were kept (*argH, cya, glpK, gnd, murC, pgm, pta,* and *purH*) to resolve this issue and reference strains were also included. Results clearly showed the existence of three principal groups, each harboring the included reference strain from one of the three species. The tree showed a very diffuse image regarding the massiliense branch and the reference strain does not cluster close to other tightly grouped *M. massiliense* strains.

Adékambi *et al.* (78), proposed that if more than 3% *rpoB* divergence between strains is found then the strains should belong to different species. The most different *rpoB* sequence between *M. massiliense* and *M. bolletii* groups was only 2.13% divergent; therefore, according to Adékambi et al. (78), criteria *M. massiliense* and *M. bolletii* do not belong to different species. The *rpoB* divergence between *M. abscessus* and *M. massiliense* and between *M. abscessus* and *M. bolletii* was exceeded by more than 3%. Almost 10% of the isolates had discordant *rpoB* and MLSA sequences, suggesting that these genes were involved in HGE processes. They believe that the *M. abscessus* group compared with the *M. massiliense/bolletii* are not entirely separate species. Hence, they propose a complete revision of the current taxonomic classification as Leao *et al.* (148), proposed before.

**Whole genome sequencing (WGS)**. WGS technologies are used to study diversity, genetic variations and solving genomic complexities (31). Recently, Gupta *et al.* (183), took advantage of the available genomic sequences (150 out of 188) of known *Mycobacterium* species to build phylogenomic trees. These trees were built using large datasets of protein sequences that included 1941 *Mycobacterium* and 135 Actinobacteria core proteins. Eight highly conserved essential proteins, present in all mycobacteria, were also included. Exhaustive genomic analyses were carried out to identify highly specific markers in the form of conserved signature indels (CSIs) and conserved signature proteins (CSPs) distinctive of the *Mycobacterium* genus. Results showed 5 clades highly supported. The *abscessus- chelonae* clade was the earliest branching lineage (represented by the *Mycobacteroides gen. nov*) that in conjunction with the *fortuitum- vaccae* clade (represented by the *Mycolicibacterium gen. nov.*) include all RGM, believed to be ancestral from which SGM evolved from. The remaining clades that include the SGM are: *Terrae*, *Triviale,* and *Tuberculosis- Simiae* represented by *Mycolocibacter gen. nov., Mycolicibacillus gen. nov.*, and the emended genus *Mycobacterium*, respectively. Besides the main five clades, smaller clades are also present. However, they will be the object of future studies.

Advances in WGS have resulted in a reduction in the full economic cost of sequencing a typical bacterial genome to as little as around 53 USD (from extracted DNA to genome sequence). Additionally, sequencing speed has increased from several weeks or days to just hours. The low cost and rapid turnaround time, combined, means that WGS can cross the divide between microbial research and the practice of diagnostic microbiology (149).

WGS offers the ultimate typing method with the potential to replace all existing methods. Novel DNA sequencing technologies, named high-throughput genome sequencing (HTGS), generate massive amounts of genetic information with increased speed, accuracy and efficiency. These new machines are called next generation because they offer an order of magnitude more sequence data for a fraction of the price of previous instruments. For example, the Solexa instrument can produce an astonishing 1 Gb of nucleotide sequence per run; it makes the technology doubly attracting and removes the need to create and propagate bacterial plasmid shotgun libraries. In addition, it reduces the time to produce samples avoiding biological bias inherent to those techniques. Our capacity to generate data will far outstrip our ability to analyze and interpret it. More trained bioinformaticians, cheaper data storage, and faster retrieval will be needed if we want to exploit the impending avalanche of genomic data (150).

The great value of WGS studying bacterial evolution, outbreaks and transmission, has been shown in recent studies (47, 151, 152). The next step will be to translate this technology from a research tool into a utility in routine diagnostic settings. In very broad terms, four main stages are required when using WGS for diagnostics; starting with detection of the pathogen in a sample, identification, testing for drug susceptibility and epidemiological typing. The obvious application of WGS data is for epidemiology, to detect laboratory cross-contamination, to define pathogen-transmission pathways, and to support outbreak investigations. Thus, highlights daily opportunities for pathogen´s infection control with a high rate of change (149). Genotypic drug susceptibility detection will complement disk susceptibility testing, which could still be necessary to detect resistance by unknown mechanisms (153). HTGS is well suited when mycobacteria grow slowly because full phenotypic susceptibility test will be measured in weeks (154).

Human error, rather than sequencing error, is often the source of mistakes in genotyping (155). Current generations of sequencers were designed for human genomes. But to be cost-effective in bacterial genomes, which are smaller, several hundred samples have to batched and individually tagged. Analyzing routinely sequenced bacteria by HTGS is not fit for clinical practice given that the time taken for the analysis would be significantly longer than WGS itself. Similarly, having dedicated bioinformaticians in every diagnostic laboratory is not realistic. Instead, analysis software for WGS is required to extract clinically relevant information in a fully automated and reliable fashion, without human intervention (149).

## DISCUSSION

Ideally, NTM identification must hold their taxonomic status, rather than only make discrimination between isolates which explains the switch proposal made from 16S rRNA to *rpoB* (a high conserved single copy gene) in this field. Combinations of housekeeping genes have been used to clarify NTM identifications. The gene or genes involved should not evolve too fast to keep the phylogenetic signal. Describing molecular epidemiology tools, in which some sequences should evolve fast enough to further better discriminate between isolates, even though belonging to the same species, is out of the scope of the present review.

NTM Species identification is mandatory to initiate an adequate antibacterial therapy. Several methods and platforms were reviewed here that can be used to identify NTM species. Some offer advantages over others, depending on laboratories and budgets. Some are in-house or home-made and others commercial. Molecular methods have shortened the time for NTM identification. Some could identify species from clinical samples, but samples need to have enough bacillary load to properly produce the identification. The *hsp65* algorithm yielded enough infor-

mation on NTM identification, but in some cases NTM complex species were not fully resolved. In some cases, *rpoB* proved to performed better clearly identifying the subspecies; specially for novel species.

In summary, some methods from low to high cost in order (after the first investment is done when required): (i) MALDI-TOF (around 3,14 USD per sample) (156), HPLC (≤6 USD per sample) (from http://www.midiinc.com/pages/mycobacterial_id.html), (ii) 16S rRNA gene sequencing, single-gene sequencing (other than 16S rRNA) (7 USD per sample including amplification cost from https://dna.macrogen.com) (157), PCR- RFLP (13 USD) (158), (iii) Line probe assays (26.58 USD) (159), (iv) multigene sequencing (x times the single-gene sequencing estimation) and WGS (53- 634 USD per two bacterial genomes) (160). From low to high discriminatory power: (i) HPLC, (i) Line probe assays, (iii) 16S rRNA sequencing and MALDI-TOF, (iv) PCR- RFLP, single-gene sequencing (other than 16S rRNA), (v) multigene sequencing (modified from van Ingen, 2015) (161) and WGS.

High-tech methods are mainly for national reference laboratories from moderate to high-income countries. The first investment is for buying the equipment and for skill acquisition, which is the most expensive part, then the price drops per analyzed sample. WGS needs fast and reliable bioinformatics tools to become a routine or widespread tool from cultures or clinical samples. The information obtained will be very helpful to reduce the time to initiate correct treatment, controlling outbreaks, and to determine the infection progress. However, still is too expensive for routine use in most laboratories (Research and Clinical microbiology laboratories).

Some people believe that WGS will become a standard tool for infection detection and control, and that it will monitor the spread and evolution of major pathogens in real time both within and outside hospitals. Bioinformatics tools are being developed to facilitate the analysis of WGS data and some are already available. Finally, according to your budget and needs, you will choose the method best suited for you depending if it is a small or big laboratory without losing systematic status during identification.

Recent reports present the annually WGS-based diagnostics as 7% cheaper than current diagnostic workflows (162).

## REFERENCES


1. **Gunaratnam PJ, Britton WJ, Hofmeyr A**. Bug breakfast in the bulletin: leprosy. N S W Public Health Bull 2013; 24: 50.
2. **WHO.** World health organization global tuberculosis report. Geneva. WHO 2014; 1-170.
3. **Sequera M, Delgado V, Araque W, Torrealba M, Núñez R, Da Mata O**, **Abadía E, Takiff H, de Waard J**. *Mycobacterium tuberculosis:* spoligotypes in the Carabobo state, Venezuela. Rev Chil Infectologia 2008; 25: 362–367.
4. **Abadía E, Sequera M, Ortega D, Méndez MV, Escalona A, Da Mata O, Izarra E, Rojas Y, Jaspe R, Motiwala A, Alland D, de Waard J, Takiff H.** *Mycobacterium tuberculosis* ecology in Venezuela: epidemiologic correlates of common spoligotypes and a large clonal cluster defined by MIRU-VNTR-24. BMC Infect Dis 2009; 9: 122-133.
5. **Zhang J, Abadia E, Refregier G, Tafaj S, Boschiroli M, Guillard B**, **Andremont A, Ruimy R, Sola C**. *Mycobacterium tuberculosis* complex CRISPR genotyping: improving efficiency, throughput and discriminative power of "spoligotyping" with new spacers and a microbead-based hybridization assay. J Med Microbiol 2010; 59: 285–294.
6. **Abadia E, Zhang J, dos Vultos T, Ritacco V, Kremer K, Aktas E**, **Matsumoto T, Refregier G, van Soolingen D, Gicquel B, Sola C**. Resolving lineage assignation on *Mycobacterium tuberculosis* clinical isolates classified by spoligotyping with a new high-throughput 3R SNPs based method. Infect Genet Evol 2010; 10: 1066–1074.
7. **Abadia E, Zhang J, Ritacco V, Kremer K, Ruimy R, Rigouts L, Gomes H, Elias A, Fauville-Dufaux M, Stoffels K, Rasolofo-**

**Razanamparany V, Garcia de Viedma D, Herranz M, Al-Hajoj S, Rastogi N, Garzelli C, Tortoli E, Suffys PN, van Soolingen D, Refrégier G, Sola C.** The use of microbead-based spoligotyping for *Mycobacterium tuberculosis* complex to evaluate the quality of the conventional method: providing guidelines for quality qssurance when working on membranes. BMC Infect Dis 2011; 11: 110-117.

8. **Bergval I, Sengstake S, Brankova N, Levterova V, Abadía E, Akhalaia M, Tuin K, Schuitema A, Panaiatov S, Bachiyska E, Herrewegh A, Schürch A, van Soolingen D, van 't Hoog A, Cobelens F, Aspindzelashvili R, Sola C, Klatser P, Anthony R.** Combined species identification, genotyping, and drug resistance detection of *Mycobacterium tuberculosis* cultures by MLPA on a bead-based array. Plos One 2012; 7: e43240. 1- 16.
9. **Gomgnimbou M, Abadia E, Zhang J, Refrégier G, Panaiotov S, Bachiyska S, Sola C.** "Spoligoriftyping," a dual-priming-oligonucleotide-based direct-hybridization assay for tuberculosis control with a multianalyte microbead-based hybridization system. J Clin Microbiol 2012; 50: 3172–3179.
10. **Sengstake S, Bablishvili N, Schuitema A, Bzekalava N, Abadia E, de Beer J, Tadumadze N, Akhalaia M, Tuin K, Tukvadze N, Aspindzelashvili R, Bachiyska E, Panaiotov S, Sola C, van Soolingen D, Klatser P, Anthony R, Bergval I.** Optimizing multiplex SNP-based data analysis for genotyping of *Mycobacterium tuberculosis* isolates. BMC Genomics 2014; 15: 572-582.
11. **Patiño M, Abadía E, Gómez S, Maes M, Muñoz M, Gómez D, Guzmán P, Méndes M, Ramirez C, España M, de Waard J, Takiff H.** *Mycobacterium tuberculosis* population structure and molecular epidemiological analysis in Sucre municipality, Miranda state, Venezuela. Invest Clin 2014; 55: 332–351.
12. **Refrégier G, Abadia E, Matsumoto T, Ano H, Takashima T, Tsuyuguchi I, Aktas E, Cömert F, Gomgnimbou M, Panaiotov S, Phelan J, Coll F, McNerney R, Pain A, Clark T, Sola C.** Turkish and Japanese *Mycobacterium tuberculosis* sublineages share a remote common ancestor. Infect Genet Evol 2016; 45: 461–473.
13. **Cook J.** Nontuberculous mycobacteria: opportunistic environmental pathogens for predisposed hosts. Br Med Bull 2010; 96: 45–59.
14. **Jagielski T, Minias A, van Ingen J, Rastogi N, Brzostek A, Żaczek A, Dziadek J.** Methodological and clinical aspects of the molecular epidemiology of *Mycobacterium tuberculosis* and other mycobacteria. Clin Microbiol Rev 2016; 29: 239–290.
15. **Timpe A, Runyon E.** The relationship of atypical acid-fast bacteria to human disease; a preliminary report. J Lab Clin Med 1954; 44: 202–209.
16. **Rindi L, Garzelli C.** Increase in non-tuberculous mycobacteria isolated from humans in Tuscany, Italy, from 2004 to 2014. BMC Infect Dis 2015; 16: 44-48.
17. **Tortoli E.** Clinical manifestations of nontuberculous mycobacteria infections. Clin Microbiol Infect 2009; 15: 906–910.
18. **Lai C, Tan C, Chou C, Hsu H, Liao C, Huang Y, Yang P, Luh K, Hsueh P.** Increasing incidence of nontuberculous mycobacteria, Taiwan, 2000-2008. Emerg Infect Dis 2010; 16: 294–296.
19. **Marras T, Daley C.** Epidemiology of human pulmonary infection with nontuberculous mycobacteria. Clin Chest Med 2002; 23: 553–567.
20. **Johnson M, Odell J.** Nontuberculous mycobacterial pulmonary infections. J Thorac Dis 2014; 6: 210–220.
21. **Park S, Kim S, Park EM, Kim H, Kwon O, Chang C,** et al. In vitro antimicrobial susceptibility of *Mycobacterium abscessus* in Korea. J Korean Med Sci 2008; 23: 49–52.
22. **Kontos F, Mavromanolakis D, Zande M, Gitti Z.** Isolation of *Mycobacterium kumamotonense* from a patient with pulmonary infection and latent tuberculosis. Indian J Med Microbiol 2016; 34: 241–244.
23. **Griffith D, Aksamit T, Brown-Elliott B, Catanzaro A, Daley C, Gordin F, Holland S, Horsburgh R, Huitt G, Iademarco M, Iseman M, Olivier K, Ruoss S, von Reyn C, Wallace R, Winthrop K.** An official ATS/IDSA statement: diagnosis, treatment, and prevention of nontuberculous mycobacterial diseases. Am J Respir Crit Care Med 2007; 175: 367–416.

24. **Arend SM, van Soolingen D, Ottenhoff T**. Diagnosis and treatment of lung infection with nontuberculous mycobacteria. Curr Opin Pulm Med 2009; 15: 201–208.
25. **Chetchotisakd P, Kiertiburanakul S, Mootsikapun P, Assanasen S, Chaiwarith R, Anunnatsiri S**. Disseminated nontuberculous mycobacterial infection in patients who are not infected with HIV in Thailand. Clin Infect Dis Off Publ Infect Dis Soc Am 2007; 45: 421–427.
26. **Scorzolini L, Mengoni F, Mastroianni CM, Baldan R, Cirillo DM, De Giusti M, Marinelli L, Cottarelli A, Fattorini L, Vullo V**. Pseudo-outbreak of *Mycobacterium gordonae* in a teaching hospital: importance of strictly following decontamination procedures and emerging issues concerning sterilization. New Microbiol 2016; 39: 25–34.
27. **Kapnadak S, Hisert K, Pottinger P, Limaye A, Aitken M**. Infection control strategies that successfully controlled an outbreak of *Mycobacterium abscessus* at a cystic fibrosis center. Am J Infect Control 2016; 44: 154–159.
28. **Ahmed I, Jabeen K, Hasan R**. Identification of non-tuberculous mycobacteria isolated from clinical specimens at a tertiary care hospital: a cross-sectional study. BMC Infect Dis 2013; 13: 493.
29. **Lim H, Park C, Park Y, Lee J, Lee S, Yang S, Yoo C, Kim Y, Han S, Yim J**. Isolation of multiple nontuberculous mycobacteria species in the same patients. Int J Infect Dis 2011; 15: e795–e798.
30. **Griffith D, Girard W, Wallace R**. Clinical features of pulmonary disease caused by rapidly growing mycobacteria. An analysis of 154 patients. Am Rev Respir Dis 1993; 147: 1271–1278.
31. **Tshikhudo P, Nnzeru R, Ntushelo K, Mudau F**. Bacterial species identification getting easier. Afr J Biotechnol 2013; 12: 5975–5982.
32. **Runyon, E. H.** Anonymous mycobacteria in pulmonary disease. Med Clin of North Am 1959; 43: 273-290.
33. **Koh W, Kwon O, Lee K**. Nontuberculous mycobacterial pulmonary diseases in immunocompetent patients. Korean J Radiol 2002; 3: 145–157.
34. **Tortoli E, Bartoloni A, Bottger E, Emler S, Garzelli C, Magliano E, Mantella A, Rastogi N, Rindi L, Scarparo C, Urbano P.** Burden of unidentifiable mycobacteria in a reference laboratory. J Clin Microbiol 2001; 39: 4058–4065.
35. **Springer B, Stockman L, Teschner K, Roberts G, Böttger E**. Two-laboratory collaborative study on identification of mycobacteria: molecular versus phenotypic methods. J Clin Microbiol 1996; 34: 296–303.
36. **Chin'ombe N, Muzividzi B, Munemo E, Nziramasanga P**. Molecular Identification of nontuberculous mycobacteria in humans in Zimbabwe using 16S ribosequencing. Open Microbiol J 2016; 10: 113–123.
37. **Turenne C, Wallace R, Behr M**. *Mycobacterium avium* in the postgenomic era. Clin Microbiol Rev 2007; 20: 205–229.
38. **Mijs W, de Haas P, Rossau R, Van der Laan T, Rigouts L, Portaels F, van Soolingen D**. Molecular evidence to support a proposal to reserve the designation *Mycobacterium avium* subsp. *avium* for bird-type isolates and "*M. avium* subsp. *hominissuis*" for the human/porcine type of *M. avium*. Int J Syst Evol Microbiol 2002; 52: 1505–1518.
39. **Turenne C, Semret M, Cousins D, Collins D, Behr M.** Sequencing of *hsp65* distinguishes among subsets of the *Mycobacterium avium* complex. J Clin Microbiol 2006; 44: 433–440.
40. **Lee S, Kim B, Kim H, Won Y, Jeon C, Jeong J, Lee S, Lim J, Lee S, Kim C, Kook Y, Kim B**. *Mycobacterium paraintracellulare* sp. nov., for the genotype INT-1 of *Mycobacterium intracellulare*. Int J Syst Evol Microbiol 2016; 66: 3132–3141.
41. **Kobayashi T, Tsuyuguchi K, Yoshida S, Kurahara Y, Ikegami N, Naito M, Sonobe S, Maekura T, Tsuji T, Minomo S, Inoue Y, Suzuki K**. *Mycobacterium abscessus* subsp. *abscessus* lung disease: drug susceptibility testing in sputum culture negative conversion. Int J Mycobacteriol 2018; 7: 69–75.
42. **Caskey S, Moore J, Rendall J**. In vitro activity of seven hospital biocides against *Mycobacterium abscessus*: Implications for patients with cystic fibrosis. Int J Mycobacteriol 2018; 7: 45–47.
43. **Falkinham J**. Nontuberculous mycobacteria from household plumbing of patients with nontuberculous mycobacteria disease. Emerg Infect Dis 2011; 17: 419–424.

44. **Thomson R, Tolson C, Carter R, Coulter C, Huygens F, Hargreaves M**. Isolation of nontuberculous mycobacteria (NTM) from household water and shower aerosols in patients with pulmonary disease caused by NTM. J Clin Microbiol 2013; 51: 3006–3011.
45. **Saiman L, Siegel J**. Infection control in cystic fibrosis. Clin Microbiol Rev 2004; 17: 57–71.
46. **Renna M, Schaffner C, Brown K, Shang S, Tamayo M, Hegyi K, Grimsey N, Cusens D, Coulter S, Cooper J, Bowden A, Newton S, Kampmann B, Helm J, Jones A, Haworth C, Basaraba R, DeGroote M, Ordway D, Rubinsztein D, Floto R**. Azithromycin blocks autophagy and may predispose cystic fibrosis patients to mycobacterial infection. J Clin Invest 2011; 121: 3554–3563.
47. **Bryant J, Grogono D, Greaves D, Foweraker J, Roddick I, Inns T, Reacher M, Haworth C, Curran M, Harris S, Peacock S, Parkhill J, Floto R.** Whole-genome sequencing to identify transmission of *Mycobacterium abscessus* between patients with cystic fibrosis: a retrospective cohort study. Lancet Lond Engl 2013; 381: 1551–1560.
48. **Tsukamura M**. A group of mycobacteria from soil sources resembling nonphotochromogens (group 3). A description of *Mycobacterium nonchromogenicum*. Igaku To Seibutsugaku 1965; 71: 110–113.
49. **Richmond L, Cummings M**. An evaluation of methods of testing the virulence of acid-fast bacilli. Am Rev Tuberc 1950; 62: 632–637.
50. **Kubica P, Silcox A, Kilburn J, Smithwick R, Beam R, Jones W, Stottmeier K**. Differential identification of mycobacteria VI. *Mycobacterium triviale* Kubica sp. nov. Int J Syst Evol Microbiol 1970; 20: 161–174.
51. **Tortoli E, Gitti Z, Klenk H-P, Lauria S, Mannino R, Mantegani P, Mariottini A, Neonakis I**. Survey of 150 strains belonging to the *Mycobacterium terrae* complex and description of *Mycobacterium engbaekii* sp. nov., *Mycobacterium heraklionense* sp. nov. and *Mycobacterium longobardum* sp. nov. Int J Syst Evol Microbiol 2013; 63: 401–411.
52. **Smith D, Lindholm-Levy P, Huitt G, Heifets L, Cook J**. *Mycobacterium terrae*: case reports, literature review, and in vitro antibiotic susceptibility testing. Clin Infect Dis Off Publ Infect Dis Soc Am 2000; 30: 444–453.
53. **Díaz N, González F, Casado I, Galán L, Rojas M, Alado J**. Lung infection by *Mycobacterium terrae*. Arch Bronconeumol 2001; 37: 96–98.
54. **Sawai T, Inoue Y, Doi S, Izumikawa K, Ohno H, Yanagihara K, Higashiyama Y, Miyazaki Y, Hirakata Y, Tashiro T, Kohno S.** A case of *Mycobacterium nonchromogenicum* pulmonary infection showing multiple nodular shadows in an immunocompetent patient. Diagn Microbiol Infect Dis 2006; 54: 311–314.
55. **Kazda J, Cooney R, Monaghan M, Quinn P, Stackebrandt E, Dorsch M, Daffé M, Müller K, Cook B, Tarnok Z.** *Mycobacterium hiberniae* sp. nov. Int J Syst Bacteriol 1993; 43: 352–357.
56. **Masaki T, Ohkusu K, Hata H, Fujiwara N, Iihara H, Yamada-Noda M, Nhung P, Hayashi M, Asano Y, Kawamura Y, Ezaki T**. *Mycobacterium kumamotonense* sp. nov. recovered from clinical specimen and the first isolation report of *Mycobacterium arupense* in Japan: Novel slowly growing, nonchromogenic clinical isolates related to *Mycobacterium terrae* complex. Microbiol Immunol 2006; 50: 889–897.
57. **Mun H, Park J, Kim H, Yu H, Park Y, Cha C, Kook Y, Kim B**. *Mycobacterium senuense* sp. nov., a slowly growing, non-chromogenic species closely related to the *Mycobacterium terrae* complex. Int J Syst Evol Microbiol 2008; 58: 641–646.
58. **Lee H, Lee S, Lee I, Yu H, Park Y, Jeong J, Lee S, Kim S, Hyun J, Kim K, Kook Y, Kim B**. *Mycobacterium paraterrae* sp. nov. recovered from a clinical specimen: novel chromogenic slow growing mycobacteria related to *Mycobacterium terrae* complex. Microbiol Immunol 2010; 54: 46–53.
59. **Zhang Z, Sun Z, Wang Z, Wen Z, Sun Q, Zhu Z, Song Y, Zhao J, Wang H, Zhang S, Guo X**. Complete genome sequence of a novel clinical isolate, the nontuberculous Mycobacterium strain JDM601. J Bacteriol 2011; 193: 4300–4301.
60. **Tasler G, Hartley W**. Foal abortion associated with *Mycobacterium terrae* infection. Vet Pathol 1981; 18: 122–125.

**61. Ngeow Y, Wong Y, Tan J, Hong K, Ng H, Ong B, Chan K**. Identification of New Genomospecies in the *Mycobacterium terrae* complex. Plos One 2015; 10: e0120789. 1-16.

**62. Brown-Elliott B, Wallace R**. Clinical and taxonomic status of pathogenic nonpigmented or late-pigmenting rapidly growing mycobacteria. Clin Microbiol Rev 2002; 15: 716–746.

**63. Schinsky M, Morey R, Steigerwalt A, Douglas M, Wilson R, Floyd M, Butler W, Daneshvar M, Brown-Elliott B, Wallace R Jr, McNeil M, Brenner D, Brown J.** Taxonomic variation in the *Mycobacterium fortuitum* third biovariant complex: description of *Mycobacterium boenickei* sp. nov., *Mycobacterium houstonense* sp. nov., *Mycobacterium neworleansense* sp. nov. and *Mycobacterium brisbanense* sp. nov. and recognition of *Mycobacterium porcinum* from human clinical isolates. Int J Syst Evol Microbiol 2004; 54: 1653–1667.

**64. Wallace R, Brown-Elliott B, Hall L, Roberts G, Wilson R, Mann L, Crist C, Chiu S, Dunlap R, Garcia M, Bagwell J, Jost K Jr**.. Clinical and laboratory features of *Mycobacterium mageritense*. J Clin Microbiol 2002; 40: 2930–2935.

**65. Han X, Dé I, Jacobson K**. Rapidly growing mycobacteria: clinical and microbiologic studies of 115 Cases. Am J Clin Pathol 2007; 128: 612–621.

**66. Lamy B, Marchandin H, Hamitouche K, Laurent F**. *Mycobacterium setense* sp. nov., a *Mycobacterium fortuitum*-group organism isolated from a patient with soft tissue infection and osteitis. Int J Syst Evol Microbiol 2008; 58: 486–490.

**67. Ucko M, Colorni A, Kvitt H, Diamant A, Zlotkin A, Knibb W**. Strain Variation in *Mycobacterium marinum* fish isolates. Appl Environ Microbiol 2002; 68: 5281–5287.

**68. Hess C, Wolock B, Murphy M**. *Mycobacterium marinum* infections of the upper extremity. Plast Reconstr Surg 2005; 115: 55e-59e.

**69. Stinear T, Mve-Obiang A, Small P, Frigui W, Pryor M, Brosch R, Jenkin G, Johnson P, Davies J, Lee R, Adusumilli S, Garnier T, Haydock S, Leadlay P, Cole S.** Giant plasmid-encoded polyketide synthases produce the macrolide toxin of *Mycobacterium ulcerans*. Proc Natl Acad Sci U S A 2004; 101: 1345–1349.

**70. Stinear T, Seemann T, Pidot S, Frigui W, Reysset G, Garnier T, Meurice G, Simon D, Bouchier C, Ma L, Tichit M, Porter J, Ryan J, Johnson P, Davies J, Jenkin G, Small P, Jones L, Tekaia F, Laval F, Daffé M, Parkhill J, Cole S.** Reductive evolution and niche adaptation inferred from the genome of *Mycobacterium ulcerans*, the causative agent of buruli ulcer. Genome Res 2007; 17: 192–200.

**71. Slany M, Makovcova J, Jezek P, Bodnarova M, Pavlik I**. Relative prevalence of *Mycobacterium marinum* in fish collected from aquaria and natural freshwaters in central Europe. J Fish Dis 2014; 37: 527–533.

**72. Trott K, Stacy B, Lifland B, Diggs H, Harland R, Khokha M, Grammer T, Parker J**. Characterization of a *Mycobacterium ulcerans*-like infection in a colony of african tropical clawed frogs (*Xenopus tropicalis*). Comp Med 2004; 54: 309–317.

**73. Mve-Obiang A, Lee R, Umstot E, Trott K, Grammer T, Parker J, Ranger B, Grainger R, Mahrous E, Small P**. A newly discovered mycobacterial pathogen isolated from laboratory colonies of *Xenopus* species with lethal infections produces a novel form of mycolactone, the *Mycobacterium ulcerans* macrolide toxin. Infect Immun 2005; 73: 3307–3312.

**74. Tobias N, Doig K, Medema M, Chen H, Haring V, Moore R, Seemann T, Stinear T.** Complete genome sequence of the frog pathogen *Mycobacterium ulcerans* Ecovar Liflandii. J Bacteriol 2013; 195: 556–564.

**75. Tortoli E, Böttger E, Fabio A, Falsen E, Gitti Z, Grottola A, Klenk H, Mannino R, Mariottini A, Messinò M, Pecorari M, Rumpianesi F.** *Mycobacterium europaeum* sp. nov., a scotochromogenic species related to the *Mycobacterium simiae* complex. Int J Syst Evol Microbiol 2011; 61: 1606–1611.

**76. Bouam A, Heidarieh P, Shahraki A, Pourahmad F, Mirsaeidi M, Hashemzadeh M, Baptiste E, Armstrong N, Levasseur A, Robert C, Drancourt M.** *Mycobacterium ahvazicum* sp. nov., the nineteenth species of the *Mycobacterium simiae* complex. Sci Rep 2018; 8: 4138- 4149.

77. **Adékambi T, Drancourt M**. Dissection of phylogenetic relationships among 19 rapidly growing *Mycobacterium* species by 16S rRNA, *hsp65*, *sodA*, *recA* and *rpoB* gene sequencing. Int J Syst Evol Microbiol 2004; 54: 2095–2105.
78. **Adékambi T, Berger P, Raoult D, Drancourt M**. *rpoB* gene sequence-based characterization of emerging non-tuberculous mycobacteria with descriptions of *Mycobacterium bolletii* sp. nov., *Mycobacterium phocaicum* sp. nov. and *Mycobacterium aubagnense* sp. nov. Int J Syst Evol Microbiol 2006; 56: 133–143.
79. **Adékambi T**. *Mycobacterium mucogenicum* group infections: a review. Clin Microbiol Infect Off Publ Eur Soc Clin Microbiol Infect Dis 2009; 15: 911–918.
80. **Lamrabet O, Medie F, Drancourt M**. Acanthamoeba polyphaga-enhanced growth of *Mycobacterium smegmatis*. Plos One 2012; 7: e29833. 1-8.
81. **Eid A, Berbari E, Sia I, Wengenack N, Osmon D, Razonable R**. Prosthetic joint infection due to rapidly growing mycobacteria: report of 8 cases and review of the literature. Clin Infect Dis Off Publ Infect Dis Soc Am 2007; 45: 687–694.
82. **Chen Y, Jou R, Huang W, Huang S, Liu K, Lay C, Chang S, Tseng C, Lai C, Su Y**. Bacteremia caused by *Mycobacterium wolinskyi*. Emerg Infect Dis 2008; 14: 1818–1819.
83. **Gupta R, Lo B, Son J**. Phylogenomics and comparative genomic studies robustly support division of the genus *Mycobacterium* into an emended genus *Mycobacterium* and four novel genera. Front Microbiol 2018; 9: 67-78.
84. **Purcaro G, Tranchida P, Dugo P, La Camera E, Bisignano G, Conte L, Mondello L**. Characterization of bacterial lipid profiles by using rapid sample preparation and fast comprehensive two-dimensional gas chromatography in combination with mass spectrometry. J Sep Sci 2010; 33: 2334–2340.
85. **Butler W, Guthertz L**. Mycolic acid analysis by high-performance liquid chromatography for identification of *Mycobacterium* Species. Clin Microbiol Rev 2001; 14: 704–726.
86. **Jost K, Dunbar D, Barth S, Headley V, Elliott L**. Identification of *Mycobacterium tuberculosis* and *M. avium* complex directly from smear-positive sputum specimens and BACTEC 12B cultures by high-performance liquid chromatography with fluorescence detection and computer-driven pattern recognition models. J Clin Microbiol 1995; 33: 1270–1277.
87. **Butler W, Jost K, Kilburn J**. Identification of mycobacteria by high-performance liquid chromatography. J Clin Microbiol 1991; 29: 2468–2472.
88. **Glickman S, Kilburn J, Butler W, Ramos L**. Rapid identification of mycolic acid patterns of mycobacteria by high-performance liquid chromatography using pattern recognition software and a *Mycobacterium* library. J Clin Microbiol 1994; 32: 740–745.
89. **Cloud J, Meyer J, Pounder J, Jost K, Sweeney A, Carroll K, Woods G**. *Mycobacterium arupense* sp. nov., a non-chromogenic bacterium isolated from clinical specimens. Int J Syst Evol Microbiol 2006; 56: 1413–1418.
90. **Cooksey R, Jhung M, Yakrus M, Butler W, Adékambi T, Morlock G, Williams M, Shams A, Jensen B, Morey R, Charles N, Toney S, Jost K Jr, Dunbar D, Bennett V, Kuan M, Srinivasan A.** Multiphasic approach reveals genetic diversity of environmental and patient isolates of *Mycobacterium mucogenicum* and *Mycobacterium phocaicum* associated with an outbreak of bacteremias at a Texas hospital. Appl Environ Microbiol 2008; 74: 2480–2487.
91. **Lane D, Pace B, Olsen G, Stahl D, Sogin M, Pace N**. Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. Proc Natl Acad Sci U S A 1985; 82: 6955–6959.
92. **Woese C, Stackebrandt E, Macke T, Fox G**. A phylogenetic definition of the major eubacterial taxa. Syst Appl Microbiol 1985; 6: 143–151.
93. **Woese C**. Bacterial evolution. Microbiol Rev 1987; 51: 221–271.
94. **Chakravorty S, Helb D, Burday M, Connell N, Alland D**. A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. J Microbiol Methods 2007; 69: 330–339.
95. **Vetrovský T, Baldrian P**. The variability of the 16S rRNA gene in bacterial genomes and its consequences for bacterial commu-

nity analyses. Plos One 2013; 8: e57923. 1-10.

96. **Dahllöf I, Baillie H, Kjelleberg S**. *rpoB*-based microbial community analysis avoids limitations inherent in 16S rRNA gene intraspecies heterogeneity. Appl Environ Microbiol 2000; 66: 3376–3380.
97. **Case R, Boucher Y, Dahllöf I, Holmström C, Doolittle W, Kjelleberg S**. Use of 16S rRNA and *rpoB* genes as molecular markers for microbial ecology studies. Appl Environ Microbiol 2007; 73: 278–288.
98. **Candela M, Vitali B, Matteuzzi D, Brigidi P**. Evaluation of the *rrn* operon copy number in *Bifidobacterium* using real-time PCR. Lett Appl Microbiol 2004; 38: 229-232.
99. **Lee C, Sieo C, Abdullah N, Ho Y**. Estimation of 16S rRNA gene copy number in several probiotic *Lactobacillus* strains isolated from the gastrointestinal tract of chicken. FEMS Microbiol Lett 2008; 287: 136-141.
100. **Fox G, Wisotzkey J, Jurtshuk P**. How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. Int J Syst Bacteriol 1992; 42: 166–170.
101. **Stackebrandt E, Goebel B**. Taxonomic Note: A place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int J Syst Evol Microbiol 1994; 44: 846–849.
102. **Park H, Jang H, Kim C, Chung B, Chang C, Park S, Song S**. Detection and identification of mycobacteria by amplification of the internal transcribed spacer regions with genus- and species-specific PCR primers. J Clin Microbiol 2000; 38: 4080–4085.
103. **Gopinath K, Singh S**. Multiplex PCR assay for simultaneous detection and differentiation of *Mycobacterium tuberculosis, Mycobacterium avium* complexes and other mycobacterial species directly from clinical specimens. J Appl Microbiol 2009; 107: 425–435.
104. **Ngan G, Ng L, Jureen R, Lin R, Teo J**. Development of multiplex PCR assays based on the 16S-23S rRNA internal transcribed spacer for the detection of clinically relevant nontuberculous mycobacteria. Lett Appl Microbiol 2011; 52: 546–554.
105. **Mwikuma G, Kwenda G, Hang'ombe B, Simulundu E, Kaile T, Nzala S, Siziya S, Suzuki Y**. Molecular identification of non-tuberculous mycobacteria isolated from clinical specimens in Zambia. Ann Clin Microbiol Antimicrob 2015; 14: 1-5.
106. **Park J, Shim T, Lee S, Lee H, Lee I, Kim K, Kook Y, Kim B**. Molecular characterization of *Mycobacterium intracellulare*-related strains based on the sequence analysis of *hsp65*, internal transcribed spacer and 16S rRNA genes. J Med Microbiol 2010; 59: 1037–1043.
107. **Stout J, Hopkins G, McDonald J, Quinn A, Hamilton C, Reller L, Frothingham R.** Association between 16S-23S internal transcribed spacer sequence groups of *Mycobacterium avium* complex and pulmonary disease. J Clin Microbiol 2008; 46: 2790–2793.
108. **Subedi S, Kong F, Jelfs P, Gray T, Xiao M, Sintchenko V, Chen S**. 16S-23S Internal transcribed spacer region PCR and sequencer-based capillary gel electrophoresis has potential as an alternative to high performance liquid chromatography for identification of slowly growing nontuberculous mycobacteria. Plos One 2016; 11: e0164138. 1-15.
109. **Tortoli E**. Impact of genotypic studies on mycobacterial taxonomy: the new mycobacteria of the 1990s. Clin Microbiol Rev 2003; 16: 319–354.
110. **De Bruyne K, Slabbinck B, Waegeman W, Vauterin P, De Baets B, Vandamme P.** Bacterial species identification from MALDI-TOF mass spectra through data analysis and machine learning. Syst Appl Microbiol 2011; 34: 20–29.
111. **Hettick J, Kashon M, Slaven J, Ma Y, Simpson J, Siegel P, Mazurek G, Weissman D.** Discrimination of intact mycobacteria at the strain level: a combined MALDI-TOF MS and biostatistical analysis. Proteomics 2006; 6: 6416–6425.
112. **Mediavilla-Gradolph M, De Toro-Peinado I, Bermúdez-Ruiz M, García-Martínez M de los Á, Ortega-Torres M, Montiel Quezel-Guerraz N, Palop-Borrás B.** Use of MALDI-TOF MS for Iidentification of nontuberculous *Mycobacterium* species isolated from clinical specimens. BioMed Res Int 2015. 2015; 854078. 1-6.
113. **Saleeb P, Drake S, Murray P, Zelazny A**. Identification of mycobacteria in solid-

culture media by matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol 2011; 49: 1790–1794.

**114. Cao Y, Wang L, Ma P, Fan W, Gu B, Ju S**. Accuracy of matrix-assisted laser desorption ionization-time of flight mass spectrometry for identification of mycobacteria: a systematic review and meta-analysis. Sci Rep 2018; 8: 4131-4139.

**115. Plikaytis B, Plikaytis B, Yakrus M, Butler W, Woodley C, Silcox V**, et al. Differentiation of slowly growing *Mycobacterium* species, including *Mycobacterium tuberculosis*, by gene amplification and restriction fragment length polymorphism analysis. J Clin Microbiol 1992; 30: 1815–1822.

**116. Telenti A, Marchesi F, Balz M, Bally F, Böttger E, Bodmer T**. Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis. J Clin Microbiol 1993; 31: 175–178.

**117. Kirschner P, Böttger E**. Microheterogeneity within rRNA of *Mycobacterium gordonae*. J Clin Microbiol 1992; 30: 1049–1050.

**118. Taylor T, Patterson C, Hale Y, Safranek W**. Routine use of PCR-restriction fragment length polymorphism analysis for identification of mycobacteria growing in liquid media. J Clin Microbiol 1997; 35: 79–85.

**119. Devallois A, Goh K, Rastogi N**. Rapid identification of mycobacteria to species level by PCR-restriction fragment length polymorphism analysis of the *hsp65* gene and proposition of an algorithm to differentiate 34 mycobacterial species. J Clin Microbiol 1997; 35: 2969–2973.

**120. Brunello F, Ligozzi M, Cristelli E, Bonora S, Tortoli E, Fontana R**. Identification of 54 mycobacterial species by PCR-restriction fragment length polymorphism analysis of the *hsp65* gene. J Clin Microbiol 2001; 39: 2799–2806.

**121. Alcaide F, Richter I, Bernasconi C, Springer B, Hagenau C, Schulze-Röbbecke R, Tortoli E, Martín R, Böttger E, Telenti A**. Heterogeneity and clonality among isolates of *Mycobacterium kansasii*: implications for epidemiological and pathogenicity studies. J Clin Microbiol 1997; 35: 1959–1964.

**122. Kim H, Kim S, Shim T, Kim M, Bai G, Park Y, Lee S, Cha C, Kook Y, Kim B**. PCR restriction fragment length polymorphism analysis (PRA)-algorithm targeting 644 bp Heat Shock Protein 65 (*hsp65*) gene for differentiation of *Mycobacterium* spp. J Microbiol Methods 2005; 62: 199–209.

**123. Ong C, Ngeow Y, Yap S, Tay S**. Evaluation of PCR-RFLP analysis targeting *hsp65* and *rpoB* genes for the typing of mycobacterial isolates in Malaysia. J Med Microbiol 2010; 59: 1311–1316.

**124. Gingeras T, Ghandour G, Wang E, Berno A, Small P, Drobniewski F, Alland D, Desmond E, Holodniy M, Drenkow J**. Simultaneous genotyping and species identification using hybridization pattern recognition analysis of generic *Mycobacterium* DNA arrays. Genome Res 1998; 8: 435–448.

**125. Lee H, Bang H, Bai G, Cho S**. Novel polymorphic region of the *rpoB* gene containing *Mycobacterium* species-specific sequences and its use in identification of mycobacteria. J Clin Microbiol 2003; 41: 2213–2218.

**126. Musser J**. Antimicrobial agent resistance in mycobacteria: Molecular genetic insights. Clin Microbiol Rev 1995; 8. 496- 514.

**127. Boor K, Duncan M, Price C**. Genetic and transcriptional organization of the region encoding the beta subunit of *Bacillus subtilis* RNA polymerase. J Biol Chem 1995; 270: 20329–20336.

**128. Kim B, Lee S, Lyu M, Kim S, Bai G, Chae G, Kim E, Cha C, Kook Y**. Identification of mycobacterial species by comparative sequence analysis of the RNA polymerase gene (*rpoB*). J Clin Microbiol 1999; 37: 1714–1720.

**129. Troesch A, Nguyen H, Miyada C, Desvarenne S, Gingeras T, Kaplan P, Cros P, Mabilat C**. *Mycobacterium* species identification and rifampin resistance testing with high-density DNA probe arrays. J Clin Microbiol 1999; 37: 49–55.

**130. Lee H, Park H, Cho S, Bai G, Kim S**. Species identification of mycobacteria by PCR-restriction fragment length polymorphism of the *rpoB* gene. J Clin Microbiol 2000; 38: 2966–2971.

**131. Kim B, Lee K, Park B, Kim S, Bai G, Kim S, Kook Y**. Differentiation of mycobacterial species by PCR-restriction analysis of DNA (342 base pairs) of the RNA polymerase gene (rpoB). J Clin Microbiol 2001; 39: 2102–2109.

**132. de Zwaan R, van Ingen J, van Soolingen D**. Utility of rpoB gene sequencing for identification of nontuberculous mycobacteria in the Netherlands. J Clin Microbiol 2014; 52: 2544–2551.

**133. Simmon K, Pounder J, Greene J, Walsh F, Anderson C, Cohen S, Petti C**. Identification of an emerging pathogen, *Mycobacterium massiliense*, by rpoB sequencing of clinical isolates collected in the United States. J Clin Microbiol 2007; 45: 1978–1980.

**134. Ben Salah I, Adékambi T, Raoult D, Drancourt M**. rpoB sequence-based identification of *Mycobacterium avium* complex species. Microbiol Read Engl 2008; 154: 3715–3723.

**135. Somoskovi A, Song Q, Mester J, Tanner C, Hale Y, Parsons L, Salfinger M**. Use of molecular methods to identify the *Mycobacterium tuberculosis* complex (MTBC) and other mycobacterial species and to detect rifampin resistance in MTBC isolates following growth detection with the BACTEC MGIT 960 system. J Clin Microbiol 2003; 41: 2822–2826.

**136. Lee A, Jelfs P, Sintchenko V, Gilbert G**. Identification of non-tuberculous mycobacteria: utility of the GenoType *Mycobacterium* CM/AS assay compared with HPLC and 16S rRNA gene sequencing. J Med Microbiol 2009; 58: 900–904.

**137. García-Agudo L, Jesús I, Rodríguez-Iglesias M, García-Martos P**. Evaluation of INNO-LiPA mycobacteria v2 assay for identification of rapidly growing mycobacteria. Braz J Microbiol Publ Braz Soc Microbiol 2011; 42: 1220–1226.

**138. Sarkola A, Mäkinen J, Marjamäki M, Marttila H, Viljanen M, Soini H**. Prospective evaluation of the GenoType assay for routine identification of mycobacteria. Eur J Clin Microbiol Infect Dis Off Publ Eur Soc Clin Microbiol 2004; 23: 642–645.

**139. Ruiz P, Gutierrez J, Zerolo F, Casal M**. GenoType *Mycobacterium* assay for identification of mycobacterial species isolated from human clinical samples by using liquid medium. J Clin Microbiol 2002; 40: 3076–3078.

**140. Richter E, Rüsch-Gerdes S, Hillemann D**. Evaluation of the GenoType *Mycobacterium* assay for identification of mycobacterial species from cultures. J Clin Microbiol 2006; 44: 1769–1775.

**141. Turenne C, Collins D, Alexander D, Behr M**. *Mycobacterium avium* subsp. *paratuberculosis* and *M. avium* subsp. *avium* are independently evolved pathogenic clones of a much broader group of *M. avium* organisms. J Bacteriol 2008; 190: 2479–2487.

**142. Devulder G, Pérouse de Montclos M, Flandrois J**. A multigene approach to phylogenetic analysis using the genus *Mycobacterium* as a model. Int J Syst Evol Microbiol 2005; 55: 293–302.

**143. Macheras E, Roux A, Ripoll F, Sivadon-Tardy V, Gutierrez C, Gaillard J, Heym B**. Inaccuracy of single-target sequencing for discriminating species of the *Mycobacterium abscessus* group. J Clin Microbiol 2009; 47: 2596–2600.

**144. Stackebrandt E, Frederiksen W, Garrity G, Grimont P, Kämpfer P, Maiden M, Nesme X, Rosselló-Mora R, Swings J, Trüper H, Vauterin L, Ward A, Whitman W.** Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. Int J Syst Evol Microbiol 2002; 52: 1043–1047.

**145. Rosselló-Mora R, Amann R**. The species concept for prokaryotes. FEMS Microbiol Rev 2001; 25: 39–67.

**146. Mignard S, Flandrois J.** A seven-gene, multilocus, genus-wide approach to the phylogeny of mycobacteria using supertrees. Int J Syst Evol Microbiol 2008; 58: 1432–1441.

**147. Macheras E, Roux A, Bastian S, Leão S, Palaci M, Sivadon-Tardy V, Gutierrez C, Richter E, Rüsch-Gerdes S, Pfyffer G, Bodmer T, Cambau E, Gaillard J, Heym B**. Multilocus sequence analysis and rpoB sequencing of *Mycobacterium abscessus* (sensu lato) strains. J Clin Microbiol 2011; 49: 491–499.

**148. Leao S, Tortoli E, Viana-Niero C, Ueki S, Lima K, Lopes M, Yubero J, Menendez M, Garcia M**. Characterization of mycobacteria from a major brazilian outbreak suggests that revision of the taxonomic status of members of the *Mycobacterium chelonae-M. abscessus* group is needed. J Clin Microbiol 2009; 47: 2691–2698.

**149. Köser C, Ellington M, Cartwright E, Gillespie S, Brown N, Farrington M, Holden M, Dougan G, Bentley S, Parkhill J, Pea-**

**cock S**. Routine use of microbial whole genome sequencing in diagnostic and public health microbiology. Plos Pathog 2012; 8: e1002824. 1-9.

**150. Loman N, Pallen M**. XDR-TB genome sequencing: a glimpse of the microbiology of the future. Future Microbiol 2008; 3: 111–113.

**151. Tettelin H, Davidson R, Agrawal S, Aitken M, Shallom S, Hasan N, Strong M, de Moura V, De Groote M, Duarte R, Hine E, Parankush S, Su Q, Daugherty S, Fraser C, Brown-Elliott B, Wallace R Jr, Holland S, Sampaio E, Olivier K, Jackson M, Zelazny A.** High-level relatedness among *Mycobacterium abscessus* subsp. massiliense strains from widely separated outbreaks. Emerg Infect Dis 2014; 20: 364–371.

**152. Wee W, Tan T, Jakubovics N, Choo S**. Whole-genome sequencing and comparative analysis of *Mycobacterium brisbanense* reveals a possible soil origin and capability in fertiliser synthesis. Plos One 2016; 11: e0152682. 1-15.

**153. Köser C, Holden M, Ellington M, Cartwright E, Brown N, Ogilvy-Stuart A, Hsu L, Chewapreecha C, Croucher N, Harris S, Sanders M, Enright M, Dougan G, Bentley S, Parkhill J, Fraser L, Betley J, Schulz-Trieglaff O, Smith G, Peacock S.** Rapid whole-genome sequencing for investigation of a neonatal MRSA outbreak. N Engl J Med 2012; 366: 2267–2275.

**154. Pallen M, Loman N, Penn C.** High-throughput sequencing and clinical microbiology: progress, opportunities and challenges. Curr Opin Microbiol 2010; 13: 625–631.

**155. Underwood A, Green J**. Call for a quality standard for sequence-based assays in clinical microbiology: necessity for quality assessment of sequences used in microbial identification and typing. J Clin Microbiol 2011; 49: 23–26.

**156. Tran A, Alby K, Kerr A, Jones M, Gilligan P**. Cost savings realized by implementation of routine microbiological identification by matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol 2015; 53: 2473–2479.

**157. Schlatter R, Matte U, Polanczyk C, Koehler-Santos P, Ashton-Prolla P**. Costs of genetic testing: supporting brazilian public policies for the incorporating of molecular diagnostic technologies. Genet Mol Biol 2015; 38: 332–337.

**158. Mondragón-Barreto M, Vázquez-Chacón C, Barrón-Rivero C, Acosta-Blanco P, Jost K, Balandrano S, Olivera-Díaz H**. Comparison among three methods for mycobacteria identification. Salud Publica Mex 2000; 42: 484–489.

**159. Kellogg J, Bankert D, Withers G, Sweimler W, Kiehn T, Pfyffer G**. Application of the Sherlock mycobacteria identification system using high-performance liquid chromatography in a clinical laboratory. J Clin Microbiol 2001; 39: 964–970.

**160. Schwarze K, Buchanan J, Taylor J, Wordsworth S**. Are whole-exome and whole-genome sequencing approaches cost-effective? A systematic review of the literature. Genet Med Off J Am Coll Med Genet 2018; 1-9.

**161. van Ingen J**. Microbiological diagnosis of nontuberculous mycobacterial pulmonary disease. In: Huitt G, Dayley C (Ed). Nontuberculous mycobacteria, an issue of clinics in chest medicine. Philadelphia- Pennsylvania. Elsevier 2015; P 47-48.

**162. Pankhurst L, Del Ojo Elias C, Votintseva A, Walker T, Cole K, Davies J, Fermont J, Gascoyne-Binzi D, Kohl T, Kong C, Lemaitre N, Niemann S, Paul J, Rogers T, Roycroft E, Smith E, Supply P, Tang P, Wilcox M, Wordsworth S, Wyllie D, Xu L, Crook D.** Rapid, comprehensive, and affordable mycobacterial diagnosis with whole-genome sequencing: a prospective study. Lancet Respir Med 2016; 4: 49–58.