The key factors contributing to the risk, diagnosis and treatment of non-tuberculous mycobacterial opportunistic infections

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Abstract

The incidence and prevalence of diseases caused by non-tuberculous mycobacteria (NTM) have been steadily increasing worldwide. NTM are environmental saprophytic organisms; however, a few strains are known to produce diseases in humans affecting pulmonary and extra-pulmonary sites. Although the environment is a major source of NTM infection, recent studies have shown that person-to-person dissemination could be an important transmission route for these microorganisms. Structural and functional lung defects and immunodeficiency are major risk factors for acquiring NTM infections. Diagnosis of NTM diseases is very complex owing to the necessity of distinguishing between a true pathogen and an environmental contaminant. Identification at the species level is critical due to differences in the antibiotic susceptibility patterns of various NTM strains. Such identification is mainly achieved by molecular methods; additionally, mass spectrometry (e.g., MALDI-TOF) is useful for NTM species determination. Natural resistance of NTM species to a wide spectrum of antibiotics makes prescribing treatment for NTM diseases very difficult. NTM therapy usually takes more than one year and requires multi-drug regimens, yet the outcome often remains poor. Therefore, alternatives to antibiotic therapy treatment methods is an area under active exploration. NTM infections are an active global health problem imposing the necessity for better diagnostic tools and more effective treatment methods.

Keywords

non-tuberculous mycobacteria • opportunistic pathogens • epidemiology • risk factors • molecular diagnostics • MALDI-TOF MS • treatment regimens • antibiotics • bacteriophages

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Introduction

Non-tuberculous mycobacteria (NTM) are close relatives of *Mycobacterium tuberculosis* (*Mtb*), the etiological agent of tuberculosis (Tb), and consist of more than 200 species and subspecies (<http://www.bacterio.net/mycobacterium.html>) present in different environmental niches [1].

NTM are an extremely heterogeneous group with many new species and subspecies added recently due to advances in molecular tools [2]. They show a high level of diversity of morphological, physiological, and biochemical features. Before the post-genomic era, NTM were classified by Runyon based on their growth on agar and pigment formation. According to the Runyon classification, bacteria growing on agar in less than seven days are called *rapidly growing mycobacteria* and bacteria which need more than seven days to grow are called *slowly growing mycobacteria* [3]. Besides high diversity, NTM share common features of

Mycolata such as a high content of guanine and cytosine bases, filamentous growth, acid-fastness, and the presence of mycolic acids [4].

The majority of NTM are saprophytic bacteria, among which a small number of species can produce diseases in humans. They are mainly opportunistic infections posing a higher risk of acquisition in immunocompromised patients and patients with pre-existing lung conditions. NTM species have been demonstrated to produce predominantly infections involving the pulmonary system, but have also been shown to cause cervical lymphadenitis in young children, disseminated diseases, skin, soft tissue, and eye infections [5, 6, 7] (figure 1).

NTM similarly to *Mtb* can survive extra- and intracellularly, grow in caseum, and produce biofilms within the host. However, differently than *Mtb*, NTM have developed mechanisms to grow in mucus, especially in patients with pre-existing chronic lung diseases [11]. NTM infections are primarily acquired from the environment through aerosols, although the precise mechanism of transmission remains unclear [12].

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Clinical diseases caused by NTM

Pulmonary disease	Lymphadenitis	Skin and soft tissue diseases	Disseminated disease	Bone and joint infections
•Common • <i>M. abscessus</i> group • <i>M. avium</i> complex • <i>M. boletii</i> • <i>M. kansasii</i> • <i>M. malmoense</i> • <i>M. massiliense</i> • <i>M. simiae</i> • <i>M. xenopi</i> •Less common • <i>M. asiaticum</i> • <i>M. celatum</i> • <i>M. chelonae</i> • <i>M. fortuitum</i> • <i>M. haemophilum</i> • <i>M. scrofulaceum</i> • <i>M. shimoidei</i> • <i>M. smegmatis</i> • <i>M. szulgai</i>	•Common • <i>M. avium</i> complex • <i>M. bohemicum</i> • <i>M. lentiflavum</i> • <i>M. malmoense</i> • <i>M. scrofulaceum</i> •Less common • <i>M. abscessus</i> group • <i>M. chelonae</i> • <i>M. fortuitum</i> • <i>M. genavense</i> • <i>M. haemophilum</i> • <i>M. kansasii</i> • <i>M. szulgai</i>	•Common • <i>M. abscessus</i> group • <i>M. chelonae</i> • <i>M. fortuitum</i> • <i>M. haemophilum</i> • <i>M. marinum</i> • <i>M. ulcerans</i> •Less common • <i>M. avium</i> complex • <i>M. immunogenum</i> • <i>M. malmoense</i> • <i>M. nonchromogenicum</i> • <i>M. smegmatis</i> • <i>M. szulgai</i> • <i>M. terrae</i> complex	•Common • <i>M. avium</i> complex • <i>M. chelonae</i> • <i>M. genavense</i> • <i>M. haemophilum</i> • <i>M. kansasii</i> •Less common • <i>M. abscessus</i> group • <i>M. celatum</i> • <i>M. conspicuum</i> • <i>M. fortuitum</i> • <i>M. immunogenum</i> • <i>M. malmoense</i> • <i>M. marinum</i> • <i>M. mucogenicum</i> • <i>M. scrofulaceum</i> • <i>M. simiae</i> • <i>M. szulgai</i> • <i>M. xenopi</i>	•Common • <i>M. avium</i> complex • <i>M. godii</i> • <i>M. haemophilum</i> • <i>M. kansasii</i> • <i>M. terrae</i> complex • <i>M. xenopi</i> •Less common • <i>M. flavescens</i> • <i>M. fortuitum</i> • <i>M. gordonae</i> • <i>M. longobardum</i> • <i>M. marinum</i> • <i>M. scrofulaceum</i> • <i>M. szulgai</i> • <i>M. ulcerans</i>

Figure 1. Clinical diseases caused by non-tuberculous mycobacteria. Common and less common isolated NTM species are listed in an alphabetical order [8, 9, 10].

Diagnosis of NTM infections is complex due to environmental exposure of individuals and the necessity of distinguishing a colonizer or contaminant from a true pathogen. The diagnosis of NTM infections requires integrated clinical, radiographic, and microbiologic data. Species-level identification is very important for choosing successful treatment and it is usually achieved by molecular methods. Mass spectrometry-based methods, such as matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), is an alternative approach enabling identification of NTM infection etiological agents.

NTM therapy is protracted (often more than one year) and requires multidrug regimens. Treatment outcome varies by patient and is dependent on the NTM species and the host status, but often remains poor [10]. Currently used regimens represent limited efficacy and often lead to severe adverse effects. NTM are naturally resistant to disinfectants and a wide spectrum of antibiotics, including most Tb drugs, posing a major challenge for effective treatment [13]. For these reasons alternatives to antibiotic therapy treatment methods have been explored in parallel.

In the last two decades, the gradual and systematic increase of NTM disease incidence has been shown worldwide [7]. Because diagnosis is complicated and treatment is very

often ineffective, NTM diseases are a growing global health problem. This article reviews the key factors contributing to the risk, diagnosis, and treatment of NTM infections.

Epidemiology

The last two decades have shown an alarming and growing trend in the incidence of non-tuberculous mycobacterial pulmonary disease (NTM-PD) worldwide. Studies from the United Kingdom showed that NTM infection rates increased from 0.9 to 2.9/100,000 from 1995 to 2006 [14]. The growing trend of NTM incidence in the United Kingdom was further supported by studies of Shah et al. [15]. Using a population-based approach, they found that infection rates in the United Kingdom rose from 5.6/100,000 in 2007 to 7.6/100,000 in 2012. This denoted an almost tenfold increase since 1995 [15]. In Denmark, the NTM-PD incidence rose from 0.6 to 1.5/100,000 from 2003 to 2008 [16]. Similar rates have been recorded in other European countries [17, 18, 19]. The incidence and prevalence of NTM-PD are most likely highly underestimated in most European countries since it is usually not a reportable condition [20]. Although NTM-PD is considered rare in European countries, it is imposing a high

economic burden due to complex diagnosis and prolonged treatment regimens [17]. Studies from the United States suggest an annual rate of NTM-PD of 1.4 to 13.9/100,000, with higher prevalence in the Southern United States and Hawaii. Furthermore, epidemiology studies consistently confirm that NTM-PD prevalence in the USA is increasing, with estimates ranging from 2.5% to 8% per year [7, 21]. A high incidence of NTM-PD was also reported in Ontario, Canada, between 2001 and 2013: 13.33/100,000 [22]. In Asia NTM-PD infections at a rate of 14.7/100,000 were estimated in the Japanese population in 2014 [23]. Among patient populations, NTM-PD rates are more dramatic in patients with pre-existing lung diseases. The overall prevalence of NTM respiratory infections in patients with cystic fibrosis (CF) ranges from 6% to 13% [24, 25]. NTM-PD in Europe is predominantly caused by *Mycobacterium avium* complex (MAC) comprised mainly of *M. avium* and *M. intracellulare*.

Several closely related species have been incorporated into the MAC based on high-throughput gene sequencing [26, 27]. *M. kansasii* is the second most common cause of NTM-PD in some European countries, including the United Kingdom, Slovakia, and the Czech Republic [28]. In Poland, the most common species isolated in clinical laboratories in 2017 were *M. kansasii*, *M. avium*, and *M. xenopi* [29]. *M. kansasii* causes lung disease, which is clinically indistinguishable from Tb. Other frequently isolated species of NTM involved in lung disease include, but are not limited to, *M. xenopi* and *M. malmoense* and the rapidly growing *M. abscessus* group (MABS) [7]. MABS group is comprised of subspecies *abscessus sensu stricto*, subspecies *massiliense*, and subspecies *bolletii*, and it was recently clustered as a separate clade called *Mycobacteroides abscessus* based on phylogenetic studies [30, 31]. MABS is the most commonly identified species in patients with chronic lung diseases, such as bronchiectasis and CF.

M. ulcerans, prevalent in sub-Saharan Africa and some parts of Australia, is a clinically important NTM causing ulcerative skin infections known as Buruli ulcers worldwide [32]. Similarly, *M. marinum*, the progenitor of *M. ulcerans*, was shown to be implicated in skin infections [33]. *M. ulcerans* and *M. marinum* are considered true pathogens since they can affect healthy immunocompetent individuals. Other species, frequently involved in skin, soft tissues, and bone diseases are *M. fortuitum*, *M. haemophilum*, and MABS (figure 1). *M. haemophilum*, which is genetically closely related to *M. leprae* and differs from all other mycobacteria by having a unique culture requirement for iron supplementation, was also shown to be involved in disseminated infections [34]. Disseminated NTM infections usually develop in patients with immunodeficiency and are most often caused by MAC species [35, 36].

Infection sources

The nearly ubiquitous presence of NTM in the environment and overlap of bacterial and human habitation makes NTM species perfect candidates for opportunistic pathogens. Natural environmental niches of NTM are lakes, streams, rivers, seawater, and soil [4]. Until recently, it was believed that the environment was the only possible source of NTM infection. NTM are believed to be naturally transmitted to human-engineered and household environments mainly through water distribution systems. For example, MAC bacteria are frequently isolated from tap water and bathrooms, the end-points of water distribution systems [12]. Particularly, shower-head biofilms have been shown to be enriched in NTM compared to main stream water [37, 38]. Significantly, MAC-based infection was shown to be more prevalent in patients whose bathrooms were contaminated with MAC bacteria. Furthermore, genotyping studies showed that clinical isolates are identical to environmental ones [12]. NTM were also found in other human-engineered environments involving water, such as swimming pools, hot tubs, rainwater tanks, cooling towers, and hospital faucets and ice machines [4]. Other common reservoirs of NTM present in the household are garden and potting soil and house dust. Interestingly, in Europe MAC bacteria and other NTM were isolated more frequently from soil than water or biofilms [12]. As an effect of their relatively hydrophobic surface, NTM are readily aerosolized. It is conceivable that aerosols formed by NTM arising from shower heads, soil, dust, and pool water can be infection sources. Survival of NTM in the environment is facilitated by the ability of these bacteria to form biofilms on surfaces. Furthermore, NTM have been shown to live within free-living amoebae where they can be protected from extreme environmental conditions [4]. This hypothesis was based on the observation that amoebae and mycobacteria are frequently isolated from the same environmental niches such as freshwater, soil, and municipal potable water systems [39]. Further, several studies demonstrated that NTM have the ability to invade amoebae and live intracellularly as endosymbionts. They can survive both within trophozoites and within cysts and replicate inside protozoa [39, 40]. However, direct evidence of the presence of mycobacteria in naturally infected amoebae remains scarce [41]. Thus, this hypothesis requires further evaluation.

The environment is also the source of *M. ulcerans* and *M. marinum* infection. The mechanism by which *M. ulcerans* is transmitted from the environment to humans is not fully understood but likely involves predatory aquatic insects, such as *Naucoris cimicoides*. These insects feed on herbivorous organisms, which are passive hosts for *M. ulcerans*. It is plausible that following the ingestion of prey contaminated with mycobacteria, they can transmit the bacteria to humans

[42]. Aquarium water containing fish infected with *M. marinum* is considered a major source of infection with *M. marinum* for humans [33].

Food products are another possible source of NTM exposure. Several studies have shown the presence of *Mycobacterium avium* subspecies *paratuberculosis* in raw and pasteurized milk and meat [43, 44].

Although household water plays an important role in spreading NTM infection, it does not explain the global increase of infections caused by these microorganisms. Most likely, other transmission routes must exist. This is further supported by the fact that the isolation of *M. abscessus*, which is the major cause of pulmonary infections in CF patients, from environmental sources is very rare [45]. Indeed, recent studies based on whole-genome analysis of a global collection of clinical species of *M. abscessus* isolated from CF patients showed that the majority of infections are acquired through indirect human-to-human transmission, potentially via fomites and aerosols [46]. Sources of NTM are summarized in table 1.

Risk groups

Though exposure to NTM through contact with shower water, soil, or dust is common in human everyday life, clinical disease manifestation occurs only in some individuals. Prominent risk factors for acquiring pulmonary NTM

infections are genetic and acquired lung diseases causing defects in lung structural and functional integrity such as CF, chronic obstructive pulmonary disease (COPD), non-CF bronchiectasis, pneumoconiosis, post-radiotherapy fibrosis, chronic pulmonary aspiration, allergic bronchopulmonary aspergillosis, previous pulmonary tuberculosis, and lung cancer [49, 50]. Additionally, genetic defects in genes related to immune responses, cystic fibrosis transmembrane conductance regulator (CFTR), cilia, and connective tissue have been found more frequently in patients with pulmonary NTM disease compared with control groups [47].

Another risk factor group includes elderly white postmenopausal women with a distinct physical phenotype characterized by slender build, pectus excavatum scoliosis, and mitral valve prolapse: described as “Lady Windermere syndrome” [51]. Similar characteristics have been also described for men as “Lord Windermere syndrome” [52]. Primary and acquired immunodeficiencies including Mendelian susceptibility to mycobacterial disease (MSMD), severe combined immunodeficiency (SCID), acquired immunodeficiency syndrome (AIDS), and hematological malignancies pose significant risks for acquiring NTM infections. Another threat is associated with immunomodulatory drugs used in autoimmune diseases and cancer treatment. Similarly, TNF therapy applied in rheumatoid arthritis patients increases the risk of NTM disease [50, 53]. Host age and gender are also significant risk factors in acquiring NTM infections. Most studies

Table 1. Sources of non-tuberculous mycobacteria [4, 12, 41, 42, 44, 47, 48]

Natural environment
Lakes, streams, rivers, ground water and seawater
Soil and dust from soil
Amoebae
Aquatic insects
Water plants and water distribution systems
Household/ Hospital environment
Plumbing systems
Tap water, shower heads, and faucets
Swimming pools, hot tubs, footbaths, hydrotherapy pools
Ice machines
Humidifiers
Dialysis centers
Heater-cooler units
Potting and garden soil
Aquarium water
Food products
Rainwater tanks and cooling towers
Indirect human-to-human transmission

Figure 2. Predisposing factors for non-tuberculous mycobacterial diseases [47, 50].

worldwide have found older patients (≥ 65 years) and women in a higher preponderance of NTM infections [7, 54]. It is worth noting that low body mass index and low nutrient intake were associated with susceptibility to pulmonary NTM disease [47]. The above-mentioned risk factors and more for acquiring NTM infection are summarized in figure 2.

NTM Diagnostics

Diagnostic criteria are summarized in several guidelines [10, 55, 56, 57]. The diagnosis of NTM-PD should be based on integrated clinical, radiographic, and microbiologic data. The most important factor is excluding contaminants or colonizers and other diseases such as tuberculosis. In histological examination, cell granulomatosis caused by NTM infections is indistinguishable from tuberculosis.

Definitive diagnosis of NTM infections should be supported by repeated isolation of NTM from several specimens from the patient or a single specimen if it is collected aseptically from a sterile body site [10]. Thus, mycobacterial culture remains the gold standard of diagnosis. The isolation of mycobacteria, especially slow-growing species, is difficult, because some species require special micronutrients

(e.g., *M. haemophilum*) or optimal pH, temperature, and oxygen levels [58, 59]. It is recommended to use both liquid and solid media simultaneously for culturing NTM from clinical samples to increase the sensitivity of mycobacteria detection. Decontamination used for environmental samples may cause certain mycobacterial species to be rendered uncultivable. Conventional identification of mycobacteria based on phenotypic characteristics and biochemical profiles is still used in low-income countries. This approach is time-consuming and not available in all clinical laboratories. Profiling of mycolic acids characteristic for each *Mycobacterium* spp. by TLC [60] and HPLC [61] is also used. Another assay is a p-nitrobenzoic acid (PNB) inhibition biochemical test, which enables differentiation between *Mtb* and NTM. Adding PNB to culture media inhibits the growth of *Mtb* but not of NTM isolates [62]. The main advantage of culture-dependent approaches is detection of viable bacteria in the sample, which increases the probability of isolation of the causative etiologic agent. However, these techniques require extended timetables. Conversely, culture-independent methods are quicker but identification of mycobacterial DNA in the clinical sample is not always proof of disease etiology.

For direct identification from clinical material, a nucleic acid amplification test (NAAT) and real-time PCR are used. Direct probe hybridization assays (e.g., AccuProbe) allow identification of *Mtb* and five other clinically important NTM

species from solid and liquid media. The disadvantage of this method is its inability to identify MABS species [63]. Line probe assays enable simultaneous identification of species in mixed cultures. INNO-LiPA MYCOBACTERIA v2 is a commercial test based on DNA probes that is capable of identifying 16 mycobacterial species [64]. Another test based on DNA probes, GenoType NTM DR, allows for identification of most clinically important species. Both *M. intracellulare* and *M. chimaera* species belonging to MAC are difficult to differentiate. The commercial molecular assay GenoType NTM-DR can distinguish between three MAC species [65]. Additionally, mutations in the *rrl* and *rrs* genes associated with macrolide and aminoglycoside resistance, respectively, can be detected with this method. A retrospective study performed on 222 clinical isolates in Slovenia using GenoType NTM DR revealed that 44.6% of the previously identified *M. intracellulare* species were *M. chimaera* [19]. The identification of species by sequencing is the most laborious method. In 16S rRNA gene sequence analysis, both conserved and variable regions of 16S rDNA amplicon are used. Due to the high similarity of mycobacterial 16S rDNA this analysis is frequently coupled with other gene sequencing; *rpoB* sequencing has better resolution on species level than 16S rRNA [66]. Other targets include: *hsp65*, *ITS*, *gyrB*, *secA*, *recA*, and *danA* [63]. Often multi-locus sequence analysis (MLSA) of several gene targets is needed for species-level identification. There are also commercial kits for 16S rRNA sequencing, such as the MicroSEQ ID system.

Whole-genome sequencing (WGS) is a promising tool which, besides identification at the species level, enables biomarker discovery [30]. WGS requires high DNA purity and quantity but additionally provides information about antibiotic resistance and is valid for epidemiologic purposes. Next-generation sequencing (NGS) technology is expensive and is not widely available in clinical laboratories but its role will likely increase after further validation and standardization.

MALDI-TOF mass spectrometry in identification of NTM

Molecular methods of mycobacteria identification are technically demanding and expensive, and not available in most clinical laboratories. As an alternative, mass spectrometry techniques- MALDI-TOF MS (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry) is used as a reliable, rapid, and cost-effective method, but this technique requires a cultivation step. Microorganism identification is based on a spectral fingerprint produced by extracted proteins. Over the last decade, MALDI-TOF MS has been used with increasing frequency in routine clinical laboratories worldwide.

Sample preparation for MALDI-TOF analysis is relatively uncomplicated; however, the lipid-rich cell envelope of mycobacteria makes it necessary to disrupt the cells before protein extraction. Different methods have been tested, including use of a special inactivation step and mechanical disruption with glass or zirconia beads [67, 68]. Recently, a method consisting of sequential freezing and mechanical cell disruption significantly improved the identification score of NTM isolates [69].

Cao et al. performed a meta-analysis of 19 studies that used MALDI-TOF for identification of clinical pathogenic mycobacteria. Among 2,593 *Mycobacterium* isolates, 85% were correctly identified to the genus level while 71% were identified to the species level. The best identification score (92%) belongs to *M. tuberculosis* isolates. For non-tuberculous mycobacteria, the identification accuracy of *M. haemophilum* was the highest at 93%, followed by *M. marinum* and others, and the lowest performance of MALDI-TOF was in *M. malmoeense* and *M. phlei* at about 50%. They concluded that the application of MALDI-TOF MS for mycobacteria identification is not satisfactory and further elaboration of MALDI-TOF databases is required [70].

Two major commercial MALDI-TOF MS identification systems—bioMérieux and Bruker—for identification of NTM were compared in the study of Brown-Elliott et al. [71]. Two large clinical laboratories in the USA utilizing different systems (VITEK bioMérieux with v3.0 Knowledge Base and Bruker Biotyper system with RUO Mycobacteria Library v5.0.0) performed identification of the same NTM isolates. The clinical isolates were previously sequenced and were analyzed as blind samples. Out of 244 isolates, Bruker Biotyper system identified 92% and bioMérieux 95% of NTM strains, to at least complex/group level. The Biotyper and bioMérieux systems identified 62% and 57%, respectively, either to the species or subspecies level. The species differentiation in *M. abscessus*, and *M. avium* complex, and also *M. mucogenium* group members was not possible. This study showed comparable results between the two commercial MALDI-TOF MS systems for NTM identification, consistent with meta-analysis previously reported [70]. *M. mucogenium* group members have similar antimicrobial susceptibility profiles and identification to the species level is not so important from a clinical point of view [72]. In the case of the *M. abscessus* complex, species identification is important due to different antimicrobial susceptibility patterns, similar to the *M. fortuitum* group. Upgrading the commercial databases or improving data processing algorithms can facilitate more precise identification in the future. Both identification systems are useful but supplemental testing would be required in some cases. Pranada et al. reported a method using a more detailed analysis of MALDI-TOF MS for accurate identification of *M. chimaera* and *M. intracellulare*, which

Figure 3. MALDI-TOF spectra of protein extracts of *Mycobacterium* spp. Expanded view of MALDI-TOF mass spectra representing *Myco-*
bacterium spp. from Polish Collection of Microorganisms (PCM): (A) *M. farcinogenes* PCM 2220, (B) *M. kansasii* PCM 2584, (C) *M. smegma-*
tis PCM 494, (D) *M. peregrinum* PCM 639, (E) *M. phlei* PCM 654, (F) *M. smegmatis* PCM 657. Colonies grown on Löwenstein-Jensen agar
were incubated at 37°C for 72 h. Spectra were acquired using extraction protocol according to El Khéchine *et al.* [67].

belong to *M. avium* complex [73]. These two species are also difficult to differentiate using conventional methods. Because of the utilization of an upgraded MALDI-TOF database it was possible to distinguish between six genotypes of *M. kansasii*, among which genotypes I and II have been associated with human disease whereas others genotypes are believed to be nonpathogenic [74]. Figure 3 shows exemplary MALDI-

TOF mass spectra of proteins extracted from several NTM species.

Apart from protein fingerprint, lipid profiles of mycobacteria obtained by MALDI-TOF mass spectrometry have strong potential and could be used for rapid species identification [75], but this technique is currently not applied in routine clinical microbiology (however, it could be adapted

Table 2. Methods used for identification of non-tuberculous mycobacterial species [57, 63, 76]

Culture-independent		Culture-dependent	
Method	Limitation	Method	Limitation
Histology	Cell granulomatosis indistinguishable from Tb	Phenotypic methods -Biochemical testing	Time consuming, does not allow identification of the newly described species
Smear microscopy	NTM not distinguishable from Mtb	Chemotaxonomical methods -HPLC of mycolic acids	Not available in all clinical laboratories
Nucleic acid amplification test -Commercial NAAT	Using for excluding Mtb	Direct probe hybridization assay	Limited number of species
Real-time PCR – in-house methods	Low sensitivity, limited range of species	Line probe assay -Commercial test	Limited number of species
Serological testing	Cross-reactivity	16S DNA sequencing Multigene sequencing -Whole genome sequencing	Limited discriminatory power Expensive, not available in clinical laboratories Highly trained staff
		MALDI-TOF MS	Database content; quality of protein extracts

in reference laboratories). Methods used for identification of NTM species are summarized in table 2.

Antibiotic treatment

As recommended by several guidelines [10, 55, 56], macrolide-based multidrug regimens guided by susceptibility testing results are the main therapeutic options for NTM infections. The use of multidrug therapy is fundamental to avoid drug resistance. In general, treatment involves the administration of clarithromycin or azithromycin in combination with at least two other antibiotics for no less than 18 months or 12 months after culture conversion [10, 55]. For lung infections with MAC, azithromycin, which is preferentially used over clarithromycin, is combined with rifampicin and ethambutol [55]. *M. kansasii* lung disease, which is usually the easiest to cure, should be treated with a regimen of either isoniazid or a macrolide combined with rifampicin and ethambutol [10]. *M. abscessus* is naturally resistant to a majority of currently available antibiotics and therefore often referred to as the “incurable nightmare” [11].

M. abscessus pulmonary disease treatment should comprise an initial phase antibiotic regimen of a macrolide administered with concurrent parenteral intravenous antibiotics (amikacin, imipenem, and tigecycline), followed by a continuation phase antibiotic treatment of nebulized amikacin and a macrolide in combination with one to three of the following oral antibiotics guided by drug susceptibility and patient tolerance: clofazimine, linezolid, minocycline or doxycycline, moxifloxacin or ciprofloxacin, and co-trimoxazole [77]. Surgical resection can be an important adjuvant to antimicrobial treatment of *M. abscessus* [55]. A study from the USA showed that patients who underwent surgery in addition to multidrug chemotherapy had higher rates of sustained culture conversion. However, surgery was also associated with a higher rate of significant morbidity [78]. The treatment of nonpulmonary NTM infections usually involves the surgical removal of the infected area or infected lymph nodes in combination with a macrolide-based regimen [10]. Though susceptibility-based treatment is recommended over empiric therapy, *in vitro-in vivo* correlation is only evident for some NTM species and drugs. A clear link has been established for macrolides and amikacin for MAC and for rifampicin and *M. kansasii* [79]. The observed discrepancy between *in vitro* drug susceptibility testing and clinical response results from the limitations of *in vitro* testing [11]. A standardized protocol from the Clinical and Standard Laboratory Institute recommends drug susceptibility testing using the broth microdilution method [80], but these culture conditions poorly represent the diverse host environments where the bacilli resides [11].

Treatment for NTM infections is extremely long, with an average duration of 18 to 24 months. The drugs and regimens are difficult to tolerate and the therapy often causes severe adverse effects which lead to treatment discontinuation or patient nonadherence [81]. Therefore, in less severe cases health professionals may choose no treatment option, and patients are kept under observation. Treatment outcome highly depends on NTM species and host health status, but usually remains poor. Whereas around 50–80% of patients with MAC pulmonary disease achieve sputum conversion [82, 83], the cure rate for lung infections caused by *M. abscessus* is usually below 50% [84, 85]. Furthermore, despite long and difficult treatment, the clinical recurrence of disease is common. For MAC pulmonary disease, recurrence occurs in 10% to 40% of patients and can represent true relapse or reinfection with a new strain of MAC [86, 87].

In summary, the main reasons for the high failure rate of NTM infection treatment include the limited number of effective therapeutics, lengthy treatment duration, and potential for severe adverse effects. Therefore, there is an urgent need for new, more efficacious and safer regimens, as well as drugs with new modes of action. Only a few therapeutics are currently under development for NTM infections and most of them are repurposed and reformulated existing antibiotics, usually compounds from Tb or malaria treatment [11].

Antibiotic resistance

As environmental bacteria, NTM are subjected to constant selective pressure from their antimicrobial-producing neighbors residing within the same niches. Therefore, NTM have developed multiple resistance mechanisms, which allow them to survive in hostile environments. Natural resistance, inducible resistance, and mutational resistance are three different resistance mechanisms by which NTM demonstrate low susceptibility to antibiotics. There have been several excellent reviews on NTM resistance mechanisms; therefore we will give only a brief summary here [88, 89]. Major factors contributing to natural resistance of NTM are a highly impermeable cell envelope, the ability to form biofilms, and expression of efflux pumps. Another mechanism of natural resistance identified in NTM is connected with polymorphism in the target gene of an antimicrobial mechanism of action. Amino acid change in the DNA gyrase GyrA of *M. avium* and *M. abscessus* and in the arabinosyl transferase EmbB of *M. abscessus* prevents fluoroquinolones [90] and ethambutol [91] binding, respectively. Further, NTM can induce expression of enzymes that modify drugs to reduce their activity. This mechanism facilitates the resistance of *M. abscessus* to β -lactams [92], aminoglycosides [93] and rifampicin [94]. Inducible or adaptive resistance is triggered

by external factors and arises under environmental pressure and, in contrast to acquired resistance, does not involve any genetic modifications. It often manifests as changes in protein or gene expression levels. An example of an adaptive resistance mechanism is *M. abscessus*'s resistance to the macrolides clarithromycin and azithromycin, which induce the expression of the ribosomal methylase Erm41, which catalyzes the methylation of the 23S ribosomal RNA (rRNA). This modification reduces the binding of these drugs to their target and attenuates their activity [95]. Acquired drug resistance involves genomic mutations in the target gene or in genes responsible for drug activation. A prolonged course of treatment is an important factor contributing to the development of acquired drug resistance in NTM. An example of this mode of resistance is macrolide resistance in *M. abscessus* and *M. avium*. A high level of resistance to clarithromycin in both species is due to mutations in the peptidyl transferase loop of the 23S rRNA (*rrl*) [82, 96]. Further, prolonged treatment with aminoglycosides leads to mutations in the 16S rRNA (*rrs*), which confer resistance to kanamycin, amikacin, and tobramycin in *M. abscessus* and *M. chelonae* [97].

Alternative treatments of NTM infections

Several alternative approaches beyond standard antibiotic therapy are under current investigation for NTM treatment. Antimicrobial peptides (AMP) are short peptides (20–60 amino acids), produced by microorganisms, humans, and other mammals. These compounds possess broad-spectrum activity against pathogenic bacteria and their mode of action is complex and diverse. AMP with anti-mycobacterial activity have been found in humans, bacteria, mycobacteriophages, and synthetically produced peptides [98]. They have been shown to display either bactericidal or bacteriostatic activity against mycobacteria. Human origin AMP such as cathelicidins, defensins, granulysins, and lactoferricins have activities against *Mtb* and a few NTM species such as *M. avium* and *M. marinum* [98, 99]. AMP can be used as a single drug or in combination with other therapeutics. A new strategy could be a combination of AMP with gold or silver nanoparticles for enhancing of killing activities [100]. The limitations of AMP are a short half-life and maintenance of activity under physiological conditions. AMP has potential as a new treatment but more work is needed for evaluation.

Another therapeutic option for NTM infection treatment is bacteriophages, viruses able to kill bacteria. Bacteriophages do not infect human cells and are specific for a strain or a species of bacteria and are effective even against multidrug-resistant strains. The phages can lyse bacteria at the site of infection and may be administered

in combination with antibiotics. To avoid immune response of the host, nonpathogenic phages should be selected for therapy. The main limitations of phage therapy are the potential of developing bacterial resistance to phages, the difficult process of phage selection, and proper formulation of phage preparation. The major obstacles to using phage therapy have been recently reviewed [101]. Though the first therapeutic use of phages took place 100 years ago [102], the data concerning mycobacteria are limited. It is estimated that about 4,200 bacteriophages can infect *Mycobacterium* spp. [103]. Few mycobacteriophages specific to *M. tuberculosis*, *M. avium*, *M. bovis*, *M. smegmatis*, and *M. ulcerans* have been studied [103]. It should be pointed out that intracellular growth of mycobacteria in macrophages or monocytes prevents easy access of phages to bacteria residing within these cells. To solve this issue, phages can be delivered inside liposomes or in nonvirulent bacteria such as *M. smegmatis* [103]. In addition, enzymes isolated from phages—such as endolysins, which can perform bacterial cell lysis—could be administered instead of whole phage particles. Moreover, phages can be modified or engineered using molecular methods. The pioneering phage treatment of *M. abscessus* infection in a CF patient after lung transplantation by a three-phages cocktail has been demonstrated by Dedrick et al. in 2018 [104]. The treatment was well tolerated and resulted in clinical improvement. It is important to note that the phages were genetically engineered and therapy was strictly personalized. Although phage therapy is a promising treatment option, it requires reference laboratories with huge collections of phages, and more experimental trials are needed to support the effectiveness and feasibility of this approach.

Iron chelators have been proposed as a potential strategy to treat mycobacterial infections. Mycobacteria, like other pathogens, need iron for proliferation and survival. Iron plays a pivotal role as a co-factor of many enzymes involved in bacterial growth and is also needed for bacterial virulence [105]. A few studies reported that adding iron chelators not only decreased *M. avium* growth, but also enhanced ethambutol activity [99]. However, the appropriate strategy of using iron chelators needs to be adapted to target the pathogen without inducing host iron deficiency. Gallium displays effects similar to iron chelators. Given that bacterial uptake systems are unable to distinguish between iron and gallium, incorporation of gallium into iron-containing proteins leads to disruption of their function. Olakanmi et al. found that gallium-containing compounds inhibit the growth of *Mtb* and MAC bacteria both within human macrophages and extracellularly in broth culture [106]. Another *in vitro* study showed that different gallium compounds, as well as nanoparticle formulations, possess inhibitory activities against *M. avium* and *M. abscessus* [107, 108]. These results suggest that iron chelator-based

treatment offers the potential for the development a novel therapy against NTM.

Nitric oxide (NO) inhalation has also been proposed as a potential treatment for NTM infections. NO possesses potent antimicrobial activity against bacteria, viruses, and fungi *in vitro* and *in vivo*. Promising results were provided by the pilot studies testing the effectiveness of nitric oxide inhalation in cystic fibrosis patients [109, 110]. Administration of high doses of NO in combination with clofazimine resulted in improved activity against *M. abscessus* [77].

Host-directed therapies are based on stimulating or modulating of immune responses to better combat mycobacteria or reduce disease symptoms. Cytokines such as IFN- γ , IL-12, GM-CSF as well as autoantibodies against IFN- γ have been tested on mice models and human patients [111]. Another strategy would involve inducing autophagy of intracellular pathogens [99]. All these host-directed therapies still need more fundamental studies as well as clinical trials to assess their real efficacy.

Prevention of NTM

Major prophylactic actions should be focused on blocking NTM transmission and developing preventive vaccines against these bacteria. NTM are environmental bacteria, and the most important preventive measure is to decrease their transmission through soil and water. Due to the extreme resistance of mycobacteria to disinfectants, chlorination is not sufficient to kill these microorganisms in water supply systems [47]. Therefore, more appropriate water treatment procedures effective against these opportunistic bacteria should be developed. Another prophylactic action could be the use of enhanced ventilation in swimming pools to lower the risk of ingestion of aerosolized biofilms formed in closed indoor areas [112]. Workers with occupational risk, exposed to soil and dust particles, should wear masks and gloves.

Currently, there are no vaccines available for NTM diseases. The BCG vaccine used against Tb may have some protection against extra-pulmonary NTM infections. There is evidence that BCG vaccination protects from NTM lymphadenitis in children, and is associated with some protection against *M. ulcerans* and *M. leprae* [113]. Research on new more effective anti-*Mtb* vaccines are ongoing and many new candidates are in development stages and in clinical trials. Moreover, some vaccine candidates against *M. avium*, *M. abscessus*, and *M. ulcerans* were tested in mouse models [47]. New vaccines for prevention as well as for immunotherapies of this difficult group of bacteria are urgently needed.

Conclusions

Increasing incidence of NTM infections, especially in industrialized countries, is alarming. As opportunistic pathogens, *Mycobacterium* spp. are extremely dangerous for elder immunodeficient patients with structural and functional lung defects, but they can also affect immunocompetent patients. Due to the ubiquitous presence of NTM in the environment, the proof that they are an etiologic agent of infection is challenging. Compared to *Mtb*, there are only a few tests enabling NTM identification. For diagnostic purposes, classical microbiological methods, as well as molecular methods, including NGS sequencing, are used. Progress in methods based on mass spectrometry such as MALDI-TOF MS may in the near future improve proper species identification, which is important for prescribing appropriate treatments. NTM are naturally resistant to anti-tubercular drugs, and require different chemotherapeutics. The therapy is prolonged and can lead to severe adverse effects, and complete pathogen eradication is not always achieved. For these reasons, alternative, nonconventional therapies are proposed: e.g., phage therapy. The most important is prophylactic interventions to reduce NTM exposure risk and the development of new diagnostics, vaccines, and therapeutics against these challenging bacteria. There is an extremely urgent need for new, more efficacious, and safe regimens and drugs with novel modes of action. However, the great diversity of NTM requires an individual approach to each species, making it a very challenging task.

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Authors' Contribution

A.G.: literature review, writing—original draft preparation, review and editing, final proofreading and approval of the version for publication; **M.P.:** literature review, writing—original draft preparation, review and editing, final proofreading and approval of the version for publication.

Conflict of Interest

The authors have no potential conflicts of interest to declare.

References

- [1] Falkinham J.O. 3rd: Environmental sources of nontuberculous mycobacteria. Clin. Chest Med., 2015; 36: 35-41
- [2] Turenne C.Y.: Nontuberculous mycobacteria: Insights on taxonomy and evolution. Infect. Genet. Evol., 2019; 72: 159-168
- [3] Runyon E.H.: Anonymous mycobacteria in pulmonary disease. Med. Clin. North Am., 1959; 43: 273-290
- [4] Pereira A.C., Ramos B., Reis A.C., Cunha M.V.: Non-tuberculous mycobacteria: Molecular and physiological bases of virulence and adaptation to ecological niches. Microorganisms, 2020; 8: 1380
- [5] Aung T.T., Beuerman R.W.: Atypical mycobacterial keratitis: A Negligent and emerging threat for blindness. J. Rare Dis. Res. Treat., 2018; 3: 15-20
- [6] Piersimoni C., Scarparo C.: Extrapulmonary infections associated with nontuberculous mycobacteria in immunocompetent persons. Emerg. Infect. Dis., 2009; 15: 1351-1358
- [7] Prevots D.R., Marras T.K.: Epidemiology of human pulmonary infection with nontuberculous mycobacteria: A review. Clin. Chest Med., 2015; 36: 13-34
- [8] Baldwin S.L., Larsen S.E., Ordway D., Cassell G., Coler R.N.: The complexities and challenges of preventing and treating nontuberculous mycobacterial diseases. PLoS Negl. Trop. Dis., 2019; 13: e0007083
- [9] Bi S., Hu F.S., Yu H.Y., Xu K.J., Zheng B.W., Ji Z.K., Li J.J., Deng M., Hu H.Y., Sheng J.F.: Nontuberculous mycobacterial osteomyelitis. Infect. Dis., 2015; 47: 673-685
- [10] Griffith D.E., Aksamit T., Brown-Elliott B.A., Catanzaro A., Daley C., Gordin F., Holland S.M., Horsburgh R., Huitt G., Iademarco M.F., et al.: An official ATS/IDSA statement: Diagnosis, treatment, and prevention of nontuberculous mycobacterial diseases. Am. J. Respir. Crit. Care Med., 2007; 175: 367-416
- [11] Wu M.L., Aziz D.B., Dartois V., Dick T.: NTM drug discovery: Status, gaps and the way forward. Drug Discov. Today, 2018; 23: 1502-1519
- [12] Nishiuchi Y., Iwamoto T., Maruyama F.: Infection sources of a common non-tuberculous mycobacterial pathogen, *Mycobacterium avium* Complex. Front. Med., 2017; 4: 27
- [13] Burgess W., Margolis A., Gibbs S., Duarte R.S., Jackson M.: Disinfectant susceptibility profiling of glutaraldehyde-resistant nontuberculous mycobacteria. Infect. Control Hosp. Epidemiol., 2017; 38: 784-791
- [14] Moore J.E., Kruijshaar M.E., Ormerod L.P., Drobniewski F., Abubakar I.: Increasing reports of non-tuberculous mycobacteria in England, Wales and Northern Ireland, 1995-2006. BMC Public Health, 2010; 10: 612
- [15] Shah N.M., Davidson J.A., Anderson L.F., Lalor M.K., Kim J., Thomas H.L., Lipman M., Abubakar I.: Pulmonary *Mycobacterium avium-intracellulare* is the main driver of the rise in non-tuberculous mycobacteria incidence in England, Wales and Northern Ireland, 2007-2012. BMC Infect. Dis., 2016; 16: 195
- [16] Andrzejak C., Thomsen V.Ø., Johansen I.S., Riis A., Benfield T.L., Duhaat P., Sørensen H.T., Lescure F.X., Thomsen R.W.: Nontuberculous pulmonary mycobacteriosis in Denmark: Incidence and prognostic factors. Am. J. Respir. Crit. Care Med., 2010; 181: 514-521
- [17] Diel R., Jacob J., Lampenius N., Loebinger M., Nienhaus A., Rabe K.F., Ringshausen F.C.: Burden of non-tuberculous mycobacterial pulmonary disease in Germany. Eur. Respir. J., 2017; 49: 1602109
- [18] Modrá H., Ulmann V., Čaha J., Hübelová D., Konečný O., Svobodová J., Weston R.T., Pavlík I.: Socio-economic and environmental factors related to spatial differences in human non-tuberculous mycobacterial diseases in the Czech Republic. Int. J. Environ. Res. Public Health, 2019; 16: 3969
- [19] Truden S., Žolnir-Dovč M., Sodja E., Starčič Erjavec M.: Nationwide analysis of *Mycobacterium chimaera* and *Mycobacterium intracellulare* isolates: Frequency, clinical importance, and molecular and phenotypic resistance profiles. Infect. Genet. Evol., 2020; 82: 104311
- [20] van der Werf M.J., Ködmön C., Katalinić-Janković V., Kummik T., Soini H., Richter E., Papaventsis D., Tortoli E., Perrin M., van Soolingen D., et al.: Inventory study of non-tuberculous mycobacteria in the European Union. BMC Infect. Dis., 2014; 14: 62
- [21] Adjemian J., Daniel-Wayman S., Ricotta E., Prevots D.R.: Epidemiology of nontuberculous mycobacteriosis. Semin. Respir. Crit. Care Med., 2018; 39: 325-335
- [22] Marras T.K., Campitelli M.A., Kwong J.C., Lu H., Brode S.K., Marchand-Austin A., Gershon A.S., Jamieson F.B.: Risk of nontuberculous mycobacterial pulmonary disease with obstructive lung disease. Eur. Respir. J., 2016; 48: 928-931
- [23] Namkoong H., Kurashima A., Morimoto K., Hoshino Y., Hasegawa N., Ato M., Mitarai S.: Epidemiology of pulmonary nontuberculous mycobacterial disease, Japan. Emerg. Infect. Dis., 2016; 22: 1116-1117
- [24] Martiniano S.L., Nick J.A., Daley C.L.: Nontuberculous mycobacterial infections in cystic fibrosis. Thorac. Surg. Clin., 2019; 29: 95-108
- [25] Skolnik K., Kirkpatrick G., Quon B.S.: Nontuberculous mycobacteria in cystic fibrosis. Curr. Treat. Options Infect. Dis., 2016; 8: 259-274
- [26] Ben Salah I., Cayrou C., Raoult D., Drancourt M.: *Mycobacterium marseillense* sp. nov., *Mycobacterium timonense* sp. nov. and *Mycobacterium bouchodurhonense* sp. nov., members of the *Mycobacterium avium* complex. Int. J. Syst. Evol. Microbiol., 2009; 59: 2803-2808
- [27] Tortoli E., Rindi L., Garcia M.J., Chiaradonna P., Dei R., Garzelli C., Kroppenstedt R.M., Lari N., Mattei R., Mariottini A., et al.: Proposal to elevate the genetic variant MAC-A, included in the *Mycobacterium avium* complex, to species rank as *Mycobacterium chimaera* sp. nov. Int. J. Syst. Evol. Microbiol., 2004; 54: 1277-1285
- [28] Hoefsloot W., van Ingen J., Andrejak C., Angeby K., Bauriaud R., Bemer P., Beylis N., Boeree M.J., Cacho J., Chihota V., et al.:

- The geographic diversity of nontuberculous mycobacteria isolated from pulmonary samples: An NTM-NET collaborative study. *Eur. Respir. J.*, 2013; 42: 1604-1613
- [29] Kwiatkowska S., Augustynowicz-Kopeć E., Korzeniewska-Kosela M., Filipczak D., Gruszczyński P., Zabost A., Klatt M., Sadkowska-Todys M.: Nontuberculous mycobacteria strains isolated from patients between 2013 and 2017 in Poland. Our data with respect to the global trends. *Adv. Respir. Med.*, 2018; 86: 291-298
- [30] Gupta R.S., 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
- [31] Tortoli E., Kohl T.A., Brown-Elliott B.A., Trovato A., Leão S.C., Garcia M.J., Vasireddy S., Turenne C.Y., Griffith D.E., Philley J.V., et al.: Emended description of *Mycobacterium abscessus*, *Mycobacterium abscessus* subsp. *abscessus* and *Mycobacterium abscessus* subsp. *bolletii* and designation of *Mycobacterium abscessus* subsp. *massiliense* comb. nov. *Int. J. Syst. Evol. Microbiol.*, 2016; 66: 4471-4479
- [32] Tai A.Y., Athan E., Friedman N.D., Hughes A., Walton A., O'Brien D.P.: Increased severity and spread of *Mycobacterium ulcerans*, Southeastern Australia. *Emerg. Infect. Dis.*, 2018; 24: 58-64
- [33] Slany M., Jezek P., Bodnarova M.: Fish tank granuloma caused by *Mycobacterium marinum* in two aquarists: Two case reports. *Biomed. Res. Int.*, 2013; 2013: 161329
- [34] Lindeboom J.A., Bruijnesteijn van Coppenraet L.E., van Soolingen D., Prins J.M., Kuijper E.J.: Clinical manifestations, diagnosis, and treatment of *Mycobacterium haemophilum* infections. *Clin. Microbiol. Rev.*, 2011; 24: 701-717
- [35] Honda J.R., Knight V., Chan E.D.: Pathogenesis and risk factors for nontuberculous mycobacterial lung disease. *Clin. Chest Med.*, 2015; 36: 1-11
- [36] Lai C.C., Lee L.N., Ding L.W., Yu C.J., Hsueh P.R., Yang P.C.: Emergence of disseminated infections due to nontuberculous mycobacteria in non-HIV-infected patients, including immunocompetent and immunocompromised patients in a university hospital in Taiwan. *J. Infect.*, 2006; 53: 77-84
- [37] Feazel L.M., Baumgartner L.K., Peterson K.L., Frank D.N., Harris J.K., Pace N.R.: Opportunistic pathogens enriched in showerhead biofilms. *Proc. Natl. Acad. Sci. USA*, 2009; 106: 16393-16399
- [38] 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
- [39] Thomas V., McDonnell G.: Relationship between mycobacteria and amoebae: Ecological and epidemiological concerns. *Lett. Appl. Microbiol.*, 2007; 45: 349-357
- [40] Drancourt M.: Looking in amoebae as a source of mycobacteria. *Microb. Pathog.*, 2014; 77: 119-124
- [41] Yu H.S., Jeong H.J., Hong Y.C., Seol S.Y., Chung D.I., Kong H.H.: Natural occurrence of *Mycobacterium* as an endosymbiont of *Acanthamoeba* isolated from a contact lens storage case. *Korean J. Parasitol.*, 2007; 45: 11-18
- [42] Marsollier L., Robert R., Aubry J., Saint André J.P., Kouakou H., Legras P., Manceau A.L., Mahaza C., Carbonnelle B.: Aquatic insects as a vector for *Mycobacterium ulcerans*. *Appl. Environ. Microbiol.*, 2002; 68: 4623-4628
- [43] Eltholth M.M., Marsh V.R., Van Winden S., Guitian F.J.: Contamination of food products with *Mycobacterium avium paratuberculosis*: A systematic review. *J. Appl. Microbiol.*, 2009; 107: 1061-1071
- [44] Shankar H., Singh S.V., Singh P.K., Singh A.V., Sohal J.S., Greenstein R.J.: Presence, characterization, and genotype profiles of *Mycobacterium avium* subspecies *paratuberculosis* from unpasteurized individual and pooled milk, commercial pasteurized milk, and milk products in India by culture, PCR, and PCR-REA methods. *Int. J. Infect. Dis.*, 2010; 14: e121-e126
- [45] Mougari F., Guglielmetti L., Raskine L., Sermet-Gaudelus I., Veziris N., Cambau E.: Infections caused by *Mycobacterium abscessus*: Epidemiology, diagnostic tools and treatment. *Expert Rev. Anti Infect. Ther.*, 2016; 14: 1139-1154
- [46] Bryant J.M., Grogono D.M., Rodríguez-Rincon D., Everall I., Brown K.P., Moreno P., Verma D., Hill E., Drijkoningen J., Gilligan P., et al.: Emergence and spread of a human-transmissible multidrug-resistant nontuberculous mycobacterium. *Science*, 2016; 354: 751-757
- [47] Chin K.L., Sarmiento M.E., Alvarez-Cabrera N., Norazmi M.N., Acosta A.: Pulmonary non-tuberculous mycobacterial infections: Current state and future management. *Eur. J. Clin. Microbiol. Infect. Dis.*, 2020; 39: 799-826
- [48] Jeon D.: Infection source and epidemiology of nontuberculous mycobacterial lung disease. *Tuberc. Respir. Dis.*, 2019; 82: 94-101
- [49] Axson E.L., Bual N., Bloom C.I., Quint J.K.: Risk factors and secondary care utilisation in a primary care population with non-tuberculous mycobacterial disease in the UK. *Eur. J. Clin. Microbiol. Infect. Dis.*, 2019; 38: 117-124
- [50] Mortaz E., Moloudizargari M., Varahram M., Movassaghi M., Garssen J., Kazempour Dizagie M., Mirsaeidi M., Adcock I.M.: What immunological defects predispose to non-tuberculosis mycobacterial infections? *Iran. J. Allergy Asthma Immunol.*, 2018; 17: 100-109
- [51] Bhatt S.P., Nanda S., Kintzer J.S.Jr.: The Lady Windermere syndrome. *Prim. Care Respir. J.*, 2009; 18: 334-336
- [52] Holt M.R., Kasperbauer S.H., Koelsch T.L., Daley C.L.: Similar characteristics of nontuberculous mycobacterial pulmonary disease in men and women. *Eur. Respir. J.*, 2019; 54: 1900252
- [53] Liao T.L., Lin C.F., Chen Y.M., Liu H.J., Chen D.Y.: Risk factors and outcomes of nontuberculous mycobacterial disease among rheumatoid arthritis patients: A case-control study in a TB endemic area. *Sci. Rep.*, 2016; 6: 29443
- [54] Mirsaeidi M., Sadikot R.T.: Gender susceptibility to mycobacterial infections in patients with non-CF bronchiectasis. *Int. J. Mycobacteriol.*, 2015; 4: 92-96

- [55] Daley C.L., Iaccarino J.M.Jr., Lange C., Cambau E., Wallace R.J., Andrejak C., Böttger E.C., Brozek J., Griffith D.E., Guglielmetti L., et al.: Treatment of nontuberculous mycobacterial pulmonary disease: An official ATS/ERS/ESCMID/IDSA clinical practice guideline. *Clin. Infect. Dis.*, 2020; 71: 905-913
- [56] Haworth C.S., Banks J., Capstick T., Fisher A.J., Gorsuch T., Laurenson I.F., Leitch A., Loebinger M.R., Milburn H.J., Nightingale M., et al.: British Thoracic Society guidelines for the management of non-tuberculous mycobacterial pulmonary disease (NTM-PD). *Thorax*, 2017; 72: ii1-ii64
- [57] Schoenfeld N., Haas W., Richter E., Bauer T., Boes L., Castell S., Hauer B., Magdorf K., Matthiessen W., Mauch H., et al.: Recommendations of the German Central Committee against Tuberculosis (DZK) and the German Respiratory Society (DGP) for the diagnosis and treatment of non-tuberculous mycobacterioses. *Pneumologie*, 2016; 70: 250-276
- [58] Forbes B.A., Hall G.S., Miller M.B., Novak S.M., Rowlinson M.C., Salfinger M., Somoskövi A., Warshauer D.M., Wilson M.L.: Practical guidance for clinical microbiology laboratories: Mycobacteria. *Clin. Microbiol. Reviews*, 2018; 31: e00038-17
- [59] Stephenson D., Perry A., Appleby M.R., Lee D., Davison J., Johnston A., Jones A.L., Nelson A., Bourke S.J., Thomas M.F., et al.: An evaluation of methods for the isolation of nontuberculous mycobacteria from patients with cystic fibrosis, bronchiectasis and patients assessed for lung transplantation. *BMC Pulm. Med.*, 2019; 19: 19
- [60] Minnikin D.E., Minnikin S.M., Parlett J.H., Goodfellow M., Magnusson M.: Mycolic acid patterns of some species of *Mycobacterium*. *Arch. Microbiol.*, 1984; 139: 225-231
- [61] Kellogg J.A., Bankert D.A., Withers G.S., Sweimler W., Kiehn T.E., Pfyffer G.E.: Application of the Sherlock Mycobacteria identification system using high-performance liquid chromatography in a clinical laboratory. *J. Clin. Microbiol.*, 2001; 39: 964-970
- [62] Sharma B., Pal N., Malhotra B., Vyas L.: Evaluation of a rapid differentiation test for *Mycobacterium tuberculosis* from other *Mycobacteria* by selective inhibition with p-nitrobenzoic acid using MGIT 960. *J. Lab. Physicians*, 2010; 2: 89-92
- [63] Huh H.J., Kim S.Y., Jhun B.W., Shin S.J., Koh W.J.: Recent advances in molecular diagnostics and understanding mechanisms of drug resistance in nontuberculous mycobacterial diseases. *Infect. Genet. Evol.*, 2019; 72: 169-182
- [64] Tortoli E., Mariottini A., Mazzarelli G.: Evaluation of INNO-LiPA MYCOBACTERIA v2: Improved reverse hybridization multiple DNA probe assay for mycobacterial identification. *J. Clin. Microbiol.*, 2003; 41: 4418-4420
- [65] Mok S., Hannan M.M., Nölke L., Stapleton P., O'Sullivan N., Murphy P., McLaughlin A.M., McNamara E., Fitzgibbon M.M., Rogers T.R.: Antimicrobial susceptibility of clinical and environmental *Mycobacterium chimaera* isolates. *Antimicrob. Agents Chemother.*, 2019; 63: e00755-19
- [66] 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
- [67] El Khéchine A., Couderc C., Flaudrops C., Raoult D., Drancourt M.: Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry identification of mycobacteria in routine clinical practice. *PLoS One*, 2011; 6: e24720
- [68] Saleeb P.G., Drake S.K., Murray P.R., Zelazny A.M.: 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
- [69] Rodriguez-Temporal D., Perez-Risco D., Struzka E.A., Mas M., Alcaide F.: Evaluation of two protein extraction protocols based on freezing and mechanical disruption for identifying nontuberculous mycobacteria by matrix-assisted laser desorption ionization-time of flight mass spectrometry from liquid and solid cultures. *J. Clin. Microbiol.*, 2018; 56: e01548-17
- [70] 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
- [71] Brown-Elliott B.A., Fritsche T.R., Olson B.J., Vasireddy S., Vasireddy R., Iakhiaeva E., Alame D., Wallace R.J., Branda J.A.: Comparison of two commercial matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) systems for identification of nontuberculous mycobacteria. *Am. J. Clin. Pathol.*, 2019; 152: 527-536
- [72] Brown-Elliott B.A., Nash K.A., Wallace R.J.Jr.: Antimicrobial susceptibility testing, drug resistance mechanisms, and therapy of infections with nontuberculous mycobacteria. *Clin. Microbiol. Rev.*, 2012; 25: 545-582
- [73] Pranada A.B., Witt E., Bienia M., Kostrzewa M., Timke M.: Accurate differentiation of *Mycobacterium chimaera* from *Mycobacterium intracellulare* by MALDI-TOF MS analysis. *J. Med. Microbiol.*, 2017; 66: 670-677
- [74] Murugaiyan J., Lewin A., Kamal E., Bakula Z., van Ingen J., Ullmann V., Unzaga Barañano M.J., Humięcka J., Safianowska A., Roesler U.H., Jagielski T.: MALDI spectra database for rapid discrimination and subtyping of *Mycobacterium kansasii*. *Front. Microbiol.*, 2018; 9: 587
- [75] Larrouy-Maumus G., Puzo G.: Mycobacterial envelope lipids fingerprint from direct MALDI-TOF MS analysis of intact bacilli. *Tuberculosis*, 2015; 95: 75-85
- [76] Cowman S., van Ingen J., Griffith D.E., Loebinger M.R.: Nontuberculous mycobacterial pulmonary disease. *Eur. Respir. J.*, 2019; 54: 1900250
- [77] Chau T., Blade K., Da Silva J., Ghaffari A., Zelazny A., Olivier K.: High efficacy of high-dose nitric oxide and its synergistic effect with antibiotics against *Mycobacterium abscessus*. *Eur. Respir. J.*, 2019; 54: OA4950

- [78] Kendall B.A., Winthrop K.L.: Update on the epidemiology of pulmonary nontuberculous mycobacterial infections. *Semin. Respir. Crit. Care Med.*, 2013; 34: 87-94
- [79] Ryu Y.J., Koh W.J., Daley C.L.: Diagnosis and treatment of non-tuberculous mycobacterial lung disease: Clinicians' perspectives. *Tuberc. Respir. Dis.*, 2016; 79: 74-84
- [80] Woods G.L., Brown-Elliott B.A., Conville P.S., Desmond E.P., Hall G.S., Lin G., Pfyffer G.E., Ridderhof J.C., Siddiqi S.H., Wallace R.J. Jr., et al.: Susceptibility testing of mycobacteria, nocardiae, and other aerobic actinomycetes [Internet]. 2nd ed. Wayne (PA): Clinical and Laboratory Standards Institute; 2011 Mar. Report No.: M24-A2
- [81] Wassilew N., Hoffmann H., Andrejak C., Lange C.: Pulmonary disease caused by non-tuberculous mycobacteria. *Respiration*, 2016; 91: 386-402
- [82] Wallace R.J.Jr., Brown-Elliott B.A., McNulty S., Philley J.V., Killingley J., Wilson R.W., York D.S., Shepherd S., Griffith D.E.: Macrolide/azalide therapy for nodular/bronchiectatic *Mycobacterium avium* complex lung disease. *Chest*, 2014; 146: 276-282
- [83] Zweijpfenning S., Kops S., Magis-Escurra C., Boeree M.J., van Ingen J., Hoefsloot W.: Treatment and outcome of non-tuberculous mycobacterial pulmonary disease in a predominantly fibro-cavitary disease cohort. *Respir. Med.*, 2017; 131: 220-224
- [84] Jarand J., Levin A., Zhang L., Huitt G., Mitchell J.D., Daley C.L.: Clinical and microbiologic outcomes in patients receiving treatment for *Mycobacterium abscessus* pulmonary disease. *Clin. Infect. Dis.*, 2011; 52: 565-571
- [85] Koh W.J., Jeong B.H., Kim S.Y., Jeon K., Park K.U., Jhun B.W., Lee H., Park H.Y., Kim D.H., Huh H.J., et al.: Mycobacterial characteristics and treatment outcomes in *Mycobacterium abscessus* lung disease. *Clin. Infect. Dis.*, 2017; 64: 309-316
- [86] Boyle D.P., Zembower T.R., Qi C.: Relapse versus reinfection of *Mycobacterium avium* complex pulmonary disease. Patient characteristics and macrolide susceptibility. *Ann. Am. Thorac Soc.*, 2016; 13: 1956-1961
- [87] Lee B.Y., Kim S., Hong Y., Lee S.D., Kim W.S., Kim D.S., Shim T.S., Jo K.W.: Risk factors for recurrence after successful treatment of *Mycobacterium avium* complex lung disease. *Antimicrob. Agents Chemother.*, 2015; 59: 2972-2977
- [88] Nessar R., Cambau E., Reytrat J.M., Murray A., Gicquel B.: *Mycobacterium abscessus*: A new antibiotic nightmare. *J. Antimicrob. Chemother.*, 2012; 67: 810-818
- [89] van Ingen J., Boeree M.J., van Soolingen D., Mouton J.W.: Resistance mechanisms and drug susceptibility testing of nontuberculous mycobacteria. *Drug Resist. Updat.*, 2012; 15: 149-161
- [90] Guillemin I., Jarlier V., Cambau E.: Correlation between quinolone susceptibility patterns and sequences in the A and B subunits of DNA gyrase in mycobacteria. *Antimicrob. Agents Chemother.*, 1998; 42: 2084-2088
- [91] Alcaide F., Pfyffer G.E., Telenti A.: Role of embB in natural and acquired resistance to ethambutol in mycobacteria. *Antimicrob. Agents Chemother.*, 1997; 41: 2270-2273
- [92] Soroka D., Dubée V., Soulier-Escrihuela O., Cuinet G., Hugonnet J.E., Gutmann L., Mainardi J.L., Arthur M.: Characterization of broad-spectrum *Mycobacterium abscessus* class A β -lactamase. *J. Antimicrob. Chemother.*, 2014; 69: 691696
- [93] Rominski A., Selchow P., Becker K., Brülle J.K., Dal Molin M., Sander P.: Elucidation of *Mycobacterium abscessus* aminoglycoside and capreomycin resistance by targeted deletion of three putative resistance genes. *J. Antimicrob. Chemother.*, 2017; 72: 2191-2200
- [94] Rominski A., Roditscheff A., Selchow P., Böttger E.C., Sander P.: Intrinsic rifamycin resistance of *Mycobacterium abscessus* is mediated by ADP-ribosyltransferase MAB_0591. *J. Antimicrob. Chemother.*, 2017; 72: 376-384
- [95] Nash K.A., Brown-Elliott B.A., Wallace R.J.Jr.: A novel gene, *erm*(41), confers inducible macrolide resistance to clinical isolates of *Mycobacterium abscessus* but is absent from *Mycobacterium chelonae*. *Antimicrob. Agents Chemother.*, 2009; 53: 1367-1376
- [96] Meier A., Heifets L., Wallace R.J.Jr., Zhang Y., Brown B.A., Sander P., Böttger E.C.: Molecular mechanisms of clarithromycin resistance in *Mycobacterium avium*: Observation of multiple 23S rDNA mutations in a clonal population. *J. Infect. Dis.*, 1996; 174: 354-360
- [97] Prammananan T., Sander P., Brown B.A., Frischkorn K., Onyi G.O., Zhang Y., Böttger E.C., Wallace R.J.Jr.: A single 16S ribosomal RNA substitution is responsible for resistance to amikacin and other 2-deoxystreptamine aminoglycosides in *Mycobacterium abscessus* and *Mycobacterium chelonae*. *J. Infect. Dis.*, 1998; 177: 1573-1581
- [98] Gutschmann T.: Interaction between antimicrobial peptides and mycobacteria. *Biochim. Biophys. Acta*, 2016; 1858: 1034-1043
- [99] Bento C.M., Gomes M.S., Silva T.: Looking beyond typical treatments for atypical mycobacteria. *Antibiotics*, 2020; 9: 18
- [100] Mohanty S., Jena P., Mehta R., Pati R., Banerjee B., Patil S., Sonawane A.: Cationic antimicrobial peptides and biogenic silver nanoparticles kill mycobacteria without eliciting DNA damage and cytotoxicity in mouse macrophages. *Antimicrob. Agents Chemother.*, 2013; 57: 3688-3698
- [101] Dąbrowska K., Abedon S.T.: Pharmacologically aware phage therapy: Pharmacodynamic and pharmacokinetic obstacles to phage antibacterial action in animal and human bodies. *Microbiol. Mol. Biol. Rev.*, 2019; 83: e00012-19
- [102] Górski A., Międzybrodzki R., Łobocka M., Głowacka-Rutkowska A., Bednarek A., Borysowski J., Jończyk-Matysiak E., Łusiak-Szelachowska M., Weber-Dąbrowska B., Bagińska N., et al.: Phage therapy: What have we learned? *Viruses*, 2018; 10: 288
- [103] Azimi T., Mosadegh M., Nasiri M.J., Sabour S., Karimaei S., Nasser A.: Phage therapy as a renewed therapeutic approach to mycobacterial infections: A comprehensive review. *Infect. Drug Resist.*, 2019; 12: 2943-2959
- [104] Dedrick R.M., Guerrero-Bustamante C.A., Garlena R.A., Russell D.A., Ford K., Harris K., Gilmour K.C., Soothill J., Jacobs-Sera D., Schooley R.T., et al.: Engineered bacteriophages for treatment

- of a patient with a disseminated drug-resistant *Mycobacterium abscessus*. *Nat. Med.*, 2019; 25: 730-733
- [105] Kelley V.A., Schorey J.S.: *Mycobacterium's* arrest of phagosome maturation in macrophages requires Rab5 activity and accessibility to iron. *Mol. Biol. Cell*, 2003; 14: 3366-3377
- [106] Olakanmi O., Britigan B.E., Schlesinger L.S.: Gallium disrupts iron metabolism of mycobacteria residing within human macrophages. *Infect. Immun.*, 2000; 68: 5619-5627
- [107] Abdalla M.Y., Switzer B.L., Goss C.H., Aitken M.L., Singh P.K., Britigan B.E.: Gallium compounds exhibit potential as new therapeutic agents against *Mycobacterium abscessus*. *Antimicrob. Agents Chemother.*, 2015; 59: 4826-4834
- [108] Choi S.R., Britigan B.E., Switzer B., Hoke T., Moran D., Narayanasamy P.: *In vitro* efficacy of free and nanoparticle formulations of gallium(III) meso-tetraphenylporphyrine against *Mycobacterium avium* and *Mycobacterium abscessus* and gallium biodistribution in mice. *Mol. Pharm.*, 2018; 15: 1215-1225
- [109] Bentur L., Gur M., Ashkenazi M., Livnat-Levanon G., Mizrahi M., Tal A., Ghaffari A., Geffen Y., Aviram M., Efrati O.: Pilot study to test inhaled nitric oxide in cystic fibrosis patients with refractory *Mycobacterium abscessus* lung infection. *J. Cyst. Fibros.*, 2020; 19: 225-231
- [110] Yaacoby-Bianu K., Gur M., Toukan Y., Nir V., Hakim F., Geffen Y., Bentur L.: Compassionate nitric oxide adjuvant treatment of persistent mycobacterium infection in cystic fibrosis patients. *Pediatr. Infect. Dis. J.*, 2018; 37: 336-338
- [111] Waterer G.: Beyond antibiotics for pulmonary nontuberculous mycobacterial disease. *Curr. Opin. Pulm. Med.*, 2020; 26: 260-266
- [112] Prevots D.R., Adjemian J., Fernandez A.G., Knowles M.R., Olivier K.N.: Environmental risks for nontuberculous mycobacteria. Individual exposures and climatic factors in the cystic fibrosis population. *Ann. Am. Thorac. Soc.*, 2014; 11: 1032-1038
- [113] Zimmermann P., Finn A., Curtis N.: Does BCG vaccination protect against nontuberculous mycobacterial infection? A systematic review and meta-analysis. *J. Infect. Dis.*, 2018; 218: 679-687