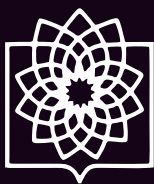
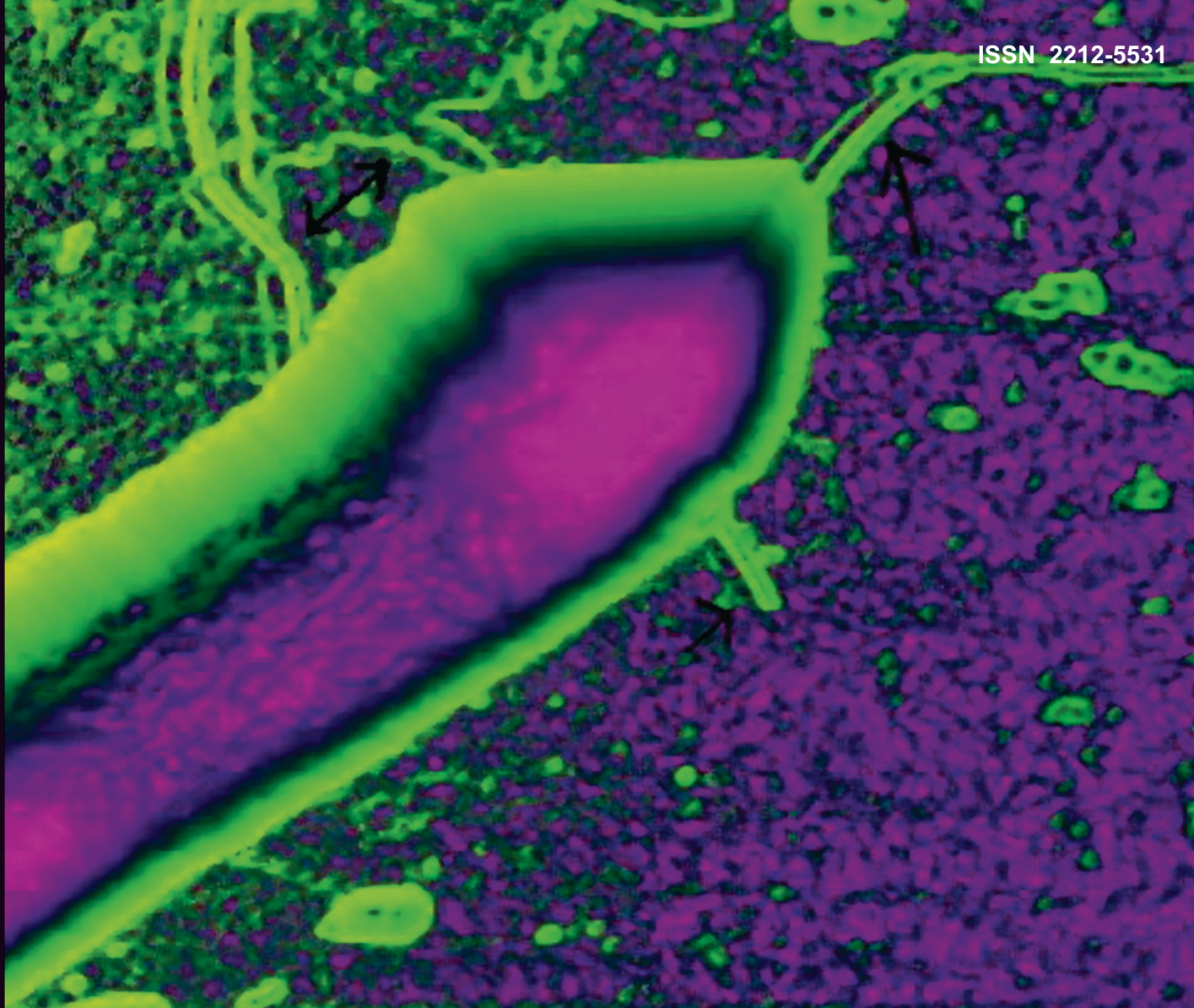


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Laboratory Diagnosis of Nontuberculous Mycobacteria in a Belgium Hospital

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Abstract

Background: Nontuberculous mycobacteria (NTM) have been identified in human pulmonary and extrapulmonary infections and are increasing globally, which makes it challenging to identify them. This article reports our experience with the laboratory identification of NTM in clinical practice among pulmonary and extrapulmonary samples received in our routine work. **Methods:** The study was conducted at the Université Catholique de Louvain at the Cliniques Universitaires Saint-Luc, Brussels, Belgium, from 2015 to 2018. A total of 386 clinical samples were collected from patients suspected of having pulmonary or extrapulmonary mycobacterial infections. Routine laboratory methods phenotypic and molecular tests were performed. **Results:** The majority of NTM species were isolated from pulmonary samples (68%). The most prevalent species identified were *Mycobacterium chimaera_intracellulare* group (32%), followed by *Mycobacterium avium* complex (21%), *Mycobacterium abscessus* complex (18%), *Mycobacterium gordonae* (9%), and *Mycobacterium chelonae* (4%). In extrapulmonary samples, *M. avium* and *M. chimaera_intracellulare* were the most frequently isolated. **Conclusion:** The species diversity of NTM found in our setting suggests the importance of the use of new modern methods for accurate identification of NTM at species level and in some case at subspecies level for the proper treatment and management of patients.

Keywords: Culture, diagnosis, matrix-assisted laser desorption ionization-time of flight, mycobacteria, sequencing

INTRODUCTION

Nontuberculous mycobacteria (NTM) refer to species of the genus *Mycobacterium* other than *Mycobacterium tuberculosis* complex and *Mycobacterium leprae*.^[1] The increasing importance of NTM in the clinical laboratory is now well recognized, and there is an increased interest in NTM identification.^[2] NTM are considered as new emerging pathogens that affect both immunocompromised and immunocompetent patients leading to an emerging public health problem.^[3] The rise in NTM isolation stresses the need for faster methods for their identification and the selection of appropriate therapy. However, NTM remain difficult to diagnose and very problematic to treat.^[4,5] The magnitude of the public health challenge associated with NTM is probably largely under-recognized because the disease caused by NTM is usually nonnotifiable. Laboratory methods play an important role in the NTM diagnosis. The NTM are grouped into slow and rapid growers. Of clinical importance is the slowly growing mycobacteria, namely the *Mycobacterium avium*

complex (MAC), *Mycobacterium kansasii*, *Mycobacterium marinum*, *Mycobacterium xenopi*, *Mycobacterium simiae*, and the rapidly growing mycobacteria, namely, *Mycobacterium abscessus*, *Mycobacterium chelonae*, and *Mycobacterium fortuitum*. In the present study, we report our experience with the laboratory identification of NTM in our routine work and give an overview of the NTM isolated in our setting.

METHODS

Study site and data collection

Retrospective analysis of suspected patients of mycobacterial infections, with clinical suspicion of pulmonary or

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extrapulmonary mycobacterial infection, attending Saint-Luc University Hospital in Brussels, Belgium, between 2015 and 2018. Data were collected retrospectively from the laboratory database for the pulmonary samples (sputum, bronchoalveolar lavage fluid, and lung tissue) and extrapulmonary samples (pleural fluid, ascitic fluid, articular liquid, gastric aspirate, bone marrow, urine, and lymph node aspirate) collected consecutively.

Sample processing and microbiological identification

Acid-fast bacilli (AFB) staining and culture for mycobacteria remain the core of our diagnostic process. Samples received are processed for AFB staining and then processed for digestion and concentration following the standard protocol.^[6] The concentrated sample was inoculated in the BACTEC MGIT 960 tube following manufacturer's instructions (Becton Dickinson) and incubated at 37°C. In parallel, three Lowenstein–Jensen medium were incubated at 37°C, 30°C, and 42°C. From the MGIT-positive tube, a smear was prepared for Ziehl–Neelsen/auramine fluorescence staining and a drop inoculated into a blood agar plates for checking any contamination (defined as growth after 48 h of incubation at 37°C). If positive for AFB, it was then subject to immunochromatographic assay (TBcID, Becton Dickinson) for MPT64 antigen detection. Those found negative for MPT64 antigen (meaning the presence of NTM) were further identified by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) MS according to the manufacturer's instructions (Bruker Daltonics GmbH Germany) and if needed by the sequencing of the *rpoB* gene. Conventional PCR was performed for partial amplification of *rpoB* gene according to Adékambi et al.^[7]

RESULTS

A total of 386 samples were enrolled during the study 2015–2018. Among them, 264 (68%) were pulmonary samples and 122 (32%) were extrapulmonary samples. Figure 1 shows the overall proportion of the different NTM species identified. The most prevalent species were *Mycobacterium chimaera_intracellulare* group (32%), followed by MAC (21%), *M. abscessus* complex (18%), *Mycobacterium gordonae* (9%), *M. chelonae* (4%), *M. xenopi* (3%), *M. kansasii* (2%), *M. neoaurum* (2%), *M. paragordoniae*, and 1% or less of *M. colombiense*, *M. peregrinum*, *M. septicum*, *M. sentence*, *M. marinum*, and *M. marseillense*. The majority of NTM species were isolated from pulmonary samples mostly coming from sputum samples (193), bronchial lavage (61), as well as from endotracheal aspirate (9), and others from strains and BACTEC myco tubes received (4) for identification. In extrapulmonary samples, the majority of NTM were identified from biopsies (79), articular liquid (14), some from urine (9), heat cooler device (5), hemoculture (5), stools (4), smears (4), bone marrow (3), ascitic puncture (3), gastric aspirate (2), or catheter (1). There was a diversity of NTM species isolated in our hospital in both pulmonary and extrapulmonary samples. *M. chimaera_intracellulare*, *M. avium*, *M. abscessus*, and *M. chelonae* were the majority species reported in

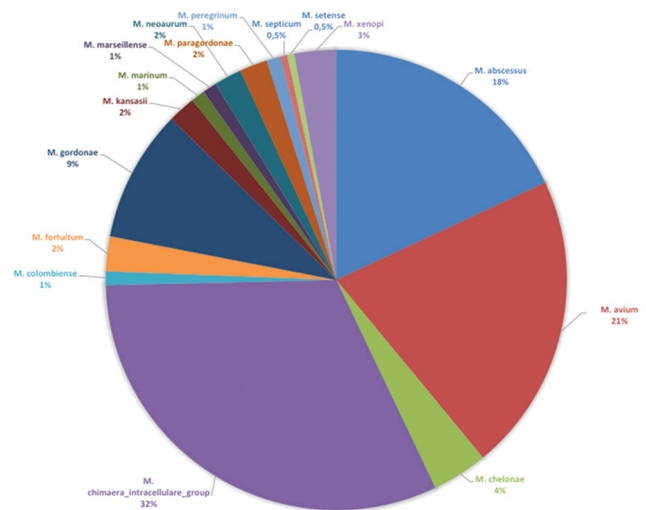


Figure 1: Distribution of nontuberculous mycobacteria species isolated

pulmonary samples [Table 1]. *M. avium* and *M. chimaera_intracellulare* were most frequently isolated in extrapulmonary samples [Table 2]. Furthermore, a nonnegligible number of biopsy samples evaluated only by *rpoB* sequencing gave negative results.

DISCUSSION

NTM are important pathogens due to their high level of drug resistance. Thus, the correct identification of NTM is essential, as their treatment varies widely between species. Nevertheless, identification is challenging due to the slow and fastidious growth of these mycobacteria. Methods used for the laboratory diagnosis of mycobacteria are continually evolving to achieve more rapid and accurate results. We present here the first retrospective laboratory-based study in the identification of clinical NTM species in a Belgium hospital in Brussels attending patients suspected of mycobacterial disease. We found that the majority of NTM were collected from respiratory samples. Furthermore, the results underline the importance of identifying NTM in suspected nonpulmonary TB patients.

We found that the most commonly isolated NTM were *M. chimaera_intracellulare* group, MAC, and *M. abscessus* complex. MAC is by far the most frequently encountered NTM pathogen in pulmonary disease in both European and worldwide countries.^[4,8,9] In an inventory study of NTM in the European Union performed by van der Werf et al. in 2014,^[10] it was shown that 99 different NTM species were identified. *M. avium*, *M. gordonae*, *M. xenopi*, *M. intracellulare*, and *M. fortuitum* were the most frequently identified. *M. gordonae* is also well known to be the most frequently recovered NTM from environmental samples and mostly considered not to be clinically relevant. However, global NTM studies are limited. The frequency of *M. chimaera* among Belgian patients has been reported in 2015;^[11] however, no cardiac infection caused by *M. chimaera* has been reported until today. Nevertheless, as also shown in our study, an important number

Table 1: Nontuberculous mycobacteria species distribution on pulmonary mycobacterial isolates

Species	Pulmonary samples					Total
	Sputum	Bronchial lavage	Aspirate Endotracheal	Strain	Flacon Bactec Myco	
<i>Mycobacterium chimaera_intracellulare</i>	47	7	1	-	-	55
<i>Mycobacterium avium</i>	18	13	1	-	-	32
<i>Mycobacterium abscessus</i>	31	2	1	-	-	34
<i>Mycobacterium chelonae</i>	5	9	-	1	1	16
<i>Mycobacterium fortuitum</i>	4	1	-	-	-	5
<i>Mycobacterium gordonae</i>	8	-	-	1	-	9
<i>Mycobacterium intracellulare</i>	1	-	-	-	-	1
<i>Mycobacterium kansasii</i>	3	-	-	-	-	3
<i>Mycobacterium paragordonae</i>	4	-	-	-	-	4
<i>Mycobacterium peregrinum</i>	1	1	-	-	-	2
<i>Mycobacterium septicum</i>	1	-	-	-	-	1
<i>Mycobacterium xenopi</i>	4	1	-	-	-	5
<i>Mycobacterium marinum</i>	-	1	-	-	-	1
<i>Mycobacterium marseillense</i>	-	-	1	-	-	1
Negative results	64	24	5	-	1	95
Total	193	61	9	2	2	264

ND: Not done

Table 2: Nontuberculous mycobacteria species distribution on extrapulmonary mycobacterial isolates

Species	Extrapulmonary samples											Total
	Biopsies	Liquid articular	urine	Heat cooler	Hemoculture	Bone marrow	Stool	Frottis	Punction ascitic	Gastric aspirate	Catheter	
<i>Mycobacterium avium</i>	5	1	1	-	1	2	-	-	-	-	-	10
<i>Mycobacterium chimaera_intracellulare</i>	2	-	-	5	-	-	1	1	-	-	-	9
<i>Mycobacterium neoaurum</i>	-	-	-	-	4	-	-	-	-	-	1	5
<i>Mycobacterium chelonae</i>	2	-	-	-	-	-	-	-	-	-	-	2
<i>Mycobacterium abscessus</i>	1	1	-	-	-	-	-	-	-	-	-	2
<i>Mycobacterium gordonae</i>	-	1	-	-	-	-	1	-	-	-	-	2
<i>Mycobacterium kansasii</i>	1	-	-	-	-	-	-	-	-	-	-	1
<i>Mycobacterium xenopi</i>	-	-	1	-	-	-	-	-	-	-	-	1
<i>Mycobacterium marinum</i>	1	-	-	-	-	-	-	-	-	-	-	1
<i>Mycobacterium setense</i>	1	-	-	-	-	-	-	-	-	-	-	1
<i>Mycobacterium marseillense</i>	1	-	-	-	-	-	-	-	-	-	-	1
<i>Mycobacterium colombiense</i>	-	-	-	-	-	-	-	1	-	-	-	1
Negative results	64	11	4	-	-	2	2	2	1	-	-	86
Total	79	14	9	5	5	4	4	4	3	2	1	122

of pulmonary infections caused by *M. chimaera_intracellulare* group are diagnosed each year in Belgium. *M. chimaera* and *M. intracellulare* are genetically very close but recently appeared to the present different epidemiological and clinical significance. Therefore, it has become important for laboratories to use adequate techniques allowing precise species identification. In our study, of the 47 *M. chimaera_intracellulare* group identified in sputum samples, 17 were *M. chimaera*, 2 were *M. intracellulare*, 1 was *M. malmoense*, and 1 was *M. xenopi* classified by *rpoB* sequencing. However, 24 were not performed to sequencing level, which is a limitation of our study. We were also limited for some samples to species results as reported in the laboratory database, which

frequently grouped *M. abscessus* complex despite their clinical subspecies differences.

In our algorithm, all clinical samples are first processed for AFB smear microscopy. AFB staining smear microscopy is important in alerting the clinician at an early stage for the presence of mycobacteria. Cultures are prone to overgrowth with other respiratory flora and copathogens which hide the presence of slow growth NTM and therefore reduce the diagnosis, particularly in cystic fibrosis (CF). To overcome this issue, different possibilities have been recommended, such as the decontamination of sputum samples with 1% or 2% N-acetyl-L-cysteine-NaOH, with a second-step employing additional agents, such as 5% oxalic acid or 1% chlorhexidine

for more heavily contaminated samples.^[12,13] A novel selective agar rapidly growing mycobacteria (RGM) medium (Newcastle upon Tyne, United Kingdom) has been proposed for the isolation of RGM from the sputum of CF patients.^[14] Furthermore, extended incubation of RGM for 28 days facilitates the isolation of slow-growing species including MAC.^[15] We have included the RGM medium in our algorithm for the samples coming from CF patients. Processing the sample soon after collection also reduces bacterial overgrowth and delays in sample processing may potentially decrease yield, which may be ameliorated by refrigeration if delay is unavoidable.^[16] Liquid culture media such as the BACTEC 960 MGIT system (Becton Dickinson) are more sensitive for the detection of NTM and offer faster time to detection than solid L-J culture.

In our experience, MALDI-TOF MS procedure involves little handling and few working hours and has definitively replaced the identification by biochemical tests, which were time-consuming and restricted to a limited number of species. However, phenotypic characteristic on solid medium such as morphology, pigmentation, and grow (rapid or slow growers) can add valuable information.^[17] The Bruker library, unfortunately, groups the complex level for the *M. tuberculosis* complex, MAC complex, *M. chimaera_intracellulare* group, and the *M. abscessus* group (*M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *bolletii*, and *M. abscessus* subsp. *massiliense*). The differentiation of these species is important since virulence differences have been observed for example between *M. avium* and *M. intracellulare* or *M. abscessus* subsp. *bolletii* that has been reported to show resistance to clarithromycin.^[18] Additional tests are thus required to be performed in these special cases. Molecular line probe assays such as the GenoType CM/AS (Hain Lifescience, Nehren, Germany) identify common NTM species although they are expensive. Although reliable, they are less used in our laboratory due to their cost and the labor involved. Furthermore, line probe assays can detect only a limited number of specified targets against which the probes are directed. However, the GenoType NTM-DR enables *M. abscessus* subspecies identification and the simultaneous determination of antibiotic resistance to macrolides and aminoglycosides, which is very useful for the adequate therapy regimen.

DNA sequencing of the 16S rRNA gene has been extensively used for the identification of NTM; however, it cannot distinguish between closely related species such as those within the *M. abscessus* complex. It is recommended to use other targets such as *rpoB* or *hsp65* genes for the identification of both rapid and slow grower mycobacteria.^[19] Whole genome sequencing is not yet currently used in routine mainly due to the costs but also for the complexity of the data analysis and the missing platform analysis. We believe that it will become soon accessible for most laboratories.

CONCLUSION

The identification of NTM at species and subspecies level is required, given differences in treatment options and

implications for patient outcome. Modern molecular tests such as MALDI-TOF and sequencing are accurate for NTM identification and have significantly improved turnaround time in our routine work.

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Conflicts of interest

There are no conflicts of interest.

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