NOVA SCHOOL OF SCIENCE & TECHNOLOGY

DEPARTAMENT OF LIFE SCIENCE

GENOMICS OF NONTUBERCULOUS MYCOBACTERIA (NTM):

DEVELOPMENT OF A LABORATORY TOOL FOR RAPID AND PRECISE DIAGNOSIS AND SURVEILLANCE OF NTM INFECTIONS

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Master in microbiology

DOCTORATE IN BIOLOGY

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Para os meus pais e para o António.

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"Happiness can be found, even in the darkest of times, if you only remember to turn on the light." (J.K Rowling, Harry Potter and the Prisoner of Azkaban).

ABSTRACT

In Portugal, the impact of nontuberculous mycobacteria (NTM), which are responsible for emerging diseases worldwide, is still unknown, and it is imperative to improve epidemiological surveillance and knowledge of antimicrobial resistance.
The main goal of this dissertation was to understand the dynamics of NTM infections in Portugal and improve their monitoring. Despite not being a notifiable disease, the data available at the LNR-TB between 2014 and 2024 showed that species belonging to the *Mycobacterium avium* (MAC) and *Mycobacterium abscessus* (MABC) complexes were the main culprits.
To enhance our genomic knowledge of these most prevalent complexes, we used whole genome sequencing (WGS) studies. The genetic characterisation and identification of resistance markers of the MABC isolates showed a progressive acquisition of resistance to different drugs in vivo and allowed us to identify "dominant circulating clones", which are associated with a worse prognosis, high resistance and cross-border dissemination.
With regard to the MAC isolates, genomic analysis made it possible to discriminate between species, subspecies and lineages, suggesting a possible need for taxonomic revision within the complex. Analysis of the resistance profiles revealed differences between species and subspecies, which reinforces the importance of accurate identification for appropriate therapeutic approaches.
In the final chapter, we intend to develop an amplicon methodology to identify and discriminate between MABC and MAC species and detect resistance mutations directly in clinical samples. Once optimised, this approach could be integrated into clinical practice, allowing for a more accurate and personalised diagnosis, reducing the time needed to make therapeutic decisions.
This study was a pioneer in the application of WGS in NTM surveillance in Portugal, reinforces the need to implement a mandatory reporting system and demonstrates the importance of integrating genomic approaches into laboratory routine to improve the diagnosis and treatment of NTM infections.

Keywords: Nontuberculous mycobacteria, *Mycobaterium avium, Mycobacterium abscessus*, Next generation sequencing, antibiotic resistance

RESUMO

Em Portugal, o impacto das micobactérias não tuberculosas (MNT), responsáveis por doença emergente a nível mundial, ainda é desconhecido, sendo imperativo melhorar a sua vigilância epidemiológica e conhecimento sobre resistência antimicrobiana.
O principal objetivo desta dissertação foi compreender a dinâmica das infeções por MNT em Portugal e melhorar a sua monitorização. Embora não sendo uma doença de notificação obrigatória, os dados disponíveis no LNR-TB entre 2014 e 2024, permitiram verificar que as espécies pertencentes aos complexos *Mycobacterium avium* (MAC) e *Mycobacterium abscessus* (MABC) foram as principais responsáveis por doença.
Para aprofundar o conhecimento genómico destes complexos mais prevalentes, recorremos a estudos de sequenciação total do genoma (WGS). A caracterização genética, e identificação de marcadores de resistência, dos isolados de MABC mostraram aquisição progressiva de resistência a diferentes fármacos *in vivo* e permitiram identificar "clones circulantes dominantes", que estão associados a um pior prognóstico, elevada resistência e disseminação transfronteiriça.
Relativamente aos isolados de MAC, a análise genómica permitiu discriminar espécies, subespécies e linhagens, sugerindo uma possível necessidade de revisão taxonómica dentro do complexo. A análise dos perfis de resistência, revelou diferenças entre espécies e subespécies, o que reforça a importância de uma identificação precisa para abordagens terapêuticas adequadas.
No capítulo final, pretendemos desenvolver uma metodologia de amplicões para identificação e discriminação de espécies de MABC e MAC, e deteção de mutações de resistência, diretamente nas amostras clínicas. Uma vez otimizada, esta abordagem poderá ser integrada na prática clínica, permitindo um diagnóstico mais preciso e personalizado, reduzindo o tempo para a tomada de decisões terapêuticas.
Este estudo foi pioneiro na aplicação do WGS à vigilância de MNT em Portugal, reforça a necessidade da implementação de um sistema de notificação obrigatória, e demonstra a importância de integrar abordagens genómicas na rotina laboratorial para melhorar o diagnóstico e o tratamento das infeções por MNT.

Palavras chave: Micobactérias não tuberculosas, *Mycobaterium avium*, *Mycobacterium abscessus,* Sequenciação de nova geração, resistência a antibióticos

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ACRONYMS

AD	Allelic difference
agMLST	Accessory-genome multi-locus sequence typing
AMK	Amikacin
AMR	Antimicrobial resistance
AZM	Azithromycin
bp	Base pairs
CF	Cystic fibrosis
CFZ	Clofazimine
CFX	Cefoxitin
cgMLST	Core-genome multi-locus sequence typing
CIP	Ciprofloxacin
CLSI	Clinical Laboratory Instituto guidelines
CLR	Clarithromycin
CT	Computerized tomography
CTAB	Cetyltrimethylammonium bromide
DCCs	Dominant circulating clones
DOX	Doxycycline
DST	Drug susceptibility testing

ENA	European Nucleotide Archive
erm	Erythromycin ribosomal methylase
gDST	Genomic drug susceptibility testing
GM-CSF	granulocyte-macrophage colony-stimulating factor
gyrB	DNA gyrase subunit B
hsp65	Heat shock protein gene
IDSA	Infectious Disease Society of America
IFN-γ	Interferon-gamma
IL-12	Interleukin-12
IMP	Imipenem
INSA	National Institute of Health
LMA	Lisbon Metropolitan Area
LNR-TB	National Reference Laboratory for Tuberculosis
LZD	Linezolid
MAC	*Mycobacterium avium* complex
MABC	*Mycobacterium abscessus* complex
MALDI-TOF-MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MIRU-VNTR	Mycobacterial interspersed repetitive units - variable number of tandem repeats
MIC	Minimum inhibitory concentration
MLST	Multi locus sequence typing
MNT	Micobactérias não tuberculosas
MOOT	Mycobacteria other than tuberculosis
MST	Minimum spanning tree
mDST	Molecular drug susceptibility testing
MXF	Moxifloxacin

NIH	Portuguese National Institute of Health
NK	Natural killer cells
NTM	Nontuberculous mycobacteria
NUTS	Nomenclature of Territorial Units for Statistics by Eurostat
pDST	Phenotypic drug susceptibility testing
pknG	Protein kinase G
PT	Portuguese
PTB	Pulmonary tuberculosis
rMLST	Ribosomal multi-locus sequence typing
rpoB	RNA polymerase β-subunit
RGM	Rapid growers
RIVM	National Institute for Public Health and the Environment
rrl	23S rRNA gene
rrs	16S rRNA gene
SGM	Slow growers
SL-HC	Single-linkage hierarchical clustering
SNP	Single nucleotide polymorphism
SNV	Single nucleotide variant sites
TGC	Tigecycline
TLR2	Toll-like receptor 2
TNF-α	Tumor necrosis factor
tNGS	Targeted next-generation sequencing
WGS	Whole genome sequencing
wgMLST	Whole genome Multi Lous Sequence typing

NOTES OF THE AUTHOR: THESIS ORGANIZATION, FORMAT AND OUTLINE

This doctoral dissertation consists of seven chapters, including an introduction, several research studies (published or in progress) and a final discussion. Its core is based on five manuscripts (listed below) that are presented as individual chapters, and one additional chapter that includes an additional study on the validation of an amplicon-based methodology that is intended to be introduced as part of the routine laboratory diagnosis. The manuscripts have been published in international peer-reviewed journals, and the corresponding chapters essentially represent what has been published. The chapters have been organised in a rational order taking into account the objectives outlined for this doctoral work. Each chapter based on a manuscript is preceded by a title page describing the publication reference and the specific contributions of the author of this doctoral thesis.
To summarise, each chapter includes the following content:

Chapter I. This chapter corresponds to the following manuscript: Carneiro, Sofia, João Paulo Gomes and Rita Macedo. 'Nontuberculous Mycobacteria: a Review.' Clin Infect Dis 7 (2023): 206. https://doi.org/10.37421/2684-4559.2023.7.206.

It consists of a general introduction that aims to introduce the reader to the state of the art on nontuberculous mycobacteria (NTM), the topics covered in this doctoral dissertation. It includes a comprehensive review of the *Mycobacterium* genus, with a particular focus on NTM, covering key aspects of taxonomy, epidemiology, pathogenesis, immune response, clinical manifestations, and the currently established diagnostic and therapeutic approaches. The chapter concludes with an overview of the major antimicrobial resistance mechanisms associated with these opportunistic pathogens.

Chapter II. Nontuberculous mycobacteria in Portugal: Trends from the last decade

This chapter corresponds to the following published manuscript: Santos A, Carneiro S, Silva A, Gomes JP, Macedo R. Nontuberculous Mycobacteria in Portugal: Trends from the last decade. Pulmonology. 2024 Jul-Aug;30(4):337-343. https://doi.org/ 10.1016/j.pulmoe.2022.01.011.

To assess which NTM species are circulating in Portugal, a retrospective study was conducted using all NTM positive cultures received at the National Reference Laboratory for Tuberculosis (LNR-TB) between 2014 and August 2024. Despite the challenges in differentiating colonization from infection, due to the absence of mandatory and standardized reporting, the study identified *Mycobacterium avium* and *Mycobacterium abscessus* complex species as the predominant causative agents of disease.

Chapter III - *Mycobacterium abscessus* complex. This chapter corresponds to the following manuscripts: Santos, A., Pinto, M., Carneiro, S., Silva, S., Rodrigues, I., Munhá, J., Gomes, J. P., & Macedo, R. (2023). Microevolution of a Mycobacteroides abscessus subsp. bolletii strain in a clinical persistent infection. Infection, Genetics and Evolution, 112, 105437. https://doi.org/10.1016/j.meegid.2023.105437; and

Carneiro, S., Pinto, M., Silva, S., Santos, A., Rodrigues, I., Santos, D., Duarte, S., Vieira, L., Gomes, J. P., & Macedo, R. (2023). Genome-Scale Characterisation of *Mycobacterium abscessus* Complex Isolates from Portugal. International Journal of Molecular Sciences, 24(20), 15402. https://doi.org/10.3390/ijms242015402'

Since our previous study identified *M. abscessus* complex (MABC) as one of the major responsible for NTM disease, we further analyzed MABC strains regarding their genomic diversity and resistance profiles. In the first study, we described a case of a chronic infection by *M. abscessus subsp. bolletii*, with progressive acquisition of resistance to multiple drugs. In the second study, using whole genome sequencing (WGS), we characterized the circulating lineages and identified genetic markers associated with antimicrobial resistance. Both these studies expanded our knowledge on MABC diversity and provided important data for improving therapeutic strategies. Additionally, we detected strains belonging to the so called dominant circulating clones (DCC), associated with worse prognosis due to their multiple drug resistance and cross-border dissemination. These findings contribute to a better understanding of MABC evolution and spread, offering essential insights for adapting therapeutic and epidemiological control strategies.

Chapter IV – *Mycobacterium avium* complex. This chapter corresponds to the following published manuscript: Carneiro, S., Pinto, M., Rodrigues, J., Gomes, JP., & Macedo, R. (2024). Genome-scale of *Mycobacterium avium* complex isolates from Portugal reveals extensive genetic diversity. https://doi.org/ 10.1016/j.meegid.2024.105682

Following the characterization of the most relevant taxonomic complexes, this chapter focuses on *Mycobacterium avium* complex (MAC). We analyzed its diversity through marker gene analysis using WGS protocols, identifying species, subspecies and lineages such as, *M. intracellulare subsp. chimaera-like*, suggesting a possible need for taxonomic revision within the group. Additionally, we assessed resistance profiles, revealing differences in antibiotic susceptibility between species and subspecies. These findings reinforce the importance of precise identification for adequate therapeutic approaches. This study complements the previous chapter, providing an integrated view of one of the most relevant NTM species in Portugal.

Chapter V - Development of a laboratory and bioinformatics-based tool for rapid diagnosis and surveillance of NTM Disease (ongoing study).
Given the need to improve NTM diagnosis, this chapter describes the development of a methodology based on targeted next generation sequencing (tNGS). The goal is to develop a rapid and sensitive approach for identification of species and subspecies as well as resistance associated mutations directly from clinical samples. The methodology was designed based on the insights gained from the previous chapters, ensuring it addresses the main challenges in NTM monitoring. Once optimized, this approach will be integrated into the LNR-TB practice, enabling a more precise and personalized diagnostics while reducing the time required for therapeutic decision-making.

Chapter VI - Final overview, concluding remarks and future directions.

The final chapter synthesizes the main findings of this dissertation, emphasizing the importance of NTM epidemiological surveillance in Portugal. We highlight the need for a national mandatory reporting system aligned with international practices, as well as the importance of integrating genomic analyses into NTM monitoring. Additionally, we propose implementing a *One Health* approach, including environmental and animal samples to better understand NTM dynamics and transmission. These initiatives will be important for improving patient outcomes and strengthening NTM surveillance in Portugal. Finally, we emphasize the potential impact of the tNGS methodology under development, which can revolutionize NTM diagnosis and monitoring, representing a significant advance in public health.

Considering the dissimilar layouts and in-text reference styles adopted by different journals where the manuscripts were published, all chapters were formatted in a unique style, with all references being cited accordingly to American Psychological Association (APA) rules. In this

regard, a single section of "References" is presented. Similarly, all the supplemental material is presented at the final of this PhD thesis, enumerated accordingly with the chapter they concern to.

1

GENERAL INTRODUCTION

This chapter was adapted from a published document in

Carneiro, Sofia, João Paulo Gomes and Rita Macedo. "Nontuberculous Mycobacteria: a Review." Clin Infect Dis 7 (2023): 206. DOI: 10.37421/2684-4559.2023.7.206

Personal Contribution

SC performed an extensive literature review and wrote the manuscript

1.1 The Genus Mycobacterium

First discovered in 1874 by Armauer Hansen, the genus Mycobacterium, with more than 220 species known to date, is the only genus of the Mycobacteriaceae family (Chalmers et al., 2020; Parte, 2018; Pfyffer, 2015). However, it was just in 1896 that the name *Mycobacterium* was established by Lehmann and Neumann (Percival & Williams, 2014; Sharma, 2020).

Bacteria from the genus *Mycobacterium* are commonly called mycobacteria and, based on their differences in the capacity to grow in vitro, epidemiology, and association with disease, they can be divided into four different groups: *Mycobacterium tuberculosis*, *Mycobacterium leprae*, *Mycobacterium ulcerans*, and Nontuberculous mycobacteria (NTM) (Forbes et al., 2018).

Mycobacteria are mostly aerobic, non-spore-forming, non-motile, resistant to acid-alcohol decolorization and colony morphology can vary from rough to smooth and from pigmented to nonpigmented (Percival & Williams, 2014; Pfyffer, 2015; Velayati & Farnia, 2017). One of their most important and unique characteristic is the composition of their cell wall, which is rich in complex lipids and contains long carbon chains (60-90 C) (Alderwick et al., 2015; Brennan, 2003; Kremer & Besra, 2004; Larsson et al., 2019; Minnikin et al., 2015; Sharma & Upadhyay, 2020). Considering the growth rate, mycobacteria can be classified into two groups: 1) the slow growers (SGM) (require more than one week to be detected on solid media); and 2) the rapid growers (RGM) (require 3-7 days to be detected on solid media). Mycobacteria also have a higher G+C content and lower copy numbers of the ribosomal operon (two copies in the RGM and one copy in the SGM) than most bacteria (Böddinghaus et al., 1990; Gupta et al., 2018).

According to pathogenicity, the genus *Mycobacterium* can also be subdivided into strict pathogens, opportunistic pathogens, and saprophyte species (Khandelwal & Dubey, 2022). Since most of them are opportunistic environmental pathogens, such as saprophytes in soil and water (Percival & Williams, 2014), the correct identification in a clinical setting is essential for diagnosis, eventual outbreak detection, and management of the putative underlying disease.

1.2 Taxonomy *Mycobacterium* Genus

Members of the genus *Mycobacterium* have many phenotypic and genomic-based characteristics that separate them from other genera (Fedrizzi et al., 2017). In 1957, Ernest Runyon proposed the first taxonomic division of mycobacteria into four groups based on the growth rate and production of a pigment (Koh et al., 2002; Velayati et al., 2019). According to this scheme,

SGM are divided in three groups: group I or photochromogen (pigmented when exposed to light), group II or scotochromogen (always pigmented) and group III or nonphotochromogen (nonpigmented). The RGM belong to group IV (Turenne, 2019; Velayati et al., 2019; Velayati & Farnia, 2017).Of note, members of the *M. avium* complex are considered nonphotochromogen, however, some isolates are capable to produce slightly pigmented colonies (Velayati & Farnia, 2017) (Table 1.1).

Table 1.1. - Runyon's classification of the Mycobacterium genus. Table adapted from Velayati (2017).

Runyon Group	Classification according to growth rate	Classification according to pigmentation	Description	Organisms (*e.g*)
I	Slow grower	Photochromogen	Cultures non pigmented in the dark and pigmented when exposed to the light	*M. kansasii* *M. marinun*
II	Slow grower	Scotochromogen	Pigmented colonies in either dark or light incubated cultures	*M. gordonae* *M. scrofulaceum*
III	Slow grower	Nonphotochromogen	Nonpigmented colonies either in cultures incubated in the dark or exposed to light	*M. avium complex* *M. tuberculosis*
	Rapid grower	-	Pigmented or nonpigmented	*M. abscessus - chelonae complex* *M. fortuitum*

Despite some limitations, the analysis of the *16S rRNA* encoding gene, which is mostly conserved between species, was used to better differentiate some *Mycobacterium* species (Böddinghaus et al., 1990; Tortoli, 2003) and supported Runyon's classification into RGM and SGM, which still continues to be used by mycobacteriologists (Forbes et al., 2018; Turenne, 2019). However, the need to improve the robustness and discrimination between different species in phylogenetic trees led to the analysis of concatenated sequences of housekeeping genes such as the 65-KDa heat shock protein gene (*hsp65*), RNA polymerase *β*- subunit (*rpoB*) and DNA gyrase subunit B (*gyrB*) (Mignard & Flandrois, 2008; Telenti et al., 1993; Tortoli, 2012). No major breakthrough was accomplished with these approaches and the introduction of

whole genome sequencing (WGS) coupled with bioinformatics tools revolutionized mycobacteria classification. Although genome-based phylogenies often do not produce very different tree topologies compared to conventional (phenotype-based) reconstructions, it is now clear that phylogenetic studies that ignore all genome-based analyses are outdated as they lack discriminatory power (Tortoli, Fedrizzi, et al., 2017).The first two studies relying on WGS data improved our understanding of mycobacteria taxonomy by validating the evident distinction between RGM and SGM: RGM assume the most ancestral branch, being the members of *Mycobacterium abscessus-chelonae* complex the most ancestral cluster, whereas species belonging to *M. terrae* complex occupy an intermediate position and SGM are clearly separated in another branch (Fedrizzi et al., 2017; Tortoli, Fedrizzi, et al., 2017) (figure 1.1).

Given the rampant advances in genomics and with the permanent release of new WGS data, it is now believed that the taxonomy of mycobacteria is well established. Still, one must realize that the genus phylogenetic tree is dynamic due to the continuous application of more robust differentiation and identification methods, leading, for example, to the inclusion of new species. In this regard, new studies have assumed some controversial opinions about the definitions of complexes/clades within the genus *Mycobacterium*. Gupta *et al*. defended the redistribution of the members of the genus Mycobacterium into five new groups: "*Tuberculosis-Simiae c*lade" that includes all of the major human pathogens, and four novel genera: the SGM *Mycolicibacterium* gen. nov. (*Fortuitum-Vaccae* clade) and *Mycolicibacillus* gen. nov. (*Triviale* clade), and the RGM *Mycobacteroides* gen. nov. (*Abscessus-Chelonae* clade) *and Mycolicibacter* gen. nov. (Terrae clade) (Gupta et al., 2018).

In contrast, Tortoli *et al*. opts for the classical nomenclature that includes 192 species, of which five (*M. chelonae-abscessus* complex, *M. fortuitum*, *M. avium*, *M. leprae* and *M. tuberculosis*) are divided into subspecies (Tindall et al., 2006; Tortoli et al., 2019).

Since this is still the most widely used approach in the scientific community, it will also be used in this review.

1.3 Nontuberculous mycobacteria - an Introduction

Nontuberculous mycobacteria, or just NTM, are also known as mycobacteria other than tuberculosis (MOTT), atypical mycobacteria or environmental mycobacteria (Forbes et al., 2018; Larsson et al., 2019). These bacteria often exhibit saprophytic, commensal, and symbiotic behaviours, and they are classified as opportunistic human pathogens (Fernandez-Rendon et al., 2012; Primm et al., 2004). They have the capacity to tolerate a wide temperature range, do not grow on standard culture media, and are resistant to many antibiotics and disinfectants (Ratnatunga et al., 2020). The cell wall, which is rich in lipids making it hydrophobic and impermeable, along with the slow growth rate, are the major characteristics that allow mycobacteria to persist in extreme environments (Falkinham et al., 2008; Farnia et al., 2019; Fernandez-Rendon et al., 2012). As such, NTM have several reservoirs, either natural or human-made,

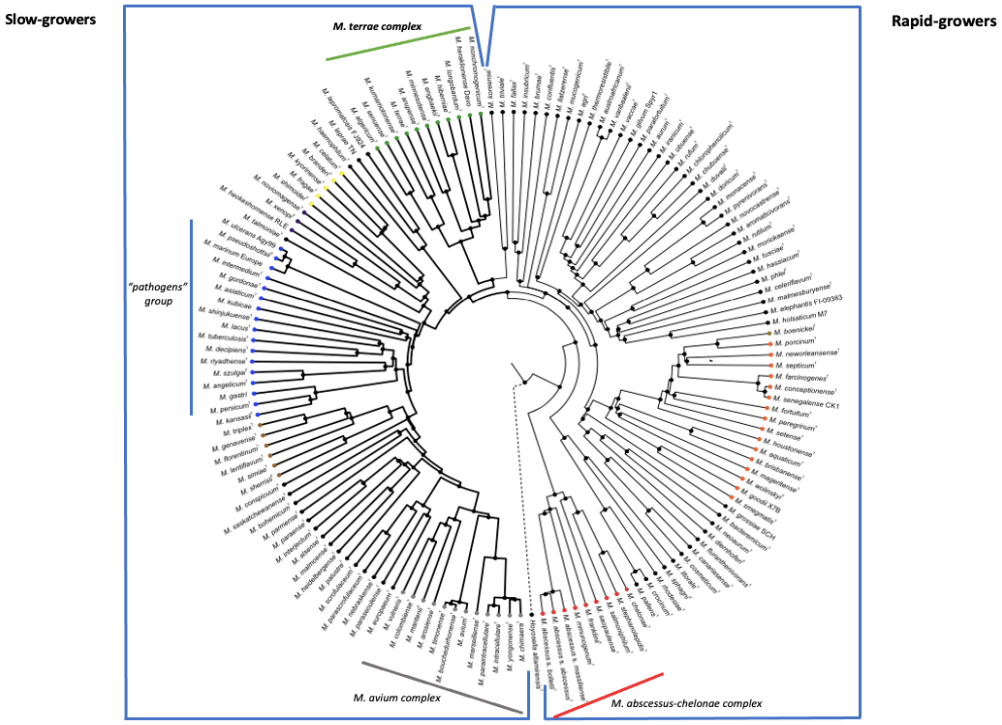

Figure 1.1 - Phylogenetic tree of Mycobacterium species. Image adapted from Tortoli et al. (2017)

such as soils, water, dust and air (Angenent et al., 2005; Falkinham et al., 2008; Falkinham, 2015; Halstrom et al., 2015; Jacobs et al., 2009; Jeon, 2019; Lahiri et al., 2014; Lande, 2019; Pereira et al., 2020; R. Thomson et al., 2013). In addition, they also have the ability to infect animals, which gives them importance in both human and veterinary medicine (Biet & Boschiroli, 2014; Jacobs et al., 2009; Zulu et al., 2021).

Unlike *M. tuberculosis*, the transmission of NTM does not seem to be person-to-person but rather trough inhalation of aerosols from infected sources and, in some cases, ingestion or trauma events (Falkinham, III, 2009). Although it has been suggested that person-to-person transmission may occur between patients with cystic fibrosis (Bryant et al., 2013, 2016), this seems to be an exception (B. E. Jönsson et al., 2007).

The environment is rich in RGM and most of them are not associated with disease. However, some species such as *Mycobacterium abscessus-chelonae* complex (MABC) (*M. abscessus subsp. abscessus*, *M. abscessus subsp. bolletii* and *M. abscessus subsp. massiliense*) and *Mycobacterium fortuitum* can cause disease. Among the SGM, the ones that are most commonly isolated and associated with disease are: *Mycobacterium avium* complex (MAC), particularly the subspecies *M. avium subsp. avium*, *M. avium subsp. intracellulare and M. avium subsp chimaera*, *Mycobacterium xenopi* and *Mycobacterium kansasii* (Hoefsloot et al., 2013; Johnson & Odell, 2014; Ratnatunga et al., 2020; Santos et al., 2024).

As NTM are ubiquitous in environment, exposure is frequent (Koh, 2017; Somoskovi & Salfinger, 2014), so the detection of these opportunistic pathogens in a clinical sample is not enough to classify them as the disease causing agents, making it difficult to clarify the clinical significance of NTM (Griffith et al., 2007).

1.3.1 Epidemiology of Nontuberculous mycobacteria

Although many studies report an increase in NTM human disease, and these are already recognized as a global health concern (Cowman et al., 2016; Donohue, 2018; McShane & Glassroth, 2015; Winthrop et al., 2020), the epidemiology of NTM is not well understood (Kendall & Winthrop, 2013; Prevots & Marras, 2015). This lack of knowledge can be attributed to several factors: 1) NTM disease is not of mandatory reporting, so most of the cases are not reported to public health authorities and, as a consequence, incidence data relies just on the number of laboratory isolates (Jeon, 2019; Larsson et al., 2019; Santos et al., 2024); 2) diagnosis of NTM requires considerable time and may be misdiagnosed as tuberculosis or other lung disorder (Chalmers et al., 2018; Maiga et al., 2012; Ryu et al., 2016); and 3) the existing diagnostic tests are unreliable and costly and no prognostic tests are available (Ratnatunga et al., 2020).

The distribution of NTM species seems to be different among geographic regions and populations (Jeon, 2019). Hoefsloot *et al.* (2013) showed that MAC is predominant worldwide, *M. avium* is predominant in North and South America and Europe, and *M. intracellulare* is most

frequently isolated in Africa and Australia. They also described that *M. xenopi* is particularly prevalent in Hungary, Croatia, Northern Italy, Ontario, and in the area close to the English Channel. *M. kansasii* appears to be more prevalent in South America, Eastern Europe and the metropolitan centers of London, Paris and Tokyo and some areas of South Africa. *M. malmoense* seems to predominate in Europe (Hoefsloot et al., 2013).

More recently, Farnia *et al.* (2019) published a systematic review on the distribution of NTM (Farnia et al., 2019). In the north and east of Europe, the most frequently found NTM in clinical samples belonged to MAC (in particular *M. avium*) and *M. gordonae*. In the south and west of Europe, the most common NTM isolated were *M. xenopi*, *M. gordonae*, and MAC, mainly *M. avium*. Similarly to the study of Hoefsloot *et al.* (2013), Farnia *et al.* (2019) also observed that in Africa and Australia *M. intracellulare* was the most frequent NTM isolated, followed by *M. avium,* and *M. kansaii* in Africa and by *M. fortuitum* in Australia. Furthermore, according to this study, in east Asia the most frequent NTM species isolated are *M. avium and M. abscessus*, in west Asia, *M. fortuitum, M. gordonae,* and *M. simiae* and, in south Asia, *M. fortuitum, M. chelonae and M. xenopi* (Farnia et al., 2019).

A recent study from Portugal showed that MAC is the most isolated NTM associated with disease, followed by MABC and *M. fortuitum*. As expected, the majority of the cases appeared in the regions with higher population density (Maekawa et al., 2011; Santos et al., 2024).

Of note, a common denominator of all of these epidemiological studies is that the prevalence of the disease likely caused by NTM seems to increase with the aging of the population, with the development of laboratory techniques, with the increase in the number of immunosuppressed patients, and with a decrease in the incidence of tuberculosis (Brode et al., 2014; Hermansen et al., 2017; Hoefsloot et al., 2013; Johnson & Odell, 2014; Prevots et al., 2010). Thus, it is clear that there is a need to create a broad approach involving both the collection of informative clinical data and more robust laboratory procedures in order to be able to estimate the true impact of these infections (Chalmers et al., 2020; Marras & Daley, 2002; Stout et al., 2016).

1.3.2 Pathogenesis and Immune response In NTM Infections

Although exposure to these bacteria is frequent, NTM disease is relatively uncommon, leading to the assumption that normal host defense mechanisms are sufficient to prevent infection. This also suggests that patients who develop NTM disease must have specific susceptibility

factors that make them vulnerable (Koh, 2017). However, the physiologic and cellular conditions that likely facilitate NTM infections are poorly understood (Honda et al., 2015). On the pathogen side, with the exception of mycolactone of *M. ulcerans,* there is no evidence of specific virulence factors among mycobacteria that may potentiate the infection (Larsson et al., 2019). It has recently been suggested that the capacity of some NTM (e.g: MAC, *Mycobacterium abscessus* and *Mycobacterium kansasii*) to change their colony morphotype from smooth to rough, contributes to their virulence, being the latest the most virulent in most of the cases, with exception of MAC where smooth colonies are the most virulent (Ferrell et al., 2022; B. Jönsson et al., 2013; Larsson et al., 2019; Nishimura et al., 2020).

M. kansasii affects mostly the lungs but it can also cause infections in lymph nodes, bone, skin and, in cases where the host has low CD4 counts (below 50/mm3), disseminated disease can be developed (Akram & Rawla, 2024; Bernard et al., 1999; McGarvey & Bermudez, 2002). This NTM species has the ability to enter macrophages, which in turn are the ideal environment for the microorganism to develop and be transported to other tissues (Le Cabec et al., 2000; McGarvey & Bermudez, 2002).

M. abscessus is the most virulent fast-growing mycobacteria (To et al., 2020). The infection shares similarities with the one caused by *M. avium* and *M. tuberculosis* (*i.e.*, formation of granulomas and persistence of infection) however, its mechanisms of transmission and establishment of disease are still not well understood (Johansen et al., 2020; To et al., 2020). It is known that the colony morphotype plays an important role in the ability of this NTM species to infect, as it allows the transition between a phenotype with a greater ability to colonize (smooth) and a more virulent and invasive phenotype (rough) (Ryan & Byrd, 2018). Both smooth and rough phenotypes can survive inside the macrophages and be maintained in loner phagosomes. The difference between these two phenotypes is that the smooth variant is usually able to prevent the activation of apoptosis and autophagy. On the other hand, when the transition to the rough variant occurs, bacterial cords formation and acidification of the phagosome begin, resulting in a massive tissue destruction leading to severe infection. The production of bacterial cords only happens in rough forms and is determinant to establish the infection (Bernut et al., 2014; Frehel et al., 1986; Johansen et al., 2020; Roux et al., 2016).

Concerning MAC infections, rough morphotypes are less pathogenic than the smooth ones, as they lack genes for glycopeptidolipids synthesis that are significant determinants for virulence (Belisle et al., 1993; Bhatnagar & Schorey, 2006; Holt & Daley, 2019). These are necessary to biofilm production, which is an important mechanism to disrupt the host's immunity leading

to settlement facilitation and resulting in invasion of bronchial epithelium (Le Cabec et al., 2000; McGarvey & Bermudez, 2002). MAC species also have the ability to survive the acidic pH of the stomach, overcome the acid barrier and gain access to the intestinal lumen (McGarvey & Bermudez, 2002). Nevertheless, some differences can be found within the species of MAC.

The phagocytes, after engulfing mycobacteria, activate a series of complex cascade reactions (Awuh & Flo, 2017); cytokines, such as interleukin-12 (IL-12), interferon-gamma (IFN-γ) and tumor necrosis factor-α (TNF- α), play a role in antimycobacterial immune response and regulation (U.-I. Wu & Holland, 2015). IL-12 production leads to natural killer (NK) cells production and t-lymphocyte proliferation (Wagner et al., 2002), which are essential for the innate immune response to *M. avium* infections (Bermudez et al., 1995). In fact, the secretion of TNF-α, IFN-γ and granulocyte-macrophage colony-stimulating factor (GM-CSF) , will stimulate infected macrophages enabling them to control the intracellular infection (Bermudez et al., 1995; McGarvey & Bermudez, 2002; Muller, 1995). Regarding *M. abscessus* infection, the rough morphotype prevents macrophages innate response (Howard et al., 2006; Nessar, Reyrat, Davidson, et al., 2011; To et al., 2020). In these cases, the immune innate response is activated by the interaction with toll-like receptor 2 (TLR2) resulting in a TNF- α release. IL-12 is also released leading to an activation and polarization of naive CD4+ T cells towards Th1 cells to produce IFN-γ (To et al., 2020). The adaptive immune response also plays an important role in the immune response against *M. abscessus* infections, as it is important for granuloma formation through recruitment of B and T cells (Dorhoi et al., 2011). The granuloma is a structure that is formed during mycobacterial infections and usually contains the infection. However, as already mentioned, *M. abscessus* has the ability to change from smooth to rough colonies resulting in granuloma collapse and dissemination of the infection (Bernut et al., 2014).

1.3.3 Disease, diagnosis and treatment

Manifestation of the disease is the reflex of an interaction between the exposure (for example, the infecting dose and duration of exposure), the microorganism (pathogenicity and virulence), and the host (immune status, genetic risk factors and prior lung disease) (Chalmers et al., 2018; Griffith & Aksamit, 2016; Y.-S. Kwon & Koh, 2016). The respiratory tract is the most frequent target of NTM, however, these bacteria have the ability to infect a wide variety of body sites (Tortoli, 2004). The most common diseases caused by NTM are pulmonary disease,

lymphadenitis, skin, soft tissue, and bone disease and disseminated disease in severely immunocompromised patients (figure 1.2) (Baldwin et al., 2019; Blanc et al., 2016; Griffith et al., 2007).

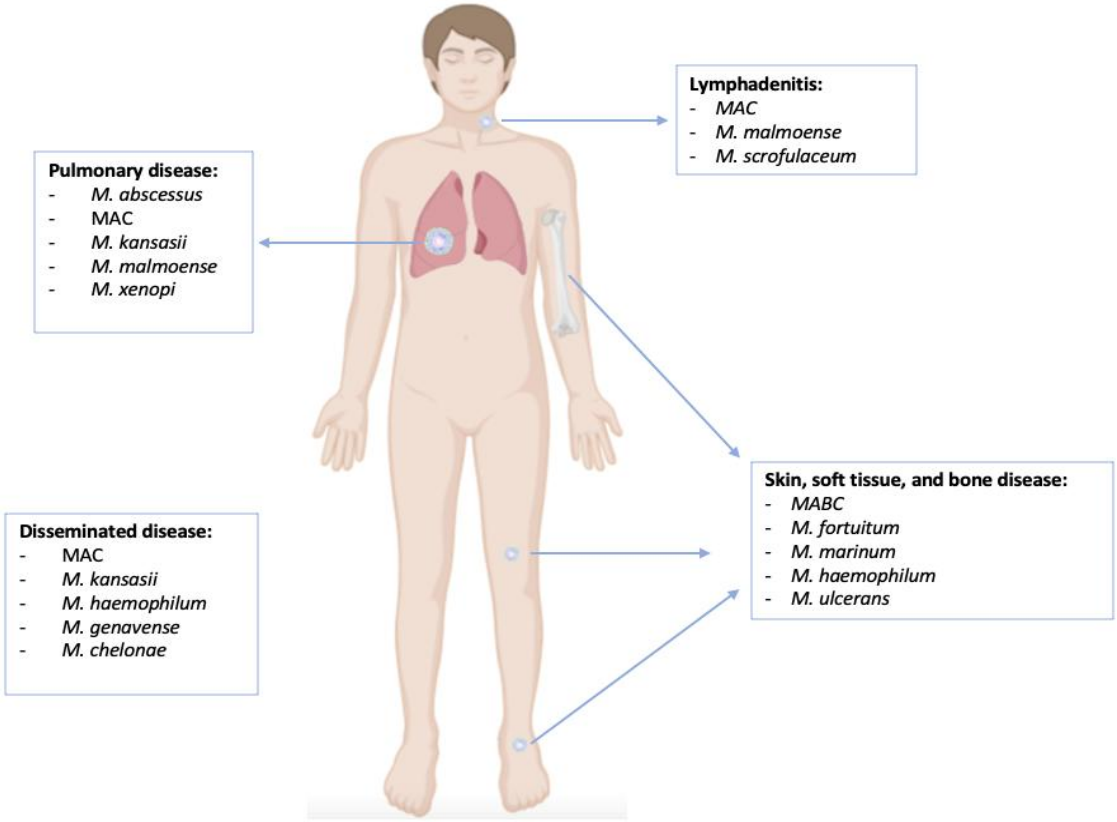

Figure 1.2 - Clinical disease more common caused by NTM. Figure adapted from Baldwin et al., 2019.

A correct and early identification of NTM is essential for the management of the disease (Larsson et al., 2019). Since the symptoms of NTM are non-specific, there is often a delay in diagnosis, resulting in disease progression and, eventually, more complicated treatment regimens with more side effects (Larsson et al., 2019; Pennington et al., 2021).

Pulmonary and extrapulmonary NTM disease are managed separately, although clinical, radiological and laboratory findings are critical in both cases (Clain & Aksamit, 2019). In either case, the diagnosis is complicated as NTM exposure is very common, although disease development is rare (Fleshner et al., 2016). Furthermore, due to the ubiquitous character of NTM, in most cases of pulmonary disease one must take into account that the presence of a NTM positive culture from respiratory sites only reflects colonization rather than the detection of the disease-causing agent. In addition, the respiratory samples are non-sterile which makes it another factor that creates uncertainty in the final diagnosis (Clain & Aksamit, 2019).

Although NTM lung disease does not reveal specific symptoms (i.e., cough, fever, fatigue, weight loss and hemoptysis), the radiological findings are suggestive of this type of infection. Typically, specific bronchiectasis with nodules or cavitation are found in nodular bronchiectasis disease and fibrocavitary disease, respectively (Ellis & Hansell, 2002; Erasmus et al., 1999; Griffith et al., 2007).

Lymphadenitis, with cervical adenitis as the clinical presentation, is common among immunocompetent children (Clain & Aksamit, 2019; Griffith et al., 2007; Larsson et al., 2019; Varma-Basil & Bose, 2019). The first symptom that is suggestive of lymphadenitis is unilateral indolent swelling that persists in time without systemic symptoms associated (Clain & Aksamit, 2019; Varma-Basil & Bose, 2019). The definitive diagnosis relies on the microbiological findings through lymph node sample analysis as these also allow to exclude tuberculosis and pyogenic lymphadenitis (Clain & Aksamit, 2019; Varma-Basil & Bose, 2019).

Skin, bone, and soft tissues disease, as the other forms of NTM infections, also demand clinical context, microbiological results, histopathology results compatible with the diagnosis and history of underling disease (Clain & Aksamit, 2019; Griffith et al., 2007). Skin and soft tissues disease may develop in immunocompromised patients after a puncture wound or a traumatic injury (Atkins & Gottlieb, 2014; Varma-Basil & Bose, 2019). Nosocomial infections are acquired due to invasive therapeutic interventions, surgeries or long term intravenous catheters (Varma-Basil & Bose, 2019). Albeit rare, NTM have been isolated from osteomyelitis cases and, in immunocompromised patients, is often a result of disseminated disease (Hirsch et al., 1996). Bone infection can occur in immunocompetent patients after an accidental trauma or surgery (Elsayed & Read, 2006; Rosenberg & Khurana, 2016). Diagnosis is difficult manly due to the lack of specific examination methods and to the various possible clinical presentations (Rosenberg & Khurana, 2016). Magnetic resonance is used to help find possible sites of infection, however, it cannot clearly differentiate infection caused by NTM or by *mycobacterium tuberculosis* (Theodorou et al., 2001). As such, for a correct diagnosis, microbiological and/or histological confirmation is mandatory (Clain & Aksamit, 2019).

Disseminated disease may appear in patients with low CD4+ T cell counts (McShane & Glassroth, 2015) and, once more, symptoms are nonspecific (e.g., fever, weight loss and abdominal pain) (Clain & Aksamit, 2019). A positive blood culture is the main differential diagnosis, but it can also be done by the isolation of NTM from another sterile sample, for example lymph node, bone marrow or liver (Clain & Aksamit, 2019).

Some genetic/ heritable and acquired disorders that compromise the lung may act as risk factors to NTM disease. Examples of acquired disorders are smoking-related emphysema, bronchiectasis as a result of another unrelated infection, use of immunosuppressives, pneumoconiosis and chronic aspiration (Chan, 2019; Dirac et al., 2012; Y. M. Kim et al., 2009). On the other hand, genetic disorders include cystic fibrosis (CF), elastin deficiency, congenital bronchial cartilage deficiency, alpha-1-antitypsin deficiency and, primary ciliary dyskinesia (Chan et al., 2007; Damseh et al., 2017; Noone et al., 2004; Nunes-Costa et al., 2016; Parr et al., 2007). Several studies indicate that NTM infections are more common in female gender, and consider older age and low body weight as risk factors for disease (Chan & Iseman, 2010; R. D. Kim et al., 2008; Mirsaeidi, Farshidpour, Ebrahimi, et al., 2014; Mirsaeidi & Sadikot, 2015; Okumura et al., 2008). In addition, an immunosuppressed status, as the one associated with human immunodeficiency virus infection, transplantation, or defects in the pathways of the IL-12, TNF-α and, IFN-γ are well-known risk factors (Amorim et al., 2010; Chan & Iseman, 2013; Honda et al., 2015; Jones & Havlir, 2002; Knoll et al., 2012; Varma-Basil & Bose, 2019). Also, in an infected individual, an immunosuppressive treatment increases the risk of progression to disease by NTM.

The clinical significance of NTM isolated in the airways is hard to measure since they are environmental bacteria, and colonization of the airways is common. For this reason, the American Thoracic Society (ATS) and the Infectious Disease Society of America (IDSA) have provided criteria for NTM disease diagnosis. It requires microbiological, clinical, and radiographic correlation, with all of these criteria of equal importance (Griffith et al., 2007) As a NTM-positive respiratory specimen from a patient who has no evidence of underlying disease may imply contamination, at least two separate positive cultures of sputum specimens collected in different time periods are required to define the case as possible disease. However, if a patient has a lung biopsy with mycobacterial histopathological features and a positive NTM culture is isolated from the biopsy sample, a single positive result is enough to consider as a potential case of disease. The same criteria apply if the patient has a positive culture of other invasively collected biological samples such as bronchial or bronchoalveolar lavage. Clinical criteria for case definition include pulmonary or systemic symptoms such as cough, sputum production, chest pain, fatigue, fever, and weight loss whereas radiographic criteria are based on chest radiographic results showing nodular or cavitary opacities or computerized tomography (CT) scan results showing bronchiectasis with multiple nodules. Even if a patient meets all the criteria necessary to be classified as having NTM disease, the pathogenicity of the NTM

isolate must be taken into account, and for low-pathogenic NTM species, recurrent positive cultures and strong clinical and radiological evidence are required for establishment of NTM disease (Daley et al., 2020; Griffith et al., 2007; Ryu et al., 2016).

Treatment regimens used in NTM infections require the prolonged use of multiple drugs, which is costly and may cause severe side effects for the patient, as these drugs usually show high toxicity (Ryu et al., 2016). As such, each treatment is unique and dependent on the individual comorbidities of each patient and the risk-benefit ratio (Aksamit & Griffith, 2019). The decision of which combination of drugs to use depends on the species and, in some cases, the subspecies of the NTM causing the disease. Although drug susceptibility testing (DST) is advised for correct treatment regimen design, correlation between *in vitro* and *in vivo* outcome is not yet well established (Brown-Elliott et al., 2012; van Ingen et al., 2012) and, as such, treatments are still mostly empirical.

Treatment regimens for SGM usually includes ethambutol, rifampicin and a macrolide and, in some severe cases, amikacin or streptomycin can also be added. Not every SGM species has defined breakpoints for DST. Members of MAC are tested for clarithromycin, linezolid, and moxifloxacin; *M. kansasii* is tested for clarithromycin, rifampicin, amikacin, ciprofloxacin, ethambutol, isoniazid, linezolid, moxifloxacin, rifabutin, streptomycin, and trimethoprim-sulfamethoxazole; and *M. marinum* is tested for amikacin, ciprofloxacin, clarithromycin, doxycycline, ethambutol, moxifloxacin, rifabutin, rifampin, and trimethoprim-sulfamethoxazole (Woods et al., 2011).

In case of infections with RGM, the treatment regimen is based on DST results that is performed for macrolides, fluroquinolones, amikacin, imipenem, tetracyclines, linezolid, and trimethoprim-sulfamethoxazole (Griffith et al., 2007).

1.3.4 Mechanisms of antimicrobial resistance

Antimicrobial resistance is a global problem underlying infections caused by bacteria, and mycobacteria are no exception. Most NTM species are resistant to a wide array of antibiotics and both natural and acquired resistance are important determinants for the treatment success (Griffith et al., 2007; Van Ingen, 2019). DST is used to determine the interplay between resistance and susceptibility during suboptimal drug exposure and selection, which helps in the establishment of appropriate treatment regimens (Van Ingen, 2019). As NTM have natural/intrinsic resistance according to species or subspecies, it is of extreme importance to identify the

NTM causing the disease in order to define the appropriate treatment regimen, even if an empiric one (Griffith et al., 2007). This natural drug resistance is conferred by mechanisms associated with the cell wall permeability, thickness, formation of granulomas or the capacity to form biofilms. These mechanisms interfere with drug uptake that allow their biotransformation, or decrease the affinity with the drug target (Ahmed et al., 2019; Falkinham, 2018; Lambert, 2002; Saxena et al., 2021; Tarashi et al., 2022; Van Ingen, 2019). The highly hydrophobic and impermeable cell wall of mycobacterial cells, which is composed by N-glycolyl muramic acid and is abundant in lipids constituted by long chain fatty acids with more than 90 carbons (Ahmed et al., 2019; Falkinham, 2018; Lambert, 2002), hampers the diffusion of hydrophilic antibiotics and nutrients through this layer (Lambert, 2002; Van Ingen, 2019). For these reason, the transport mechanisms across membrane is essentially controlled by porin channels (Lambert, 2002; Van Ingen, 2019; van Ingen et al., 2012). It is the activity of these porins that determines the susceptibility to hydrophilic (*e.g* norfloxacin and β- lactam) and hydrophobic (e.g vancomycin and rifampicin) antibiotics (Danilchanka et al., 2008; De Moura et al., 2021; Stephan et al., 2004). In addition, NTM also possess efflux systems (*e.g*. P55, tetV and tap that confer resistance to aminoglycosides and tetracyclines) that avoid the accumulation of drugs inside the cell (De Rossi et al., 1998; Pereira et al., 2020; Ramón-García et al., 2006; Van Ingen, 2019). These efflux pumps are substrate-specific, but others transport a wide range of substrates, conferring resistance to multiple drugs at once (Nasiri et al., 2017). Another efflux pump system has been described in MAC: MmpL5/MmpS5, which confers resistance to clofazimine and bedaquiline (Alexander et al., 2017).

The persistence of a multidrug-resistant phenotype is possible due to the existence of several genes and systems involved in cell wall maintenance such as the protein kinase G (pknG), and *asnB* in *M. smegmatis*, and the *mtrAB* in *M. smegmatis* and *M. avium* (van Ingen et al., 2012). Whenever a disruption in these genes is observed, there will usually occur a decrease in hydrophobicity of the mycobacterial cell wall, leading to increased susceptibility to lipophilic antibiotics (*e.g*. macrolides, rifamycins, and penicillins) (Cangelosi et al., 2006; H. T. Nguyen et al., 2010; Ren & Liu, 2006; Wolff et al., 2009).

As mentioned before, the formation of biofilms and granulomas are two additional mechanisms to promote antimicrobial resistance in NTM (Saxena et al., 2021; M.-L. Wu et al., 2018). The lipid-rich extracellular matrix of the biofilm works as a barrier, which does not allow the penetration of drugs (M.-L. Wu et al., 2018). Furthermore, in biofilms, horizontal gene transfer

is potentiated due to the interactions between the bacteria that form the biofilm layers, promoting the spread of drug resistance (Faria et al., 2015). Moreover, some genes are expressed differently when the bacteria grows in biofilms (Casadevall & Pirofski, 2009). For example, it is assumed that the increased chlorine resistance noted in *M. avium* and *M. intracellulare* cells when these bacteria grow in biofilms is due to changes in the cell wall, which in turn results from changes in the structure of mycolic acid (Steed & Falkinham, 2006).

Changes in gene expression may lead to modifications in the binding sites of antibiotics. This is the case of the erythromycin ribosomal methylase (*erm*) gene that confers resistance to macrolides by methylating the bacterial ribosome, thereby blocking the binding site for macrolides (Choi et al., 2012; Stout & Floto, 2012). The erm gene has been described in *M. abscessus spp. abscessus*, *M. abscessus spp. bolletii, M. fortuitum*, and *M. porcinum* (Bastian et al., 2011; Brown-Elliott et al., 2012; Nash et al., 2009). In *M. abscessus spp. massiliense* the treatment with macrolides can still be an option as inducible macrolide resistance does not occur in this subspecies (S. Y. Kim et al., 2015; Koh et al., 2016).

On the other hand, acquired resistance appears mainly due to spontaneous mutations in chromosomal genes, especially during antibiotic treatment (Nasiri et al., 2017; Tarashi et al., 2022) and, due to this type of resistance, multidrug-resistance pathogens are rapidly increasing worldwide (S.-Y. Kim, Han, et al., 2017). Mutations in 23S rRNA gene (*rrl*) and in 16S rRNA gene (*rrs*) are responsible for resistance to macrolide and aminoglycoside respectively, in MABC and MAC clinical isolates (Bastian et al., 2011; Brown-Elliott et al., 2013; Meier et al., 1996; Olivier et al., 2017; Prammananan et al., 1998; M.-L. Wu et al., 2018). Rifamycin resistance is acquired by a mutation in the rpoB gene encoding the β-subunit of RNA polymerase resulting in a blockage of RNA synthesis (Levin & Hatfull, 1993; Miotto et al., 2017). These mutations have been reported in MAC and *M. kansasii* (Brown-Elliott et al., 2012; Klein et al., 2001; Obata et al., 2006). The expression of *arr* gene in *M. smegmatis* and *M. abscessus* it is also associated with a reduced efficacy of rifamycins such as rifampicin and rifabutin (Baysarowich et al., 2008; Quan et al., 1997).

2

NONTUBERCULOUS MYCOBACTERIA IN PORTUGAL: TRENDS FROM THE LAST DECADE

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Personal Contribution

SC contributed to the design of the study, experimental work, literature review and wrote the manuscript

2.1 Abstract

Introduction and objectives: Nontuberculous mycobacteria (NTM) are opportunistic human pathogens found in the environment. The transmission seems to be associated with inhalation of aerosol droplets, ingestion or trauma events. Recent studies indicate that NTM disease is increasing worldwide, however, the true clinical impact of NTM infections is difficult to determine due to challenges in discriminating between disease and colonization as they are ubiquitous in the environment. In addition, understanding the epidemiology of NTM is difficult and has not yet been established. In this work, we used a country NTM representative collection from the National Reference Laboratory for Tuberculosis (NRL-TB) of the National Institute of Health (INSA), to characterize the circulation trends of NTM species in Portugal and the most affected regions, contributing to a better understanding of the NTM epidemiology.

Material and methods: We conducted a nationwide retrospective study where all individuals with positive NTM cultures at the NRL-TB of the INSA from 2014 to December 2020 were included. Positive cultures were identified using *GenoType Mycobacterium CM/AS* (Hain Lifescience) according to manufacturer's instructions, or *hsp65* DNA sequencing as previously described. Social-demographic data from patients were also analyzed and patients classified into 3 groups according only to microbiological data, "definite NTM disease", "NTM colonization" and, "possible NTM disease".

Results: In the period 2014-2020, the NRL-TB performed 50397 cultures. Among these, 1118 cultures were NTM positive retrieved from 944 patients. Most of our cases were in patients whose mean age was 64±15.9 years, and no significant differences between gender was observed, although more frequent in male patients. Overall, from the 944 cases, we were able to identified 93 "definite NTM disease" cases and 79 "possible NTM disease". *Mycobacterium avium* complex (MAC) (40,8%), *Mycobacterium abscessus-chelonae* complex (MABC) (9,6%) and *Mycobacterium fortuitum* (6,3%) were responsible for most of the infections. The geographical distribution of NTM cases varied significantly and was possible to observe that was independent of population density. The region were most cases occurred was Lisbon Metropolitan Area (31,9%), followed by North (25,3%) and Centre (24,4%), however North region has the highest number of "definite NTM disease" cases (n=33).

Conclusions: This is the first national wide epidemiological study on this subject, contributing to a better understanding of NTM dynamics in Portugal. MAC was the NTM species

responsible for the majority of infections and, LMA the region with the highest number of cases. It was also possible to conclude that the number of NTM isolates is independent of the demography of the region

2.2 Introduction

Nontuberculous mycobacteria (NTM) represent a group of environmental bacteria that are commonly found in soil and water and that belong to the genus *Mycobacterium* (Falkinham, 2016; Griffith et al., 2007; Primm et al., 2004). Until recently, NTM were considered transient colonizers in humans, but their association with disease is now well recognized and accepted as a growing problem. It predominantly occurs in immuno-compromised individuals and patients with underlying disease such as bronchiectasis and cystic fibrosis (Honda et al., 2015; Prevots et al., 2017). Diseases caused by NTM can be very diverse, with pulmonary and extrapulmonary manifestations, and can also mimic infections caused by *Mycobacterium tuberculosis*. Repeated exposure to environments colonized by NTM is thought to be the mode of transmission, with inhalation of contaminated aerosols or inoculation by trauma being the usual forms of infection (Honda et al., 2018). Outbreaks due to NTM are also reported in the literature (Ahmed et al., 2020; Buser et al., 2019; Lyman et al., 2017). The number of infections by NTM is increasing worldwide, which is mainly associated with an aging population, a rise in the proportion of immunosuppressed patients, an increased awareness of the disease, and the development of improved culture techniques (Hermansen et al., 2017; Johnson & Odell, 2014). However, it is difficult to assess the true clinical impact of NTM infections due to the difficulty in discriminating b tween disease and colonization. Also, as NTM disease is not notified, data are not routinely collected, and incidence rates are based mainly on the numbers of NTM isolated. Although several epidemiological studies have already been carried out (Hermansen et al., 2017; Hoefsloot et al., 2013; Rindi & Garzelli, 2016; Spaulding et al., 2017; J. Wu et al., 2014), they lack geographic and temporal representation, not allowing a characterization of the distribution of species or origin of infection. Thus, epidemiological surveillance studies are mandatory in order to provide local epidemiological data useful for the management of patients. In Portugal, there are very few studies on NTM infections (Durão et al., 2019; Hoefsloot et al., 2013; Rocha et al., 2020) and incidence and prevalence of the disease is still unknown. Although a laboratory surveillance network for tuberculosis is already established, including laboratories with heterogeneous competences (peripheral, hospital, private sector

and the National Reference Tuberculosis Laboratory - NRL-TB), this network is not being currently used for NTM detection and management. Consequently, the identification of NTM infections is affected and is dependent on the importance given to certain clinical symptoms or cultural isolations. Since the awareness of these infections is rising, the NRL-TB continuously receives suspicious clinical samples or culture isolates from laboratories that do not have the capability to perform NTM identification without an official recommendation or guideline. Using the large and country representative NTM collection that is centralized in the NRL-TB of the Portuguese National Institute of Health, including diverse associated metadata, we aim to characterize the circulation trends of mycobacterial species in Portugal. This will contribute to a better understanding of NTM epidemiology and, eventually, to the development of guidelines to identify and manage NTM infections

2.3 Materials and methods

2.3.1 Data source and study design

This is a nationwide retrospective study enrolling samples and data retrieved at the NRL-TB of the Portuguese National Institute of Health (INSA), from all patients with a positive NTM culture from 2014 to December 2020. NTM positive cultures were defined as those with at least one mycobacterial culture growing a NTM species, independently of the body site that was sampled for culture. Only the first positive NTM test per patient in the dataset was analyzed. If a patient was culture-positive for more than one NTM species during the study period, both NTM were included. We classified all mycobacterial species into 14 groups: Mixed (that includes all samples in which more than one NTM species was identified), *Mycobacterium abscessus-chelonae* (MABC), *M. fortuitum*, *M. genavense*, *M. gordonae*, *M. kansasii*, *M. lentiflavum*, *M. mucogenicum*, *M. scrofulaceum*, *M. simae*, *M. xenopi*, *Mycobacterium avium* complex (MAC), *Mycobacterium spp*, and, others. Social-demographic data include age, gender, region of requesting hospital and local of residence of the patients. Geographic regions were defined according to Portuguese NUTS (Nomenclature of Territorial Units for Statistics by Eurostat). Mainland Portugal is divided in 5 regions at NUT II level, namely the North, Centre, Lisbon Metropolitan Area (LMA), Alentejo and Algarve, enrolling a total of 18 districts. NTM species identification was carried out using *GenoType Mycobacterium CM/AS* (Hain Lifescience) according to manufacturer´s instructions, or hsp65 DNA sequencing as previously described (Saifi et al., 2013).

2.3.2 Case definition

Since the NRL-TB does not have access to clinical data, classification of NTM disease was only based on microbiological data using previously validated definitions described elsewhere.3,10,19 Accordingly, patients were classified to have "definite NTM disease" if they had one of the following: (1) more than three positive specimens; (2) three positive specimens, including at least one obtained by bronchoscopy; (3) one positive sample from a biopsy from any body site. Cases were considered as "NTM colonization" if they had only one positive sample (exception for biopsies); the remaining patients were classified as "possible NTM disease" (Andréjak et al., 2010; Griffith et al., 2007; Hermansen et al., 2017).

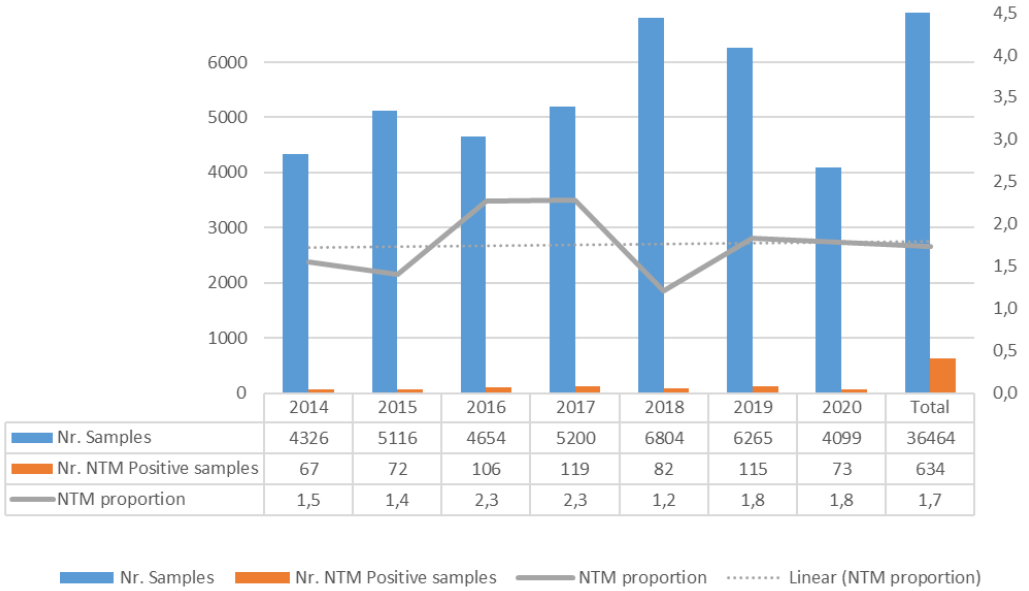
2.3.3 Statistics

The quantitative variables associated with data from patients and cultures were described as mean and standard deviation, whereas the qualitative variables were described as numbers and percentages. Means and proportions were compared with the t Student and $\chi 2$ tests, respectively. The p values $<.05$ were considered significant.

2.4 Results

During the study period, the NRL-TB performed 50397 cultures. Of these, 4296 (8.5%) were culture positive for mycobacteria, from which 1118 (26%) were NTM positive retrieved from

944 individuals. The annual number of positive NTM isolations in the Portuguese population remained stable during this period (Figure 2.1).



	2014	2015	2016	2017	2018	2019	2020	Total
Nr. Samples	4326	5116	4654	5200	6804	6265	4099	36464
Nr. NTM Positive samples	67	72	106	119	82	115	73	634
NTM proportion	1,5	1,4	2,3	2,3	1,2	1,8	1,8	1,7

Figure 2.1 - Number of positive NTM isolations in Portugal during 2014-2020.

The majority of the isolates (96.4%, n = 910) were identified to the species/complex level and a total of 24 different NTM species were encountered. Figure 2.2 present the frequency of NTM species isolated. For seven patients (0.74%) more than one NTM species was isolated in same sample.

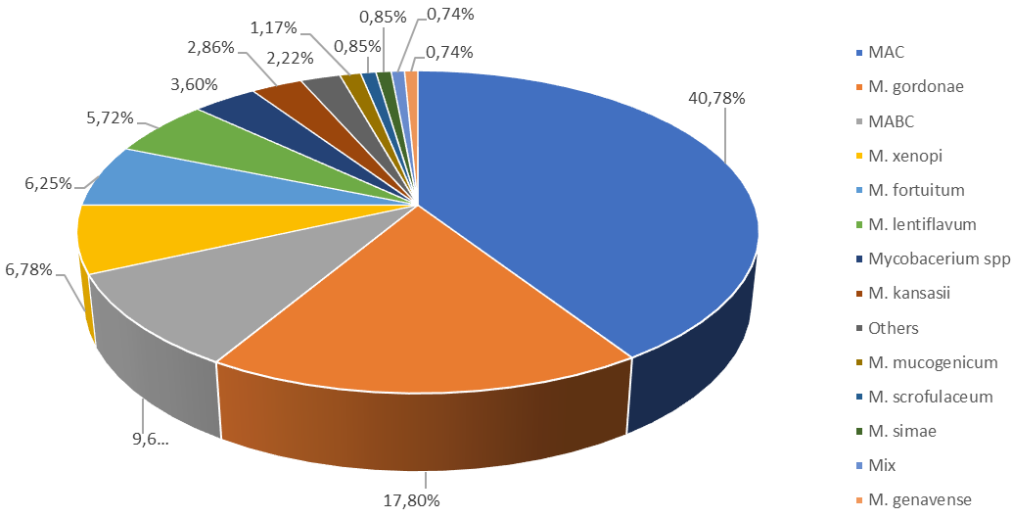


Figure 2.2 - Distribution of NTM species isolated from patients in Portugal from 2014-2020. *Others refer to the following species: *M. asiaticum, M. goodii, M. interjectum, M. malmoense, M. paraffinicum, M. peregrinum, M. shimoidei, M. terrae complex, M. triplex, M. triviale, and M. marinum.*

The five most commonly identified species in the Portuguese population were MAC (n = 385, 40.8%), *M. gordonae* (n = 168, 17.8%), MABC (n = 91, 9.6%), *M. fortuitum* (n = 59, 6.3%) and *M. lentiflavum* (n = 54, 5.6%). These five species accounted for 79.9% of all NTM identified. Using microbiological criteria, 93 (9.8%) of the cases were classified as "definite NTM disease", 79 (8.4%) as possible "NTM disease" and 772 (81.8%) as "NTM colonization" (Table 2.1).

Table 2.1 - Frequency of NTM according to disease category, age and gender.

NTM species	Total	Definite NTM disease	Possible NTM disease	Colonization	Age Mean±SD	Gender male (%)
MAC	385	61	35	289	64,8±14,5	53,2
MABC	91	16	2	73	61,0±18,9	56
M. fortuitum	59	3	3	53	63,7±17,0	64,4
M. gordonae	168	0	11	157	65,2±16,7	62,5
M. lentiflavum	54	2	8	44	66,0±17,3	50
Others	187	11	20	156	63,0±15,5	68,4
Total	944	93	79	772	64,1±15,9	58,6

*MAC - Mycobacterium avium complex

**MABC - *Mycobacterium abscessus-chelonae complex*

Regarding the infection site, 475 were pulmonary samples, 15 were extrapulmonary and for 628 samples no data was available. Overall, NTM were most frequently isolated in men, but male to female ratio varied according to the species isolated. MAC was the responsible for the majority of "definite NTM disease" cases, followed by MABC (Figure 2.2, Table 2.1).

The median patient age was 64.1±15.9 years old. Age-standardized rates did not reveal significant differences between age groups (Table 2.2).

Among the adult population aged 15 years and above, most of the NTM patients identified were in the 65 age group (n = 500; 53%). The majority of the cases were in male patients (n = 553; 58.6%) and MAC was the most prevalent NTM isolated (n = 385; 40.8%).

We did not observe definite cases in children under 15 years old (Table 2.2) and three out of the four cases in this age group were caused by *M. gordonae*. *M. lentiflavum*, which was also a frequent NTM isolated (5.6%), originated two definitive cases in two elder male patients (>70 years old) from LMA and North regions.

Table 2.2 - Distribution of NTM positive cases by age and gender.

Age group	NTM positive Patients (n)	Gender male	(%)	Definite cases (n) (all NTM)	Most Prevalent NTM (%)
< 15 yr	4	75		0	M. gordonae (50)
15-19 yr	3	0		1	MABC** (33.3)
20-24 yr	9	33.3		2	MAC* (44.4)
25-29 yr	8	37.5		3	MABC** (62.5)
30-34 yr	25	64.0		3	MAC* (40.0)
35-39 yr	21	52.4		2	MAC* (52.4)
40-44 yr	42	59.5		2	MAC* (28.,6)
45-49 yr	58	72.4		6	MAC* (46.5)
50-54 yr	63	60.3		7	MAC* (38.0)
55-59 yr	91	56.0		13	MAC* (45.0)
60-64 yr	92	57.6		10	MAC* (41.3)
≥65 yr	500	59.0		43	MAC* (41.6)
unkown	28	44.8		1	MABC** (20.6)
Total	944	58.6		93	MAC* (40,7)

*MAC - Mycobacterium avium complex

**MABC - *Mycobacterium abscessus-chelonae complex*

Distribution of NTM disease differed significantly by geographic area. The majority of the cases occurred in LMA (31.9%) followed by North (25.3%) and Centre (24.4%) regions (Figure 2.3, Figure 2.4).

Algarve and Alentejo accounted for 6.3% and 3.7% of the cases and the autonomous regions of Madeira and Azores for 0.7% and 2.0%, respectively (data not shown). A fine-tuned analysis by region allowed the identification of some districts with higher number of cases, such as Setubal (21.3%) and Lisbon (10.6%) (both from LMA), Viseu (9.6%) (Centre), and Vila Real (9.2%) (North) (Figure 2.3). Given that we observed that the regions with the highest number

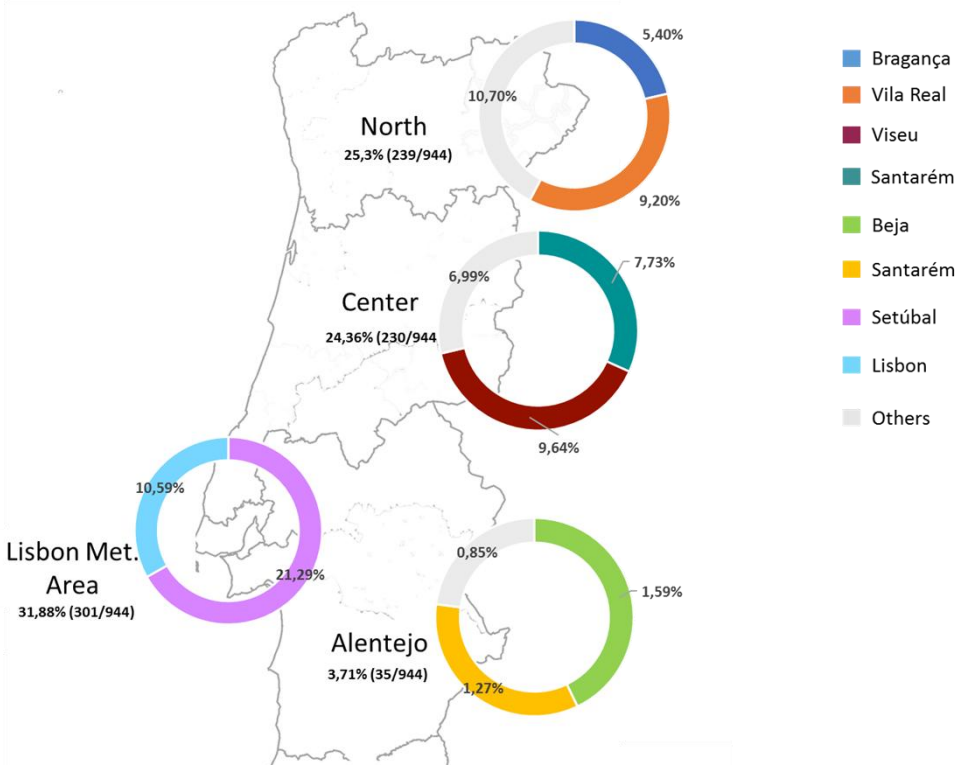
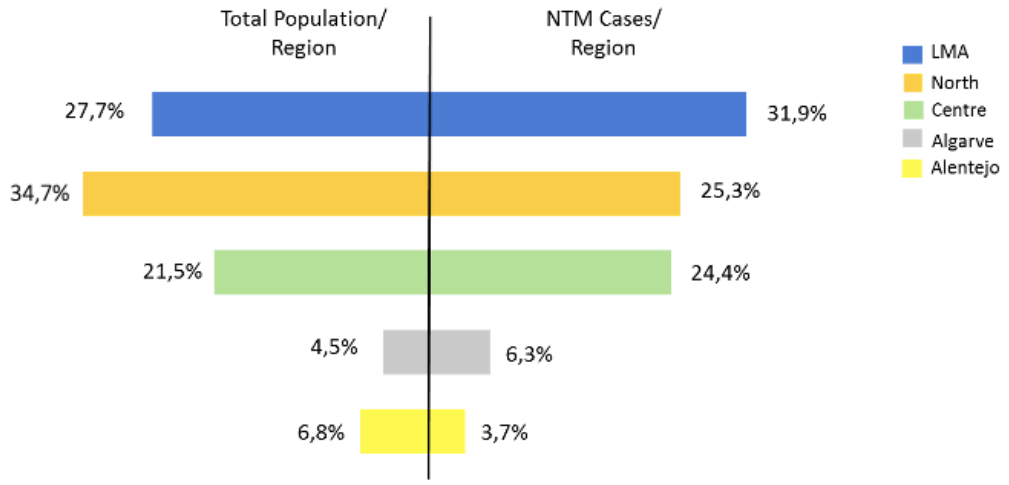


Figure 2.3 - Distribution of NTM cases by Portugal mainland between 2014-2020.

of NTM cases corresponded to those with the highest population density (North, LMA and Center), we tested the hypothesis of association between these variables and concluded that they are not associated. In fact, for example, the North region, which has a higher population density than the LMA region, registered significant fewer cases of NTM ($p < .05$) than the latter, suggesting that cases of NTM are independent of demographics (Figure 2.4). Another highly illustrative example stands for the comparison between Alentejo and Algarve regions, where the former has a higher population density but a considerably lower number of NTM cases ($p < .05$). As already mentioned (Table 2; Figure 2.1) MAC (40.8%), MABC (9.6%) and *M.*



Region	Nr of Population	%	Nr of NTM cases	%
LMA	2871133	27,7%	301	31,9%
North	3588701	34,7%	239	25,3%
Centre	2227912	21,5%	230	24,4%
Algarve	467495	4,5%	59	6,3%
Alentejo	704934	6,8%	35	3,7%
Total (Portugal)	10347892		944	

Figure 2.4 - Demography of the NTM cases analyzed; comparison of the total number of population (left side of the figure) with the number of NTM cases (right side of the figure) by region.

fortuitum (6.3%) were the NTMs that showed more association with disease. To better understand the dynamics of these infections, we analyzed these three groups of NTM individually. All of these were mainly found in male patients over 35 years old, with a predominance in the 65 age group. MAC was more frequent in the North region (38.4%) while MABC and *M. fortuitum* were predominant in LMA (Table 2.3). North region showed the highest number of "definite NTM cases" (n = 33) (table 2.4).

Table 2.3 - Number of patients infected with MAC (*Mycobacterium avium* complex), MABC (*Mycobacterium abscessus-chelonae* complex) and *M. fortuitum* by gender and region of isolation.

	MAC N (%)	MABC N (%)	*M. fortuitum* N (%)
Total	385 (40.8%)	91 (9.6%)	59 (6.3%)
Age category			
< 15 yr	0 (0%)	1 (1.1%)	0 (0%)
15-34 yr	14 (3.6%)	8 (8.8%)	3 (5.1%)
35-64 yr	152 (39.5%)	27 (28.6%)	22 (37.3%)
≥ 65 yr	211 (54.8%)	49 (53.8%)	33 (55.9%)
Unknown	8 (0.26%)	6 (6.6%)	1 (1.7%)
% male	205 (53.2%)	50 (54.9%)	38 (64.4%)
Region			
North	148 (38.4%)	16 (17.6%)	9 (15.3%)
Centre	87 (22.6%)	18 (19.8%)	14 (23.7%)
LMA	97 (25.2%)	29 (31.9%)	19 (32.2%)
Alentejo	13 (3.4%)	5 (5.5%)	1 (1.7%)
Algarve	12 (3.1%)	10 (11%)	8 (13.6%)
Açores	7 (1.8%)	3 (3.3%)	6 (10.2%)
Madeira	2 (0.5%)	1 (1.1%)	0 (0%)
Unknown	19 (4.9%)	9 (9.9%)	2 (3.4%)

Table 2.4 - Distribution of the most prevalent NTM species by case classification and region of isolation.

	North	Centre	LMA	Alentejo	Algarve	Açores	Madeira
Total infections (N)	239	230	301	35	59	19	7
MAC* N (%)	148 (61.9)	87 (37.8)	97 (32.2)	13 (37.1)	12 (20.3)	7 (36.8)	2 (28.6)
MABC** N (%)	16 (6.7)	18 (7.8)	29 (9.6)	5 (14.3)	10 (16.9)	3 (15.8)	1 (14.3)
M. fortuitum N (%)	9 (3.8)	14 (6.1)	19 (6.3)	1 (2.9)	8 (13.6)	6 (31.6)	0
Definite Cases (N)	33	14	28	3	5	6	1

*MAC - Mycobacterium avium complex

**MABC - *Mycobacterium abscessus-chelonae complex*

2.5 Discussion

In order to describe the trends in the circulation of nontuberculous mycobacterial species in Portugal, we analyzed NTM isolates from patients who resorted to a Portuguese hospital between 2014 and 2020 and had microbiological species identification at the NRL-TB at INSA. This is the first national wide epidemiological study on this subject. More than 50 000 cultures were analyzed, yielding 1118 NTM positive samples from 944 patients. The annual number of NTM isolations presented only slightly differences between 2014 and 2020 (figure 2.1), which

is expected for countries with a low TB incidence (Freeman et al., 2007; Hermansen et al., 2017). In Portugal, TB incidence has been steadily declining since 2000 (Direção Geral da Saúde, 2020). Nevertheless, the increasing number of NTM described worldwide (Y.-M. Lee et al., 2021; Rindi & Garzelli, 2016; J. Wu et al., 2014) is justified by an aging population, comorbidities, and enhanced clinical and laboratory capabilities (Hermansen et al., 2017; Hoefsloot et al., 2013; Prevots et al., 2017; Rindi & Garzelli, 2016; Spaulding et al., 2017). Based on our dataset, the most commonly represented disease-associated species in Portugal is MAC (40.8%) followed by MABC (9.6%) and *M. fortuitum* (6.3%). It should be noted that *M. gordonae* also stands out for its considerable representation (17.8%). These results are in line with a previous NTM-Net European study enrolling samples isolated in 2008, including data from Portugal, which showed that MAC (40%) was predominant, followed by rapid growers (MABC and *M. fortuitum*, 22.0%) and *M. gordonae* (14%) (Hoefsloot et al., 2013). Despite MAC and MABC are well known pathogenic NTM, most of our cases appear as "NTM colonization". This may be explained by the lack of clinical information that, especially for these two NTM species, could be sufficient to reclassify these cases as "possible NTM disease". Based on our experience, a single isolation of one of these agents is sufficient for the treatment decision, which leads us to believe that symptoms and radiological evidence suggestive of NTM infection were present. On the other hand, although *M. gordonae* is considered a non-pathogenic environmental "contaminant" (Griffith et al., 2007; R. M. Thomson & Yew, 2009; Winthrop & Roy, 2020), in our dataset it was responsible for 11 cases of "possible NTM disease". Again, the classification of these cases is hampered by the absence of clinical information, which would be fundamental for their potential reclassification. In fact, recent literature has demonstrated that *M. gordonae* could be pathogenic, causing infection, not only in immunocompromised hosts, but also in immunocompetent patients (Chang et al., 2021). To fully understand the dynamics of these infections in our population, clinical based studies of NTM infections are crucial. As described in other studies, (Andréjak et al., 2010; Hermansen et al., 2017; Mirsaeidi, Farshidpour, Allen, et al., 2014) most of the cases enrolled in the present study were male patients and the median patient age was 64.1±15.9 years. This finding reinforces the fact that NTM disease is related with older patients with possible associated comorbidities. However, our study lacks this information, since we do not have access to clinical data of the patients. The number of patients under 15 years old was low (n = 4) and we did not find any definite or possible NTM disease case. We observed that the distribution of NTM differed significantly by geographic area and

was not dependent on the population density ($p < .05$). LMA enrolled the majority of the cases, followed by North and Centre. This asymmetric distribution of NTM cases was also observed at the district level. For example, Porto, which is the second Portuguese district with the most population, shows up ranked in 8th place in terms of NTM cases, with only 3.4% of the cases. Setubal, Lisbon, Viseu and Vila Real were the Portuguese districts with the highest number of cases (21.3%, 10.6%, 9.7% and 9.2% of the cases, respectively). As human-to-human transmission is not considered to be the main cause of NTM spread (Griffith et al., 2007; Le Dantec et al., 2002), and given that in our dataset the number of cases was independent of demography, further studies are needed to identify possible NTM environmental reservoirs, human risk factors, such as associated pathologies (cystic fibrosis, HIV/AIDS and other immunosuppressive diseases/therapy) or health care access issues. Case definition for NTM disease is still not consensual as there are no universal guidelines for its classification. Normally, the American Thoracic Society (ATS) guidelines for NTM management are used (Griffith et al., 2007). They rely on both clinical and laboratory data for definition of a NTM disease case, and laboratory data alone is not sufficient to differentiate between disease and colonization. The majority of the published studies on this subject lack clinical information as they are performed by reference laboratories. As such, for classification purposes, guidelines have been adapted, using larger datasets and combinations of different types of samples collected for analysis (respiratory, biopsies, etc) (Andréjak et al., 2010; Hermansen et al., 2017; Marras et al., 2013; Prevots et al., 2017). We opted for this approach and defined three categories based on the type and number of patient samples collected: "definite NTM disease", "possible NTM disease" and "NTM colonization". In Portugal, most of the cases enrolled in this study (772 out of 944) were classified as colonization, regardless of pathogenicity, as we only had one positive isolation. Since there are no universal guidelines, or even national guidelines or recommendations, for diagnosis and follow-up of NTM patients, the majority of these cases were not further investigated. Thus, they were clinically undervalued, or treated accordingly, based on the NTM identification on a single sample. On the other hand, most of NTM cases are initially screened for TB and positive cultures that were negative for *M. tuberculosis* are disposed without further analysis. This absence of standardization in NTM screening and lack of awareness is also of prejudice for NTM management. Another problematic issue of NTM disease is that it is not a notifiable disease, which hampers a proper determination of its incidence and prevalence. As such, the real burden of NTM disease is underestimated worldwide although some countries are able to gather NTM cases from its TB programs (Ahmed et al., 2020; Shah et al., 2016). From

our point of view, this relatively simple approach could be the starting point for the establishment of a more robust surveillance system. In Portugal, the TB surveillance system (clinical and laboratory based), already established since 1995 (Direção Geral da Saúde, n.d.), could be used for this purpose, thus contributing to a more comprehensive view of the NTM scenario, and providing a better categorization of the cases. In summary, our study contributed to a better understanding of the dynamics of the NTM infections in Portugal. From 2014 to 2020 about 1000 NTM isolations were identified, which allowed us to classify 172 of the cases as "definite NTM disease" or "possible NTM disease", mostly among patients over 65 years old. We also observed that the occurrence of NTM cases was not dependent on the population density and may be associated with still undisclosed environmental determinants and/or population health factors. We highlight the need for the establishment of a systematic approach to diagnose NTM disease and uniform reporting to assess the real NTM disease epidemiology. Future studies are needed to address species-specific environmental niches, the genetic variability of the circulating strains and the identification of species and resistance markers as a way to enhance NTM importance as an emerging health problem.

2.6 Final considerations

The data available in published studies are predominantly derived from centralized national laboratory reports, and the trends in NTM disease/ infection remain heterogenous (Dahl et al., 2022). Despite these limitations, there has been a global rise in the number of NTM Infections/ disease, being the most commonly reported NTM species those belonged to MAC and MABC complex (Dahl et al., 2022; Marshall et al., 2024).

According to the study mentioned above, in Portugal, MAC and MABC are the species most commonly responsible for human's disease. The same was observed based on data collected between 2021 and August 2024, a period not covered by the published study. During this period, the LNT-TB in Lisbon received 426 positive NTM cultures, of which 186 identified as MAC species and 78 as MABC species.

Contrary to most data in the literature, which suggest rising case numbers, our findings show a stable trend In Portugal (139 cases in 2021, 100 in 2022, 102 in 2023 and, until August, we counted with 85 cases in 2024). However, it is Important to note that these figure may be influenced by the fact that NTM disease is not notifiable, making it difficult to access the real data. Furthermore, the reported cases reflect only those diagnosed by the LNR-TB in Lisbon.

Due to the observed high number of cases observed especially in countries where TB rates are declining, it is crucial to understand the dynamic of these opportunistic bacteria (Hermansen et al., 2017).

NTM infections are not restricted to specific geographic regions or populations, though they are most prevalent among immunosuppressed patients and older population (Bhanushali et al., 2023; Hermansen et al., 2017). The differences in incidence rates of NTM disease may be the result of different complex interplay of genetic, environmental and health system-related issues (Maurya et al., 2015). This highlights the urgent need to understand how environmental determinants, genetic predispositions, and the timing of diagnosis/ and beginning of therapy can be critical for the prevention and management of these Infections (Spaulding et al., 2017).

To better understanding the dynamics of NTM infections, it is necessary to first assess the characteristics of the circulating strains. For this purpose, we conducted a detailed analysis of the species within the two complex most responsible for causing disease: MABC and MAC.

3

MYCOBACTERIUM ABSCESSUS COMPLEX

Personal Contribution

SC contributed to the design of the study, experimental work, literature review and wrote the manuscript

3.1 General Introduction to the chapter

The *Mycobacterium abscessus complex* (MABC) was initially comprised by the *M. abscessus* and *M. chelonae* species but, developments in molecular biology, allowed the recognition of *M. abscessus* as a single species (Lopeman et al., 2019). Nowadays, MABC is composed by three subspecies, *M. abscessus* subsp. *massiliense, M. abscessus* subsp. *bolletii* and, *M. abscessus* subsp. *abscessus* (Tortoli et al., 2016). The incidence of these opportunistic pathogens are increasing worldwide, with particular relevance in patients with cystic fibrosis (Johansen et al., 2020; B. E. Jönsson et al., 2007). MABC transmission occurs predominantly through contaminated environmental sources, however, person-to-person transmission also appears to occur (Aitken et al., 2012; Ruis et al., 2021). Inhalation of aerosols or contact with contaminated medical equipment has been identified as one of the main routes of transmission, especially in hospital and healthcare environments (Bryant et al., 2016; Viana-Niero et al., 2008).

According to the latest 2020 ATS/ERS/ESCMID/IDSA guidelines, treatment for MABC disease should be based on susceptibility to macrolides. In this case, a combination of one or two drugs such as amikacin (AMK), imipenem (IMP), cefoxitin (CFX) or tigecycline (TGC) is advised, along with two oral drugs such as azithromycin (AZM), clofazimine (CFZ) and linezolid (LZD), with the addition of inhaled AMK. For macrolide-resistant MABC strains, the guidelines recommend a combination of two or three injectable drugs (AMK, IMP, CFX, TGC) and two or three oral drugs (usually CFZ and LZD, and possibly others, depending on susceptibility), also adding inhaled AMK (Daley et al., 2020). However, the successful treatment of MABC infections is largely hindered by the acquired and intrinsic antibiotic resistance characteristics of this opportunistic bacteria (Griffith, 2011; van Ingen et al., 2012; Victoria et al., 2021). Furthermore, MABC can assume two morphotypes: smooth, when the strains begin to colonize the lung epithelium and form biofilms; and rough, when the strains acquire a more virulent variant and cause invasive lung infections (Degiacomi et al., 2019; T. Q. Nguyen et al., 2024).

In light of the above, correct identification of resistance and the infecting species are essential in order to optimise treatments and mitigate the impact of NTM infections. Furthermore, it is imperative to improve reporting and surveillance systems in order to optimize the access of clinical data to better understand transmission and resistance dynamics. The future of managing these infections will likely depend on new approaches and enhanced collaboration between public health authorities, clinicians and researchers.

In order to maintain the original structure of the scientific publications that are included in this thesis, we will use the different nomenclatures *Mycobacterium* and *Mycobacteroides* to refer to the species *Mycobacterium abscessus* since both are accepted for the taxonomic definition of this complex.

This chapter was adapted from the following published documents

Santos, A., Pinto, M., **Carneiro, S**., Silva, S., Rodrigues, I., Munhá, J., Gomes, J. P., & Macedo, R. (2023). Microevolution of a *Mycobacteroides abscessus subsp. bolletii* strain in a clinical persistent infection. Infection, Genetics and Evolution, 112, 105437. https://doi.org/10.1016/j.meegid.2023.105437

3.2 Microevolution of *Mycobacteroides abscessus subsp. bolletii* strain in a clinical persistent Infection - Abstract

Mycobacteroides abscessus complex (MABC), a fast-growing nontuberculous mycobacterium, is emerging as a significant infectious disease threat, due to both intrinsic and acquired resistance mechanisms to antibiotics and disinfectants and the need for extensive and multidrug regimens for treatment. Despite the prolonged regimens, outcomes are poor and persistence cases have been reported. Here, we describe clinical, microbiologic and genomic features of a *M. abscessus subsp. bolletii* (*M. bolletii*) strain consecutively isolated from a patient within an eight-year infection period. From April 2014 to September 2021, the National Reference Laboratory for Mycobacteria received eight strains isolated from a male patient. Species identification, molecular resistance profile and phenotypic drug susceptibility were determined. Five of these isolates were recovered for further in-depth genomic analysis. Genomic analysis confirmed the multidrug resistant pattern of the strain and also other genetic changes associated with adaptation to environment and defence mechanisms. We highlight the identification of new mutations in locus MAB_1881c and in locus MAB_4099c (*mps1* gene), already described as associated with macrolides resistance and morphotype switching, respectively. Additionally, we also observed the emergence and fixation of a mutation in locus MAB_0364c that appeared at a frequency of 36% for the 2014 isolate, 57% for the 2015 isolate and 100% for the 2017 and 2021 isolates, clearly illustrating a fixation process underlying a microevolution of the MAB strain within the patient. Altogether these results suggest that the observed genetic alterations are a reflection of the bacterial population's continuous adaptation and survival to the host environment during infection, contributing to persistence and treatment failure.

3.2.1 Introduction

The species *Mycobacteroides* (or *Mycobacterium*) *abscessus* (MABC) is a fast growing nontuberculous mycobacterium (NTM) known to be associated with an increasing number of disease cases worldwide (Johansen et al., 2020). It is particularly difficult to eradicate, due to both its intrinsic resistance mechanisms (i.e., innate, not acquired during the course of antibiotic exposure) to antibiotics and disinfectants and to the need for extensive and multidrug regimens for treatment. Although transmission routes are not yet well established and MAB infections are

assumed to be acquired from the environment, recent studies demonstrate that most individuals are infected with one of several dominant circulating clones, which suggests a possible global transmission (Diricks et al., 2022; Ruis et al., 2021). How these clones emerged and spread throughout the globe is still unclear, and person-to-person transmission presumably through indirect mechanisms is a possibility (Aitken et al., 2012; Bryant et al., 2013; Ruis et al., 2021). Genomic studies are contributing to a better understanding of this emerging species, mostly on its epidemiology, antimicrobial resistance and adaptation mechanisms. Moreover, these studies recently validated the subdivision of MAB complex into three subspecies: 1) *M. abscessus subsp. abscessus* (*M. abscessus*), the most common subspecies of the complex; 2) *M. abscessus subsp. massiliense* (*M. massiliense*) and 3) *M. abscessus subsp. bolletii* (*M. bolletii*) (Victoria et al., 2021). All of these subspecies are opportunistic pathogens and although genetically related, they show differences in intrinsic drug resistance, association with clinical disorders and mode of transmission (Griffith, 2014; Lewin et al., 2021; Tan et al., 2017). For example, *M. bolletii* is unfrequently isolated from clinical samples, but is often associated with multidrug resistance (Tan et al., 2017). Nevertheless, bacteria from the MAB complex are considered one of the most resistant mycobacteria, and the majority of the strains harbour a highly dynamic open pan-genome that may also contribute to their evolution and adaptation to stressful environments including within the host (Johansen et al., 2020; Victoria et al., 2021). The manifestation of the disease is the reflex of complex interactions between host (immune status, genetic risk factors and prior lung disease), pathogen (pathogenicity and virulence) and environmental determinants such as the infecting dose and duration of exposure (Johansen et al., 2020). MAB often cause severe respiratory, skin, and mucosal infections and have recently emerged as major pathogens, greatly affecting people with cystic fibrosis (Griffith et al., 2007; Koh, Jeong, et al., 2017). In this context, a correct and early identification of the MAB subspecies, with clinical, radiological and laboratory findings is essential for the management of the disease. Since the symptoms are non-specific there is often a delay in diagnosis, resulting in disease progression and eventually leading to more complicated treatments. These regimens require the prolonged use of multiple drugs, which is costly and may cause severe adverse reactions for the patient as these drugs usually show high toxicity (Griffith et al., 2007; Koh, Jeong, et al., 2017). For MAB-complex associated infections, the American Thoracic Society has recommended an empiric regimen comprising a combination of a macrolide [clarithromycin

(CLR) or azithromycin (AZM)], an aminoglycoside [amikacin (AMK)], and a β-lactam [cefoxitin (FOX) or imipenem], for a period of culture-free for more than one year (Griffith et al., 2007). Although it is assumed that nearly 50% of the disease cases are successfully resolved (Johansen et al., 2020; Kreutzfeldt et al., 2013), with the patient showing a clearance of both symptoms and microbiological isolations of the agent, cases of disease persistence have been reported (Esther et al., 2010; Harris et al., 2012; Kreutzfeldt et al., 2013; Lewin et al., 2021). In the present study, we aim to describe clinical, microbiologic and genomic features of a *M. bolletii* strain consecutively isolated from a patient within an eight year infection period. The analysis of the long-term genomic evolution of this strain may contribute to a better understanding of the mechanisms underlying the persistence of these infections.

3.2.2 Materials and methods

2.3.2.1 Clinical case description

In the beginning of 2013 (Figure 3.1), a 63 year old male patient attended to a hospital consultation presenting moderate respiratory symptoms that included cough, bloody sputum and atypical chest pain. Clinical history included a benign prostatic hyperplasia and pulmonary tuberculosis (PTB) at the age of 7.

After imaging exams, he was referred with lower left lobe bronchiectasis. Bronchoalveolar lavage analysis from March 2013 revealed the presence of Alcohol-Acid Fast Bacilli, and the patient was clinically diagnosed with PTB and treated accordingly (i.e., two months of isoniazid PO 250 mg/day, rifampicin PO 500 mg/day pyrazinamide PO 1250 mg/day and ethambutol PO 750 mg/day – HRZE, followed by four months of isoniazid PO 250 mg/day and rifampicin PO 750 mg/day – HR). Of note, M. tuberculosis isolation in culture was never achieved. After PTB treatment completion, in April 2014, a follow-up sputum sample was collected leading to the first isolation of *M. bolletii*. The worsening of the symptoms (fatigue, cough, purulent bloody sputum, atypical chest pain and bronchiectasis in the lower left and upper right lobes) led to

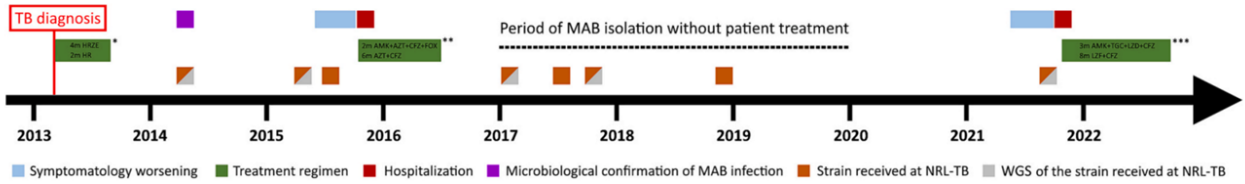


Figure 3.1 - Timeline of the persistent *Mycobacteroides abscessus subsp. bolletii* infection from 2013 to 2022. *Four months isoniazid PO (250 mg/day), rifampicin PO (500 mg/day), pyrazinamide PO (1250 mg/day), ethambutol PO (750 mg/day), followed by two months isoniazid PO (250 mg/day), rifampicin PO (500 mg/day); **Two months amikacin IV (750 mg/day), azithromycin PO (500 mg/day), clofazimine PO (100 mg/day), cefoxitin IV (12 g/day), followed by six months azithromycin PO (500 mg/day), clofazimine PO (100 mg/day); ***Three months amikacin IV (750 mg/day), tigecycline IV (50 mg/day), clofazimine PO (100 mg/day), linezolid PO (600 mg/day), followed by seven months linezolid PO (600 mg/day), clofazimine PO (100 mg/day). TB – tuberculosis; MAB – *Mycobacteroides abscessus subsp. bolletii*; WGS – Whole-genome sequencing; NRL-TB – National Reference Laboratory for Mycobacteria. HRZE – Isoniazid, rifampicin pyrazinamide and ethambutol; HR – Isoniazid and rifampicin; AMK – amikacin; AZT – azithromycin; CFZ – clofazimine; FOX – cefoxitin; TGC – tigecycline; LZD – linezolid.

hospitalization in November 2015 where he was first treated for a MAB infection (Figure 3.1). The treatment, that included AMK IV 750 mg/day, AZM PO 500 mg/day, clofazimine (CFZ) PO 100 mg/ day and FOX IV 12 g/day during hospitalization (two months) followed by six months of AZM PO 500 mg/day and CFZ PO 100 mg/day in ambulatory (Figure 3.1), was completed after eight months with mild clinical improvement. From 2017 up to 2019, although no worsening of clinical symptoms was observed, the *M. bolletii* continued to be isolated and a new treatment was proposed but the patient declined it. During 2021 the patient's symptoms worsened again leading to a bilateral pulmonary emphysema and a new hospitalization, in a respiratory isolation unit, in November. A second MAB-directed treatment was initiated (Figure 3.1) including AMK IV 750 mg/day, tigecycline (TGC) IV 50 mg/day, linezolid (LZD) PO 600 mg/day and CFZ PO 100 mg/day during hospitalization (three months), and LZD PO 600 mg/day plus CFZ PO 100 mg/day for seven months in ambulatory. Since then, no other MAB strains were isolated, and in September 2022 the patient was considered cured and the treatment regimen was interrupted.

3.2.2.2 Microbiological characterization of MAB Isolates

From April 2014 to September 2021, the National Reference Laboratory for Mycobacteria (NRL-TB) received eight strains isolated from this patient. Species identification and molecular resistance profile were determined using *GenoType Mycobacterium* CM® and *GenoType* NTMDR® (Hain Lifescience). Drug susceptibility testing (DST) was performed according to

the Clinical and Laboratory Standards Institute (Woods et al., 2011, CLSI 2021). Minimum inhibitory concentrations (MICs) of AMK, FOX, ciprofloxacin (CIP), CLR, DOX, LZD, moxifloxacin (MXF) and CFZ were determined (Table 3.1), and *M. peregrinum* ATCC 700686 strain was used as the reference strain for quality control purposes. Briefly, the inoculums were prepared in cation-adjusted Mueller-Hinton broth, incubated at 30 ^{o}C and examined after 72 h. If visible growth in the control tube was observed, the MIC of each antibiotic was recorded and interpreted accordingly; otherwise the tubes were re-incubated and the readings repeated at day four or five. To ensure detection of inducible macrolide resistance, the incubation for CLR was maintained until day 14, unless resistance (MIC ≥8 μg/mL) was recognized earlier.

Table 3.1 - Antibiotics used, Minimal inhibitory Concentrations (MIC) tested and interpretive criteria for Susceptibility Testing of Rapidly Growing Mycobacteria

		Interpretive criteria		
Antibiotics	**MIC (μg/mL)**	**Susceptible**	**Intermediate**	**Resistant**
Amikacin	16; 32; 64	≤ 16	32	≥ 64
Cefoxitin	16; 32; 64; 128	≤ 16	32 - 64	≥ 128
Ciprofloxacin	1; 2; 4	≤ 1	2	≥ 4
Clarithromycin	2; 4; 8	≤ 2	4	≥ 8
Doxycycline	1; 2; 4; 8	≤ 1	2 - 4	≥ 8
Linezolid	8; 16; 32	≤ 8	16	≥ 32
Moxifloxacin	1; 2; 4	≤ 1	2	≥ 4

3.2.2.3 Whole genome sequencing and bioinformatics analysis

Five of the isolates collected from 2014 up to 2021 were recovered for further genomic analysis (of note, three of the strains were unrecoverable for further analysis). Genomic DNA was extracted as previously described (Somerville et al., 2005), and subjected to Nextera XT library preparation (Illumina, USA) prior to paired-end sequencing (2 × 250 bp or 2 × 150 bp) on either a MiSeq or a NextSeq 550 or 2000 instrument (Illumina, USA), according to the manufacturer's instructions. Genomes were assembled using INNUca v4.2.2 (https://github. com/B-UMMI/INNUca), an integrative bioinformatics pipeline for read quality analysis, species

identification, and de novo genome assembly and improvement (Llarena et al., 2018) (see Table 3.2 for genome assembly details). Final draft genomes were annotated using Bakta v1.2.2 (Schwengers et al., 2021), using default parameters. Multi Locus Sequence Typing (MLST) was performed upon query and sequence submission to the PubMLST database (https://pubmlst.org/organisms /mycobacteroides-abscessus-complex). To evaluate the genetic microevolution and adaptation of this strain within its host, quality processed reads were individually mapped against the annotated draft genome of the first isolated strain (Mabs_012014) and single nucleotide polymorphism (SNP) calling was performed using Snippy v4.5.1 (https://github.com/tseemann/snippy) (Snippy-Seemann, 2023), with a minimum per base coverage of 10 and minimum proportion of reads differing from the reference of 70%. As such, mutations found at 100% in frequency were defined as "fixed mutations", and those found at a frequency below 70% as "emerging mutations". A draft core-SNP-based phylogeny was constructed using parsnps v1.2 (Treangen et al., 2014), enrolling 114 publicly available *M. bolletii* genomes (see figure 3.2 for details) and the GD91 genome as reference sequence (Genbank accession #CP065265.1). Whenever raw reads datasets were available, public genomes were also assembled using INNUca v4.2.2. For reference purposes, quality processed reads were also mapped to the reference genome ATCC 19977 (#CU458896.1), in order to report, when possible, the identified targeted regions relative to this reference, both for position and locus tag. Additionally, genomes were screened for known genetic markers associated with antimicrobial resistance (Halloum et al., 2017; Hurst-Hess et al., 2017; Johansen et al., 2020; Pryjma et al., 2017; Soroka et al., 2014).

Table 3.2 - Clinical, microbiologic and genomic characterization of the strains received at National Reference Laboratory for Mycobacteria during 2014–2021

	Mabs_012014	**Mabs_012015**	**Mabs_022015**	**Mabs_012017**	**Mabs_022017**	**Mabs_032017**	**Mabs_012018**	**Mabs_012021**
Year of isolation	2014	2015	2015	2017	2017	2017	2018	2021
Sample type	sputum	sputum	sputum	BAL	BAL	sputum	sputum	sputum
Colony morphotype	smooth	smooth	smooth	smooth	smooth	rough	rough	rough
mDST								
MA	R	R	R	R	R	R	R	R
AG	S	S	S	S	S	S	S	
pDST								
FOX	-	-	S	S	-	S	-	S
AMK	-	-	S	S	-	S	-	R
CLR	-	-	-	R(3)*	-	R(5)*	-	R(6)*
DOX	-	-	-	R	-	R	-	R
LZD	-	-	-	S	-	S	-	S
CIP	-	-	R	R	-	R	-	R
MXF	-	-	-	I	-	I	-	R
gDST	I303Q and L304M *embB* (*MTB_Rv3795*) for ethambutol; *erm(41)* T28 (MAB_2297) for macrolides; *gyrA*-Ala74 (MAB_0019) and *gyrB* Arg482 e Asn499 (MAB_006) for fluoroquinolones; presence of *arr_mab* gene (MAB_0591) for rifamycin group; presence of *bla_mab* gene (MAB_2875) for *β-lactams*; presence of *Mab_ApH* (MAB_2385) for streptomycin; presence of *MabTetX* (MAB_1496c) enzyme for tetracycline.							
Genome Statistics								
Deph coverage	72.49	78.14	-	118.08	-	82.54	-	88.04
# of contigs	132	84	-	70	-	64		37
Genome size (bp)	4,873,488	4,857,614	-	4,859,582	-	4,857,984	-	4,859,417
ENA accession	**ERR10554471**	**ERR10554469**	**-**	**ERR10554472**	**-**	**ERR1054468**	**-**	**ERR10554470**

R – resistant; S – sensitive; I – intermediate resistance; mDST – molecular drug susceptibility testing- Hain Lifescience®; pDST – phenotypic drug susceptibility testing; gDST – genomic determinants of resistance present in all strains; MA – macrolides; AG – aminoglycosides; FOX – cefoxitin; AMK – amykacin; CLR – clarithromycin; DOX – doxycycline; LZD – linezolid; CIP – ciprofloxacin; MXF – moxifloxacin; BAL – bronchoalveolar lavage; ENA – European Nucleotide Archive. *number of days of incubation until macrolides resistance recognition (MIC >8 μg/mL).

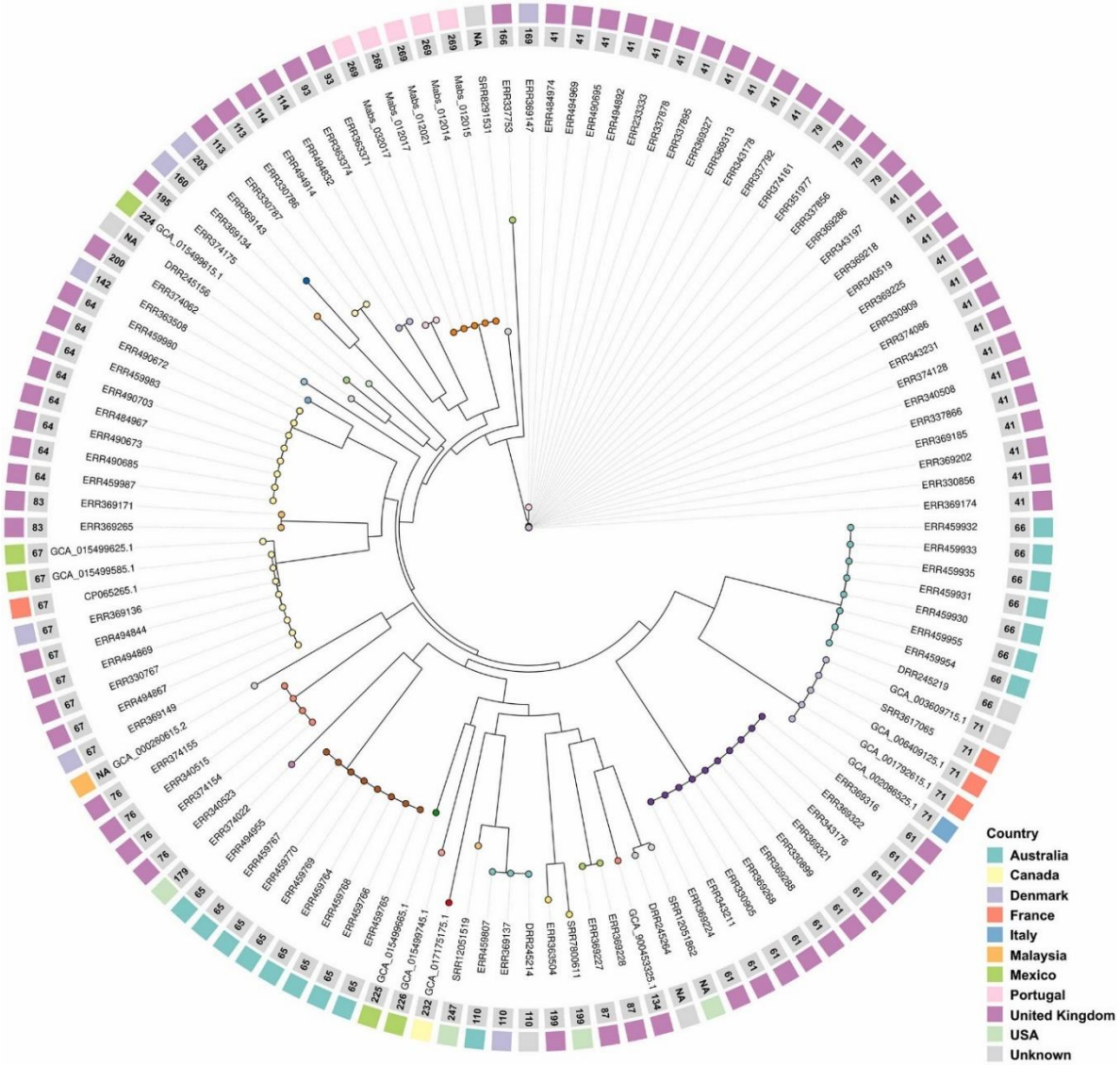


Figure 3.2 - *Mycobacteroides abscessus subsp. bolletii* core-SNP-based phylogeny, enrolling 114 publically available genomes and the five novel genomes from the present study. Analysis was performed using GD91 genome as reference sequence (Genbank accession #CP065265.1). Total core-genome analysed was 76% of the reference genome.

3.2.3 Results

All isolates were identified as *M. bolletii* and belonging to a novel MLST profile, ST269. Clinical and microbiologic features of the isolates are summarized in Table 3.2. Molecular DST (mDST) using NTM-DR Kit allowed the detection of the *erm (41)* T28 type for all isolates, suggesting resistance to macrolides. Phenotypic DST (pDST), performed for four isolates (Mabs_012015, Mabs_012017, Mabs_032017 and Mabs_012021), revealed a consistent resistance pattern, with

all of them being resistant to CLR, DOX, CIP and MXF and susceptible to LZD. Minor differences were observed for aminoglycosides resistance, as the strain isolated in 2021 was phenotypically (but not genetically) resistant to AMK. Additionally, we screened the sequenced genomes for already described specific genetic determinants known to confer resistance. All five isolates revealed the same mutation resistance pattern (see gDST in Table 3.2) except for novel mutations in MAB_1881c (encoding a TetR family transcriptional regulator). These alterations were only present in Mabs_012017 and Mabs_012021 isolates, namely a 12 bp deletion and a SNP leading to protein truncation, respectively (Supplementary Table S1).

Of note, we observed that during 2017 the morphotype of the *M. bolletii* colonies switched from smooth to rough (Table 3.2). The integration of the novel genome sequences with publicly available *M. bolletii* genomes revealed a high genetic diversity within the analysed dataset (Figure 3.2), with genomes differing by an overall mean pairwise distance of 17,285 SNPs in a total of 138,450 analysed variant sites (total core-genome analysed was 76% of the reference genome). In fact, novel *M. bolletii* genomes were genetically distant from those that are publicly available, with the closest genome (ERR374175) differing by 13,083 SNPs, in the applied analysis. In-depth genomic analysis of the novel *M. bolletii* genomes revealed a strong similarity among them (Figure 3.2), with a discreet emergence of mutations from 2014 up to 2021, indicating that the prolonged infection was caused by a single evolving strain (Figure 3.3). The isolate collected in 2021 (Mabs_012021) was distinguishable from the 2014 isolate (Mabs_012014) by 6 indels and 11 SNPs. The majority of the SNPs (i.e., 8 out of the 11) corresponded to non-synonymous mutations and one of them was found to be fixed (i.e., 100% frequency) in all isolates from 2017 onwards (MAB_0364c, an ATPase similar to *badF/badG/bcrA/bcrD* type), but also in both isolates from 2014 and 2015, at frequencies of 35.7% and 57.4% respectively (Figure 3.3), suggesting a microevolution of the MAB strain within the patient. Furthermore, we observed three distinct genetic alterations, including a large 37 bp deletion and 1 bp indels targeting an homopolymeric tract, each in a different isolate (Mabs_012015, Mabs_032017, Mabs_012021), in gene MAB_4099c, a probable non–ribosomal peptide synthetase (*mps1* gene). Still, most identified mutations targeted genes involved in

drug resistance, defence mechanisms and adaptation to starvation and oxidative stress (Figure 3.3, Supplementary Table S1).

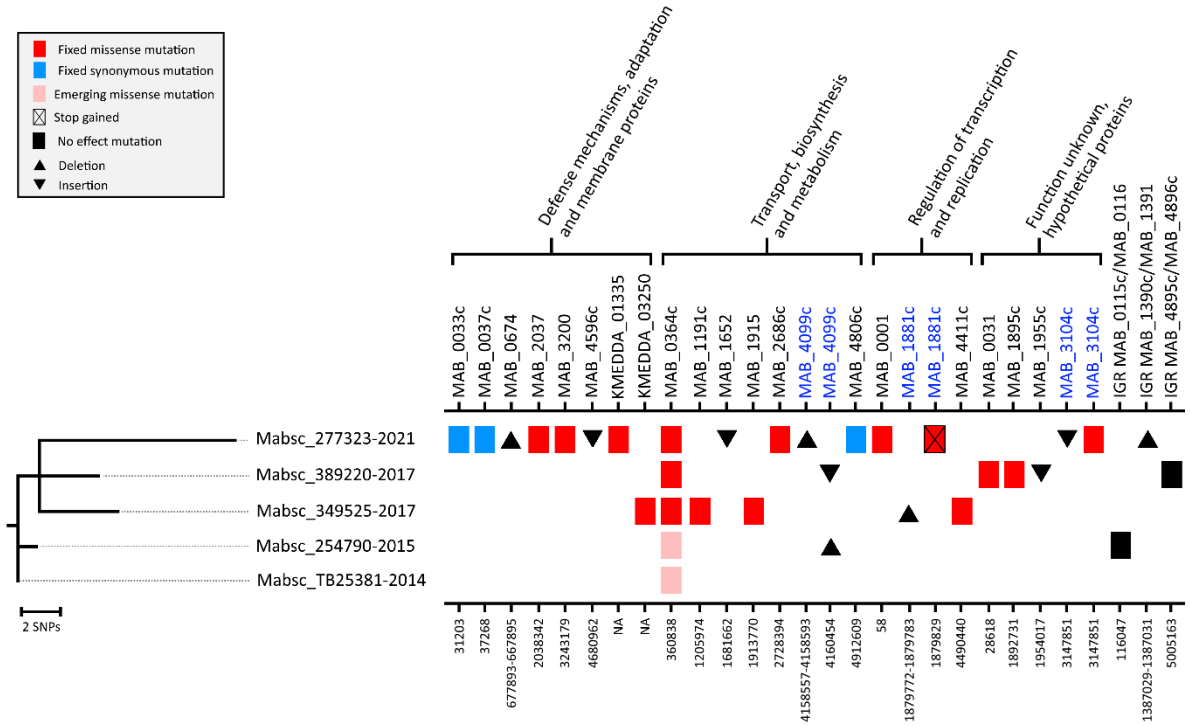


Figure 3.3 - Phylogeny of the persistent infection-causing *Mycobacteroides abscessus subsp. bolletii* isolates and detailed within patient mutational dynamics. Fixed mutations refer to single nucleotide polymorphisms (SNPs) or indels observed with a frequency ≥ 70%. Emerging mutations refer to SNPs or indels observed with a frequency < 70%. Gene designations (upper text) refer to the locus tags of the *M. abscessus* ATCC 19977 reference genome (#CU458896.1), with the exception of KMEDDA_01335 and KMDDA_03250 which refer to the draft annotation of Mabs_012014. Lower text refers to the SNP position in *M. abscessus* ATCC 19977. Genes targeted by mutations more than once are highlighted in blue. IGR – Intergenic region. NA – Not applicable. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2.4 Discussion

Mycobacteroides abscessus infections are a growing problem worldwide and despite prolonged multidrug treatment regimens, treatment outcomes are generally poor, and low conversion rates are reported (Griffith et al., 2007; Johansen et al., 2020; Koh, Jeong, et al., 2017). In Portugal, MAB is responsible for near 10% of NTM infections (Santos et al., 2024) and little is known about the clinical outcomes, recurrence or persistent rates. Here we describe one case of a chronically infected patient, enrolling the evolution of the infecting strain for over eight years (Figure 3.1). During the infection period, the patient received two MAB-directed treatments and, although both regimens included the recommended antibiotics, the species *M. bolletii* is

resistant to macrolides and treatments stopped before the one-year-treatment regimen recommended (Griffith et al., 2007; Koh, Jeong, et al., 2017). The genomic analysis of the multiple isolates confirmed the multidrug resistant pattern of the strain. Regarding macrolide resistance, besides the known *erm* (T28) type, we also observed two novel alterations in MAB_1881c (encoding a *TetR* family transcriptional regulator), an already described CLR resistance genetic target (B. Li et al., 2020). Nevertheless, as *M. bolletii* strain are resistant to macrolides due to *erm* gene (Johansen et al., 2020), the effect of these MAB_1881c alteration would not be expressed phenotypically. Still, their emergence reflect bacterial host adaptation, either due to continued macrolide exposure during treatment or as a consequence of a persistent infection as previously described (Bryant et al., 2021). Moreover, it allowed the identification of a more complete antibiotic susceptibility profile, even for antibiotics that are not usually phenotypically tested. All isolates presented mutations in genes conferring resistance to ethambutol, streptomycin, tetracyclines, fluoroquinolones, rifamycins and *β-lactams* (Table 3.2). The exception was observed for AMK, as the 2021 isolate appeared as phenotypically resistant. Although the search for already described mutation markers (Hurst-Hess et al., 2017; Prammananan et al., 1998; Pryjma et al., 2017; Rominski, Selchow, et al., 2017) was not successful, we cannot discard that this phenotype may be provided by a new, not yet described resistance mechanism. As the majority of the MAB treatments are initiated empirically, with the American Thoracic Society/Infectious Diseases Society of America (ATS/IDSA) recommending multidrug macrolide based therapy (Griffith et al., 2007), inducible macrolide and aminoglycoside resistance is a reality and further complicates treatment (Johansen et al., 2020; Pryjma et al., 2017). As such, a genomic approach, which allows a complete genetic resistance profile of the strain causing the infection, may enable a more patient-oriented treatment with better outcomes. During the eight years of disease evolution, and despite clinical follow-up, the patient refused therapy for about four years (2017–2021), even when maintaining respiratory symptoms and the laboratory isolations of the infecting strain. Thus, it is likely that, in this period, optimal conditions were created for the bacteria to evolve and adapt, which was observed both phenotypically and genetically. In fact, not only did the infecting strain acquire a rough morphotype at the end of 2017, which is believed to be associated with a more virulent phenotype (Table 3.2, Figure 3.3) (Lewin et al., 2021; Pawlik et al., 2013; Ryan & Byrd, 2018; Victoria et al., 2021), but also resistance to antibiotics increased from this point on (to AMK and MXF) as well as the emergence of mutations. We observed three distinct mutations in

locus MAB_4099c (Figure 3.3), a probable non–ribossomal peptide synthethase (*mps1* gene). Alterations in *mps1* have already been described as being implicated in morphotype switching (Johansen et al., 2020; Pawlik et al., 2013). A detailed genetic analysis of this locus revealed a 1 bp deletion and 1 bp insertion in a poly(G) tract for the 2015 and the last 2017 isolate, respectively, and a truncating 37 deletion for the 2021 isolate. Of note, the insertion observed in the 2015 isolate did not cause any visible phenotypical alteration. However, considering that poly(G) tracts are frequently associated with phase variation mechanisms underlying the ON/OFF state of proteins (Cayrou et al., 2021; Davidsen & Tønjum, 2006; Esson et al., 2016; Orsi et al., 2010), it is reasonable to speculate that the switch from the smooth to the rough colony morphotype here observed, may rely on the production/activity of the non–ribossomal peptide synthetase, as the three described genetic alterations may hypothetically underlie this enzymatic state. Nevertheless, although the identified alterations are likely triggering morphotype switching, as previously described in this genetic region (Johansen et al., 2020; Pawlik et al., 2013), its biological effect should undergo experimental confirmation. In fact, to date, only genetic changes in the locus *gpl*, that encodes proteins involved in synthesis or export of Glico-Peptido-Lipids, were confirmed as responsible for morphotype switching. One of these mutations was observed in MAB_4099c gene, namely an insertion of GC in 5′ region that is responsible for arrest of the whole operon and phenotypical alteration from smooth to rough (Johansen et al., 2020; Pawlik et al., 2013). Most of the identified mutations targeted genes involved in drug resistance, defence mechanisms and adaptation to starvation and oxidative stress (Figure 3.3, Supplementary Table S1). The comparative genome analysis of five isolates identified 25 genes with genetic alterations, the vast majority of them appearing in the 2017 and the 2021 isolates, with special emphasis in the latter. This enabled us to chronologically identify the emergence and fixation of genetic alterations in the genome of the infecting strain during the persistent infection (Figure 3.3), likely illustrating an evolutionary adaptation scenario. Curiously, we could observe the chronological evolutionary emergence and fixation of one of these mutations in the locus MAB_0364c. In fact, this SNP (Supplementary Table S1) was identified at a frequency of 36% for the 2014 isolate, 57% for the 2015 isolate and 100% for the 2017 and 2021 isolates clearly illustrating a fixation process (Figure 3.3) underlying a microevolution of the MAB strain within the patient. MAB_0364c encodes an ATPase similar to badF/badG/bcrA/bcrD type that was already described in other bacteria, more particularly environmental bacteria/ opportunistic pathogens (Winsor et al., 2008) as being involved in

Benzoyl-CoA pathway, which is an intermediate in the anaerobic metabolism of aromatic compounds, allowing survival and adaptation to low oxygen environments (Breese et al., 1998; Egland et al., 1997). Altogether, these results suggest that the observed genetic alterations are a reflection of the bacterial population's continuous adaptation and survival to the host environment during its persisting infection. This follows previous observation that within-host evolution of *M. abscessus*, influenced by chronic infection, drives pathogenic adaptation (Bryant et al., 2021). Still, there are two points that may constitute a limitation of our study, namely: we did not have access to the patient's original biological samples (i.e., prior to strain isolation) and strain isolation with subsequent laboratory propagations may have not allowed the authentic representation of the whole MAB population infecting the patient; and the laboratory loss of three strains, which hampered the complete scenario of this strain's evolution during the persistent infection. Ultimately, we highlight the need for similar studies for a better understanding of the mechanisms involved in adaptation and survival of this challenging bacteria in the context of the human-pathogen arms race.

This chapter was adapted from the following published documents

Carneiro, S., Pinto, M., Silva, S., Santos, A., Rodrigues, I., Santos, D., Duarte, S., Vieira, L., Gomes, J. P., & Macedo, R. (2023). Genome-Scale Characterization of *Mycobacterium abscessus* Complex Isolates from Portugal. International Journal of Molecular Sciences, 24(20), 15402 https://doi.org/10.3390/ijms242015402

3.3 Genome-Scale Characterization of *Mycobacterium abscessus* Complex Isolates from Portugal - Abstract

The *Mycobacterium abscessus* complex (MABC) is an emerging, difficult to treat, multi-drug resistant nontuberculous mycobacteria responsible for a wide spectrum of infections and associated with an increasing number of cases worldwide. Dominant circulating clones (DCCs) of MABC have been genetically identified as groups of strains associated with higher prevalence, higher levels of antimicrobial resistance, and worse clinical outcomes. To date, little is known about the genomic characteristics of MABC species circulating in Portugal. Here, we examined the genetic diversity and antimicrobial resistance profiles of 30 MABC strains isolated between 2014 and 2022 in Portugal. The genetic diversity of circulating MABC strains was assessed through a gene-by-gene approach (wgMLST), allowing their subspecies differentiation and the classification of isolates into DCCs. Antimicrobial resistance profiles were defined using phenotypic, molecular, and genomic approaches. The majority of isolates were resistant to at least two antimicrobials, although a poor correlation between phenotype and genotype data was observed. Portuguese genomes were highly diverse, and data suggest the existence of MABC lineages with potential international circulation or cross-border transmission. This study highlights the genetic diversity and antimicrobial resistance profile of circulating MABC isolates in Portugal while representing the first step towards the implementation of a genomic-based surveillance system for MABC at the Portuguese NIH.

3.3.1 Introduction

Species of the *Mycobacterium abscessus* complex (MABC; which include subspecies *abscessus*, subsp. *massiliense*, and subsp. *bolletii*) are the most reported rapidly growing nontuberculous mycobacteria (NTM) responsible for severe respiratory, skin, and mucosal infections (Johansen et al., 2020; M.-R. Lee et al., 2015; To et al., 2020). As NTM are environmental organisms, MABC infections are assumed to occur after exposure to a contaminated environment. Although infections are more likely to occur indirectly via fomite contamination or long-living infection aerosols, person-to person transmission in cystic fibrosis (CF) patients may also occur (Bryant et al., 2013, 2016; Falkinham, III, 2009; Harris et al., 2014; Tortoli, Kohl, et al., 2017). For example, a genomic study focused on isolates from hospitalized patients at a CF center showed

that 31.5% of patients were infected with the same (or a very similar) strain of MABC, suggesting nosocomial transmission (Bryant et al., 2013). The same observations regarding possible person to-person transmission were also demonstrated in another study conducted in a CF center in Seattle (Aitken et al., 2012). Given the increase in MABC cases reported worldwide (Pierre-Audigier et al., 2005; Prevots et al., 2010), in 2016, Bryant and colleagues demonstrated the usefulness of whole-genome sequencing (WGS) in the study of MABC isolate transmission models, revealing the existence of dominating circulating clones (DCCs), defined by clusters of genetically related isolates of *Mycobacterium massiliense* or *Mycobacterium abscessus* (Bryant et al., 2016). These have been associated with the majority of human infections, higher antibiotic resistance patterns, and worse clinical outcomes (Bryant et al., 2013, 2016). In addition, these isolates have genomic characteristics that allowed this NTM to evolve from an environmental organism to a true pulmonary pathogen (Bryant et al., 2013, 2016). Additionally, MABC species are emerging multidrug-resistant pathogens with particular clinical relevance, causing lung disease and mainly affecting immunocompromised people (Johansen et al., 2020). Due to their innate and acquired resistance to a wide range of antimicrobials, and the limited therapeutic options (Nessar et al., 2012), MABC infections are challenging and sometimes impossible to treat (Daley et al., 2020). Several genetic targets have already been associated with resistance (Nasiri et al., 2017), such as mutations in the ribosomal RNA genes *rrs* (16S rRNA) and *rrl* (23S rRNA) resulting in resistance to amikacin and macrolides, respectively (Bronson et al., 2021). In *M. abscessus* and *Mycobacterium bolletii*, the *erm*(41) gene is responsible for an inducible innate resistance to macrolides, whilst isolates of *M. massiliense* have this gene inactivated and can therefore be susceptible (Nash et al., 2009). This emphasizes the need to correctly identify the MABC subspecies in order to predict resistance phenotypes and possible treatment outcomes (H.-Y. Kim et al., 2010). Currently, there are no defined guidelines for standard therapy, and treatment regimens are based on a multidrug approach with long-term administration, which can lead to toxicity and the emergence of new resistance phenotypes (Chew et al., 2021; Jarand et al., 2011; Jeong et al., 2017; Kasperbauer & De Groote, 2015; B. Li et al., 2020). For this reason, drug susceptibility testing (DST), following the Clinical Laboratory Standards Institute Guidelines (CLSI), should always be performed to guide treatment regimen design (Griffith & Daley, 2022; Woods et al., 2011). However, given the poor correlation between in vitro data and clinical efficacy results (Griffith, 2014), i.e., laboratory issues that lead to low accuracy in phenotypic prediction, WGS-based approaches may be key to the

prediction of resistance through the screening of multiple resistance-associated genetic markers and, consequently, supporting treatment regimen definitions, as has been demonstrated for other bacterial pathogens (Dookie et al., 2022; Jeukens et al., 2019; NIHR Global Health Research Unit on Genomic Surveillance of AMR, 2020). In this context, in the present study, we characterized the genetic diversity and antimicrobial resistance profiles (using molecular, genomic, and phenotypic approaches) of 30 MABC isolates (collected between 2014 and 2022) from the National Reference Laboratory for Mycobacteria (NRL-TB) of the Portuguese National Institute of Health (NIH). For this purpose, we applied a gene-by-gene approach (wgMLST) in order to understand the genetic similarities between Portuguese strains and those identified in other countries. Additionally, we performed DST for seven antimicrobials and screened isolates for mutations that are possibly associated with antimicrobial resistance using molecular and genomic approaches.

3.3.2 Materials and Methods

3.3.2.1 Sample collection and characterization

Between 2014 and 2022, the NRL-TB obtained 56 MABC cultures from 38 patients. Metadata such as age, gender, residence (according to the Nomenclature for Territorial Units level II (NUT II)), and date of diagnosis were collected. The patient dataset consisted of 19 males, 18 females, and 1 of unknown sex, with a median age of 64 18 years. The majority of these patients were from the Lisbon Metropolitan Area (LMA; 34.2%), followed by the North Region (18.4%) and the Algarve (13.2%). Alentejo and the Central Region accounted for two patients each (5.3%), and the Azores Autonomous Region accounted for one patient (2.6%). For 21.7% of the patients (8/38), the region of residence was not available. In total, 30 MABC isolates retrieved from 23 patients were successfully recovered and included in this study for further analysis (Supplementary Table S2). More than one MABC isolate from the same patient was included in the analysis when the isolation date was six or more months apart. For molecular and genomic approaches (*hsp65* gene sequencing, genotype kits, and WGS), total DNA was extracted from solid/liquid cultures using the cetyltrimethylammonium bromide (CTAB) procedure for total nucleic acid extraction, as previously described (National Institute for Public Health and the Environment (RIVM), n.d.), with slight adjustments: (i) after lysozyme addition, suspensions were incubated overnight at 37 C; and (ii) after SDS/proteinase K solution was added,

suspensions were incubated at 65°C until complete dissolution. MABC subspecies were identified using *GenoType Mycobacterium* CM/NTM-DR (Hain Lifescience, Germany) or *hsp65* Sanger sequencing, as previously described (Hain Lifescience, n.d.-a, n.d.-b; Saifi et al., 2013). Phenotypic antibiotic susceptibility testing (pDST) was performed on 27 strains (3 isolates could not be recovered for further pDST), according to the Clinical and Laboratory Standards Institute (CLSI), for amikacin (AMK), cefoxitin (FOX), ciprofloxacin (CIP), clarithromycin (CLR), doxycycline (DOX), linezolid (LZD), and moxifloxacin (MXF) (Woods et al., 2011, CLSI 2019). Molecular antibiotic susceptibility testing (mDST) was carried out for all 30 strains using the Genotype Mycobacterium NTM-DR Kit (Hain Lifescience, Germany), for macrolide and aminoglycoside resistance prediction, according to the manufacturer's instructions (Hain Lifescience, n.d.-b).

3.3.2.2 Whole-Genome Sequencing, Genome Assembly, and Characterization

Isolates' total genomic DNA was subjected to Nextera XT library preparation (Illumina, San Diego, CA, USA) before paired-end sequencing (2 250 bp or 2 150 bp) on either an MiSeq, NextSeq 550, or NextSeq 2000 instrument (Illumina, USA) according to the manufacturer's instructions (Supplementary Table S2). All genome sequences were assembled using the INNUca v4.2.2 pipeline (https://github.com/B-UMMI/INNUca; accessed on 20 September 2023), an integrative bioinformatics pipeline for read quality analysis and de novo genome assembly (Llarena et al., 2018). Briefly, read quality analysis and improvement were performed using FastQC v0.11.5 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/; accessed on 20 September 2023) and Trimmomatic v0.38 (Bolger et al., 2014) (with sample-specific read trimming criteria determined automatically based on FastQC report), respectively. Genomes were assembled with SPAdes v3.14.0 (Bankevich et al., 2012) and subsequently polished using Pilon v1.23 (Walker et al., 2014) with Quality Assurance/Quality Control statistics being monitored and reported throughout the analysis. Species confirmation and contamination screening were assessed using Kraken v2 (Wood & Salzberg, 2014) (with the Standard-16 database for 12 September 2022, available at https://benlangmead.github.io/aws-indexes/k2; accessed on 20 September 2023) for both raw reads and final polished assemblies. Additionally, polished genomes were also screened for contamination with the ribosomal Multi-Locus Sequence Typing (rMLST) tool (available at PubMLST;

https://pubmlst.org/species-id; accessed on 20 September 2023). For data integration, all genomes (N = 1797) used for the core-genome MLST (cgMLST) created and validated by Diricks et al., 2022 (Diricks et al., 2022) were retrieved from public databases (when reads were available, de novo assembly was performed). Additionally, read datasets (N = 6145) identified as MABC were retrieved from the European Nucleotide Archive (ENA). Assembly was performed using the INNUca v4.2.2 pipeline, and genomes were excluded when they did not meet at least one of the following criteria: (i) final assembly was not identified as MABC or was flagged as contaminated in Kraken or rMLST species classification; (ii) the final assembly size was 6 Mbp; (iii) the maximum number of contigs was >250 in the final assembly; and (iv) samples were identified as duplicates with different ENA run accession numbers. A total of 7065 public genomes were included in downstream analysis (Supplementary Table S3). For antimicrobial-resistance-associated genetic marker screening, quality processed reads were individually mapped against the ATCC 19977 reference genome sequence (Gen bank accession number CU458896.1) using snippy v4.5.1 (https://github.com/tseemann/ snippy; accessed on 20 September 2023). All screened genetic markers are described in Supplementary Tables S2 and S4.

3.3.2.3 Gene-by-gene Analysis

For gene-by-gene analysis, two panels of loci were retrieved (30 June 2022) from the RIDOM-Nomenclature Server (https://www.cgmlst.org/ncs; accessed on 20 September 2023) developed by Diricks and colleagues (Diricks et al., 2022). These panels include 2904 loci from the "*Mycobacteroides abscessus* cgMLST" schema and an additional 1986 loci from the "*Mycobac teroides abscessus* Accessory" schema (agMLST). In the present study, we adapted both locus panels into a whole-genome MLST (wgMLST) schema for chewBBACA v3.1.0 (Silva et al., 2018) using the PrepScheme module and a training file generated by Prodigal v2.6.3 from the ATCC19977 reference genome (RefSeq accession number GCF_000069185.1). After allele calling with chewBBACA on 7095 genomes (i.e., public dataset plus PT genomes), loci called in less than 10% of the dataset were removed from the schema. This resulted in the removal of 45 and 64 loci from the cgMLST and agMLST schemas, respectively (yielding a final schema size of 2859 for cgMLST and 4781 for wgMLST). Exact and inferred matches were used to construct an allelic profile matrix, where other allelic classifications (see

https://github.com/B-UMMI/chewBBACA/wiki; accessed on 20 September 2023) were assumed as "missing" loci. Genomes with less than 95% loci called in the cgMLST schema (i.e., fewer than 2717 loci called) were removed for subsequent phylogenetic inferences (Diricks et al., 2022). This only occurred for 17 out of the 7095 genomes under analysis (Supplementary Table S3), including 1 PT genome (PTNTM_0019). Additionally, we used the 1795-genome dataset described in Diricks et al., 2022 in order to classify our PT genomes into DCCs. Briefly, after allele calling using the cgMLST schema with a tolerance of 1% missing data per locus, we took advantage of the ReporTree v2.0.3 software (Mixão et al., 2023) to determine cluster stability regions within our dataset and compared these with the previously described cut-off for the DCC classification of 250 ADs (Diricks et al., 2022). PT genomes were then integrated into the dataset and classified based on their phylogenetic placement after minimum spanning tree (MST) generation using Grapetree v1.5.0 with the MSTv2 algorithm (Zhou et al., 2018). To increase the resolution power of the analysis with the wgMLST schema (which may be key for discriminating cases during epidemiological investigations), we used ReporTree (Mixão et al., 2023), which allows maximizing the shared genome between samples in a dynamic manner, i.e., for each subset of strains under comparison, the maximum number of shared loci between them is automatically used for tree construction. As such, MSTs were generated for the complete PT genomic dataset, and we used the ReporTree's zoom cluster-of-interest option in order to recalculate the MSTs containing each PT genome and their respective closest 15 public genomes, based on the global allelic distance matrix, to assess their genetic relatedness to other internationally reported isolates.

3.3.3 Results

3.3.3.1 Portuguese Dataset Characterization

The 30 MABC isolates enrolled in the present study were retrieved from 23 patients (12 males, 10 females, 1 of unknown sex) with a median age of 69 16 (Table 3.3). Regarding region of residence, 13 patients were from the Lisbon Metropolitan Area (LMA), three were from the Algarve, one was from the North Region, and one from the Central Region. For five patients, the region of residence was unknown. Molecular methods allowed the identification of ten isolates as *M. abscessus*, six as *M. massiliense*, ten as *M. bolletii*, and four as MABC species (no differentiation was possible). WGS analysis allowed for the differentiation of all 30 isolates at

the subspecies level: 16 isolates as *M. abscessus*, six as *M. massiliense*, and eight as *M. bolletii* (Table 3.3). Throughout this manuscript, we will use the identification results provided by the WGS approach.

Table 3.3 - Summarized characterization of the Portuguese MABC Isolates dataset.
DDC - Dominant Circulating Clone; LMA - Lisbon Metropolitan Area; UNK - Unkowon

Isolate ID	WGS-based taxonomic classification	DCC classification	Collection year	Patient ID	Region of residence	Gender	Age Group
PTNTM_0001	*M. bolletii*	Non-DCC	2014	Patient 01	Algarve	Male	[65-70[
PTNTM_0002	*M. bolletii*	Non-DCC	2015	Patient 01	Algarve	Male	[65-70[
PTNTM_0003	*M. bolletii*	Non-DCC	2017	Patient 01	Algarve	Male	[65-70[
PTNTM_0004	*M. bolletii*	Non-DCC	2017	Patient 01	Algarve	Male	[65-70[
PTNTM_0005	*M. bolletii*	Non-DCC	2021	Patient 01	Algarve	Male	[70-75[
PTNTM_0006	*M. abscessus*	Non-DCC	2021	Patient 06	LMA	Female	[50-55[
PTNTM_0007	*M. abscessus*	Non-DCC	2022	Patient 17	LMA	Female	[75-80[
PTNTM_0008	*M. abscessus*	DCC4	2019	Patient 11	LMA	Female	[70-75[
PTNTM_0009	*M. bolletii*	Non-DCC	2020	Patient 09	LMA	Female	[65-70[
PTNTM_0010	*M. massiliense*	DCC3a	2021	Patient 03	UNK	Male	[40-45[
PTNTM_0011	*M. bolletii*	Non-DCC	2021	Patient 13	UNK	Male	UNK
PTNTM_0012	*M. massiliense*	DCC6	2021	Patient 14	North	Male	[60-65[
PTNTM_0013	*M. abscessus*	Non-DCC	2016	Patient 10	LMA	Female	[65-70[
PTNTM_0014	*M. abscessus*	DCC1	2022	Patient 04	UNK	Male	UNK
PTNTM_0015	*M. abscessus*	Non-DCC	UNK	UNK	UNK	UNK	UNK
PTNTM_0016	*M. abscessus*	Non-DCC	2017	Patient 21	LMA	Male	[70-75[
PTNTM_0017	*M. bolletii*	Non-DCC	2019	Patient 22	Algarve	Female	[25-30[
PTNTM_0018	*M. abscessus*	Non-DCC	2019	Patient 08	Centre	Female	[80-85[
PTNTM_0019	*M. abscessus*	Non-DCC	2020	Patient 20	UNK	Female	UNK
PTNTM_0020	*M. abscessus*	Non-DCC	2020	Patient 19	LMA	Male	[30-35[
PTNTM_0021	*M. massiliense*	DCC3a	2015	Patient 05	LMA	Male	[75-80[
PTNTM_0022	*M. abscessus*	DCC1	2015	Patient 15	LMA	Male	[75-80[

PTNTM_0023	*M. abscessus*	DCC1	2016	Patient 07	LMA	Male	[55-60[
PTNTM_0024	*M. abscessus*	DCC1	2017	Patient 16	LMA	Female	[65-70[
PTNTM_0025	*M. massiliense*	Non-DCC	2018	Patient 02	LMA	Male	[85-90[
PTNTM_0026	*M. massiliense*	Non-DCC	2018	Patient 02	LMA	Male	[85-90[
PTNTM_0027	*M. abscessus*	Non-DCC	2019	Patient 18	LMA	Female	[75-80[
PTNTM_0028	*M. abscessus*	DCC1	2022	Patient 04	UNK	Male	UNK
PTNTM_0029	*M. abscessus*	DCC1	2022	Patient 12	Algarve	Male	[70-75[
PTNTM_0030	*M. massiliense*	DCC3a	2021	Patient 03	UNK	Male	[40-45[

3.3.3.2 Antimicrobial Susceptibility Profiling

The molecular and phenotypic antimicrobial resistance (mDST and pDST) profiles of all Portuguese (PT) isolates are presented in Figure 3.4 (the MICs of pDST are shown in Supplementary Table S2). According to the mDST results, 29 out of the 30 isolates carried the wild-type *erm(41)*T28 genetic marker, which is known to be responsible for induced resistance to macrolides. Of note, although all PT *M. massiliense* strains carry the wild-type *erm(41)*T28, these subspecies are known to have a deletion in *erm*, which can only be detected with gDST (see the materials and methods section), rendering it inactive, which can result in a macrolide susceptible phenotype. One *M. abscessus* isolate (PTNTM_0027) did not present the wild-type *erm(41)*T28, in agreement with the pDST results. No resistance-associated mutations in the *rrs* gene were detected through mDST, suggesting potential susceptibility to aminoglycosides. pDST performed on 27 isolates showed that most isolates were susceptible to amikacin (AMK) (Figure 3.4), with only three isolates showing an intermediate phenotype (two *M. bolletii* and one *M. massiliense*). In contrast, the majority of isolates were resistant to doxycycline (DOX) 77.78%; 21/27), fluoroquinolones (85.19%; 23/27), and clarithromycin (CLR;74.07%; 20/27).

Although CLR is one of the current antimicrobial drugs of choice for treatment, all *M. bolletii* isolates,14 out of 16 *M. abscessus* and only one *M. massiliense* (PTNTM_0030), were resistant (Figure 3.4). The majority of the isolates (88.89%; 24/27) were resistant to more than two antimicrobials. Noteworthily, one isolate was resistant to all type *erm(41)*T28, in agreement with

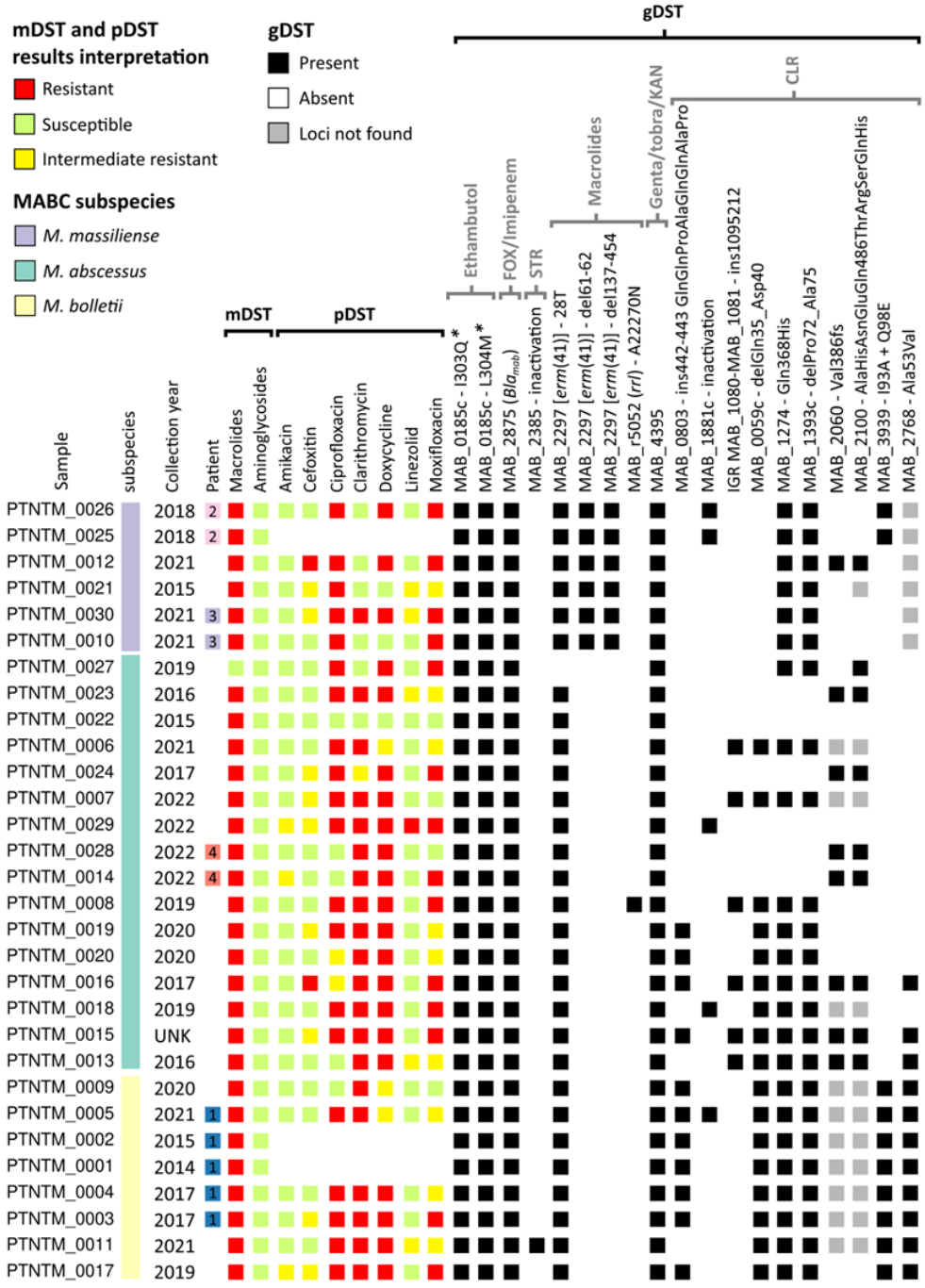


Figure 3.4 - Phenotypic, molecular and genotypic antimicrobial resistance screening of Portuguese *Mycobacterium abscessus* complex isolates. Gene designations refer to the locus tags of the *M. abscessus* ATCC19977 reference genome. *Positions relative to *M. tuberculosis* reference genome H37Rv.

the pDST results. No resistance-associated mutations in the *rrs* gene were detected through mDST, suggesting potential susceptibility to aminoglycosides antimicrobials tested (PTNTM_0029) and one isolate (PTNTM_0022) was fully susceptible (Supplementary Table S2). WGS data were used to screen a total of 48 resistance-associated genetic markers (gDST) for different classes of antimicrobials (Figure 3.4; a detailed list of markers is presented in Supplementary Table S4). Regarding aminoglycosides, the resistance-associated mutations A1374N and A1375N in the *rrs* gene (MAB_r5051) were not observed in any isolate. On the other hand, all isolates carried the aminoglycoside phosphotransferases MAB_3637c, MAB_4910c, MAB_0951, and MAB_0327, which are believed to be linked to resistance in *M. smegmatis* and hypothesized to have the same function in MABC (Nessar et al., 2012). Only one isolate (PTNTM_0011) presented a deletion in MAB_2385 (encoding a Poly(A) in 3-O-phosphotransferase), suggesting streptomycin susceptibility (Dal Molin et al., 2018). All isolates carried the MAB_4395 gene, coding for a 2-N-acetyltransferase, which has been described to be associated with a higher MIC for gentamicin, tobramycin, and kanamycin (Rominski, Selchow, et al., 2017). Additionally, all our isolates also carried the MAB_4532c gene (*eis2*) described, in vitro, as being responsible for impairing hygromycin and AMK activity through structural modifications (acetylation of the primary amine of aminoglycosides) (Dubois et al., 2019; Ung et al., 2019). The ethambutol resistance associated mutations I303Q and L304M in the *embB* gene (MAB_0185c) (Alcaide et al., 1997) were detected in all isolates. Potential reduced susceptibility to lactams (i.e., imipenem and cefoxitin) was observed in all isolates, due to the presence of β-lactamase *BlaMab* (MAB_2875). Resistance to thioacetazone can be conferred by the presence of a complex efflux pump, *MmpS5-MmpL5* (MAB_4383c/MAB_4382c) (Halloum et al., 2017; Richard et al., 2018). Most MABC isolates had this efflux pump, except for all *M. massiliense* isolates and two *M. abscessus* isolates (PTNTM_0006 and PTNTM_0027), suggesting a potential increased susceptibility to thioacetazone for the latter (Supplementary Table S2). Although 85% of isolates were phenotypically resistant to ciprofloxacin (CIP) and/or moxifloxacin (MXF) (Figure 3.4), no fluoroquinolone resistance associated mutations in *gyrA* and *gyrB* were detected (Supplementary Table S2). Regarding macrolide resistance, only one isolate (PTNTM_0027) showed an *erm(41)*C28 genotype associated with macrolide susceptibility, which is concordant with the mDST and pDST results. *M. massiliense erm(41)* deletion leading to gene inactivation was confirmed in all isolates via WGS. The isolate PTNTM_0008 also showed the macrolide resistance associated mutation A2270N in the *rrl*

gene (Mizzi et al., 2022) (Figure 3.4). Other resistance markers in the *rrl* gene (G795A, A2271N, G2281N, or A2293N) were screened for, but none were found in any of our isolates (Supplementary Table S2). PT isolates were additionally screened for recently proposed novel genetic markers potentially associated with CLR resistance (B. Li et al., 2020). All our isolates presented at least one mutation in one of these genes (Figure 3.4 and Supplementary Table S2), suggesting their potential impact on CLR decreased susceptibility. Of note, in some cases, pDST was not even concordant among same-patient isolates. In particular, isolates from patient 3 showed differences in phenotypic susceptibility for FOX, CLR, DOX, and LNZ, and isolates from patient 4 for AMK and MXF, while presenting the same analyzed resistance-associated genetic markers.

3.3.3.3 MABC Phylogenetic Analysis

Reconstruction of the global core-genome Multi-Locus Sequence Typing (cgMLST)-based phylogeny presented by Diricks and colleagues (Diricks et al., 2022) (Figure 3.5) allowed us not only to assess the 250 allelic differences (ADs) cut-off for DCC classification but also to classify our own isolates. In fact, clustering analysis at all AD thresholds on this dataset revealed a large cluster stability interval ranging from 95 to 297 ADs in the MST, where the proposed DDC classification thresholds fall. Noteworthily, an earlier stability region was also observed

ranging from 56 to 93 ADs, which could potentially be used for long-term genomic surveillance of circulating MABC strains. After successfully reproducing the schema, we were able to classify eleven Portuguese MABC isolates into different DCCs (Figure 3.4 and Table 3.3).

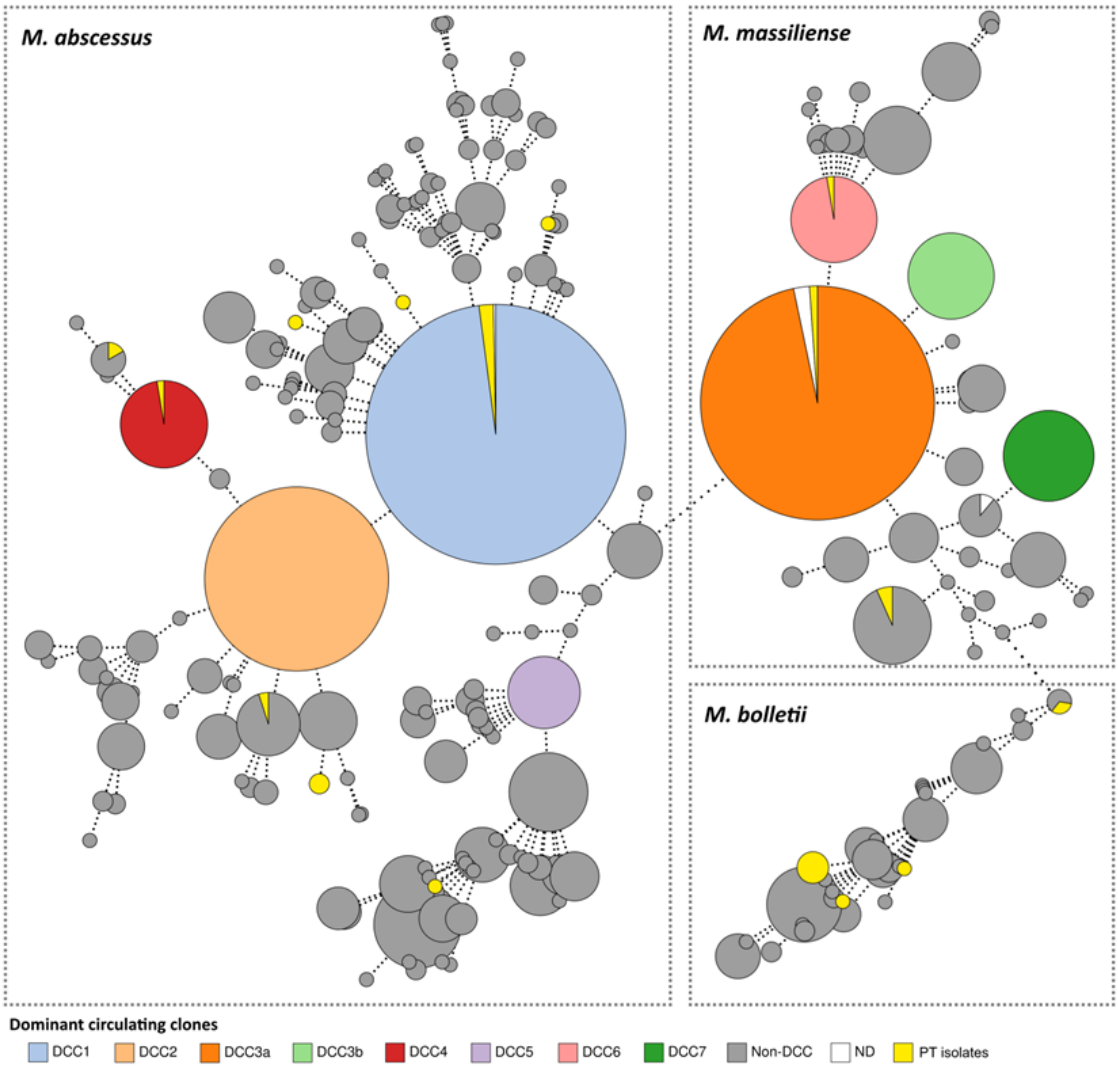


Figure 3.5 - Phylogenetic integration of the Portuguese isolates into the MABC dataset described by Diricks et al., 2022. Minimum spanning tree was constructed using Grapetree (Mixão et al., 2023) based on the allelic matrix of 1815 isolates and 2648 loci generated with the MABC cgMLST schema, with a site inclusion tolerance of 1% missing data. The size of each node proportionately corresponds to an isolate/genome or group of isolates and nodes are coloured according to the DCC classification. PT isolates are highlighted in yellow. For visualization purposes, nodes have been collapsed at the DCC defining 250 AD threshold described by Diricks et al., 2022. Dashed lines connecting nodes represent AD above 100. ND – Not determined.

As such, the isolates PTNTM_0014, PTNTM_0022, PTNTM_0023, PTNTM_0024, PTNTM_0028, and PTNTM_0029 were classified into DCC1, the isolates PTNTM_0010, PTNTM_0021, and PTNTM_0030 were classified into DCC3a, the isolate PTNTM_0008 was

classified into DCC4, and the isolate PTNTM_0012 was classified into DCC6. To take advantage of the adapted wgMLST schema, we applied a dynamic approach on our PT genomic dataset to allow us to maximize the number of shared loci, thus increasing the sensitivity of the analysis. The global genetic diversity of the PT isolates is presented in Figure 3.6, which clearly shows that most isolates are highly divergent, suggesting the circulation of a wide range of MABC strains in Portugal. In fact, 11 isolates presented more than 100 ADs from their closest isolate, with most differing in more than 2000 ADs. Moreover, isolates from the same patient were closely linked (maximum of 16 ADs) in four distinct genetic clusters (Figure 3.6), while also being genetically distant from other isolates (minimum of 43 ADs observed between PTNTM_0014 and PTNMT_0024). Of note, the genetic cluster composed of isolates PTNTM_0001 up to PTNTM_0005 corresponds to an in-depth studied persistent infection by *M. bolletii* observed in a single patient from 2015 to 2021 (Santos et al., 2023).

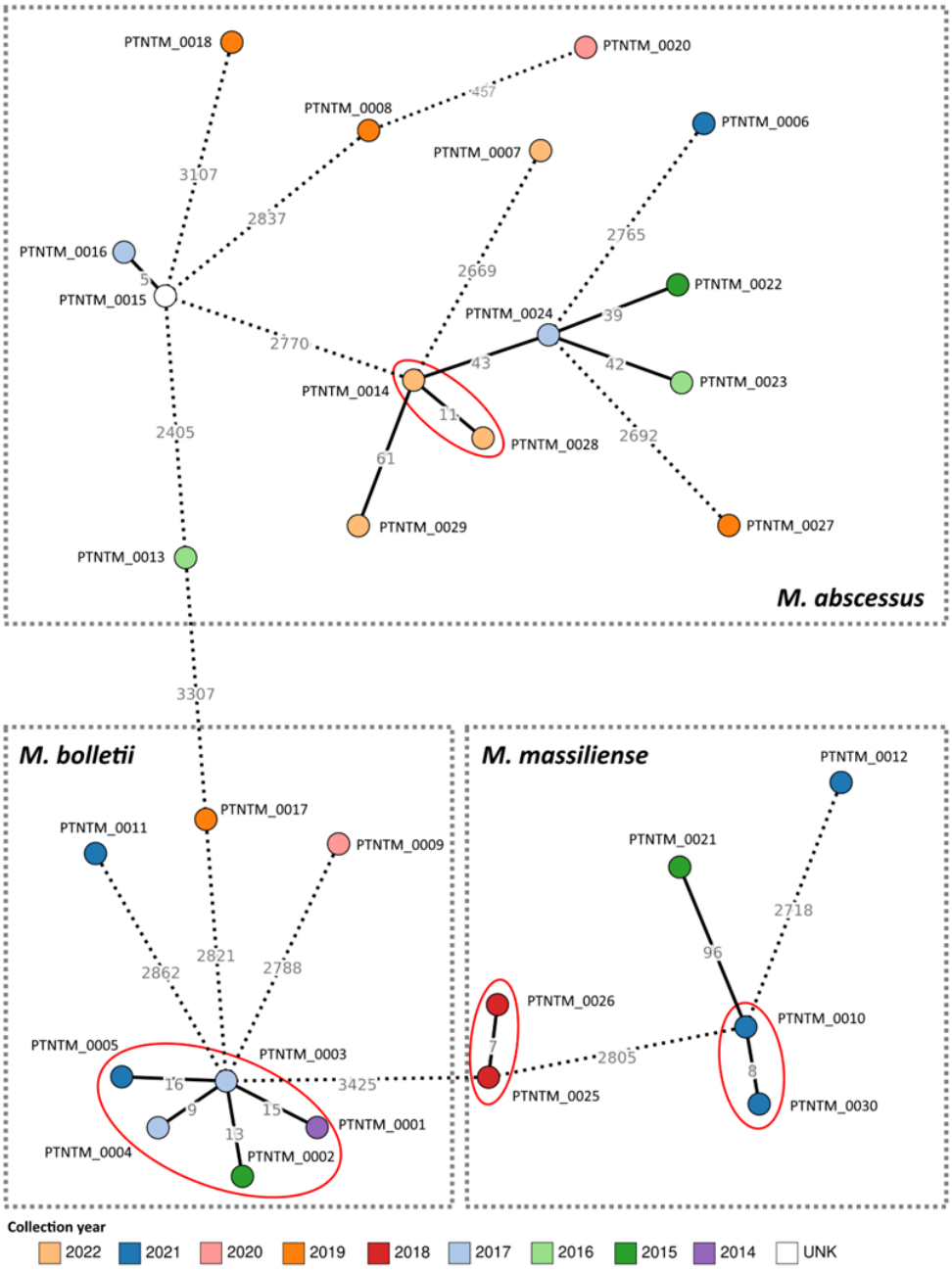


Figure 3.6 - Global phylogeny of the MABC Portuguese isolates based on an gene-by-gene approach. Minimum spanning tree was constructed using Grapetree (Mixão et al., 2023) based on the allelic matrix of 29 isolates and 3458 loci generated with the MABC wgMLST schema, with a site site inclusion tolerance of 1% missing data. Each node corresponds to an isolate/genome and has been coloured according to its diagnosis year. Isolates belonging to the same patient are highlighted with red circles. Dashed lines connecting nodes represent AD above 100. UNK – Unknown.

Taking advantage of the large number of available genomic data, we performed an in-depth clustering analysis of the PT isolates by highlighting the 15 closest MABC isolates within the global dataset (Figure 3.7). Using a conservative approach, i.e., considering isolates linked at

<50 ADs within each zoomed-in cluster (close to the identified stability region; see above), twelve PT isolates presented more than 50 ADs to the closest international genome (Figure 3.7 A, C, E, G, L), suggesting that these strains are yet to be reported in other countries. With the exception of PTNTM_0022 and PTNTM_0027 (Figure 3.7 K, N), all remaining PT isolates (ten isolates) integrate multi-country genetic clusters composed of isolates from at least three countries. Curiously, for six PT isolates, the closest genome in the dataset was collected from the United Kingdom (Figure 3.7 B, D, I–K, N). Of note, ten PT isolates (which included all same-patient isolates, PTNTM_0001 up to PTNTM_0005, from Figure 3.7 A) presented more than 2300 ADs from the closest isolate in the analyzed dataset (PTNTM_0006, PTNTM_0007, PTNTM_0009, PTNTM_0011, and PTNTM_0018).

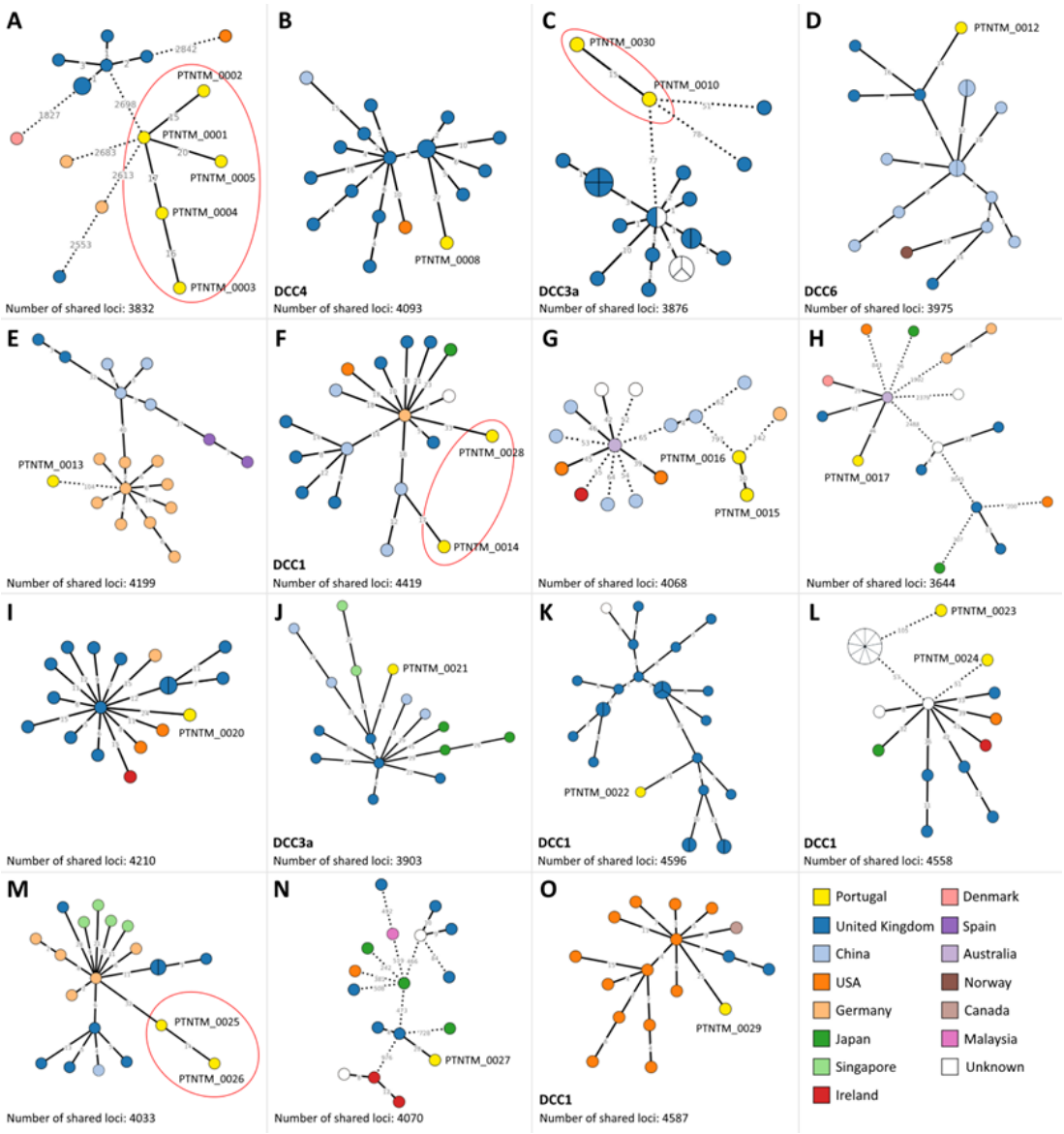


Figure 3.7 - Genetic clusters including PT isolates and the 15 closest isolates of the used public dataset of 7078 genomes. (A-O) correspond to different genetic clusters enrolling PT isolates. Minimum spanning trees were constructed using Grapetree (Mixão et al., 2023) based on the allelic matrices generated with the MABC wgMLST schema, with a site inclusion tolerance of 1% missing data. At the bottom of each panel, the number of loci under analysis for each subset, i.e., shared by all isolates, is indicated. Each node corresponds to an isolate/genome and has been coloured according to its country of origin. Isolates belonging to the same patient are highlighted with red circles. Dashed lines connecting nodes represent AD above 50.

3.3.4 Discussion

Infections caused by MABC species are increasing worldwide (Johansen et al., 2020) and are particularly challenging to treat, mainly due to resistance to several commonly used antimicrobials, leading to multidrug regimen treatments and often to treatment failure (Abdelaal et al., 2022; Koh et al., 2014). In the present study, we used molecular, phenotypic, and genomic approaches to characterize MABC isolates circulating in Portugal between 2014 and 2022. WGS allowed for the subspecies classification of all isolates, showing a higher discriminatory power when compared with the molecular methodologies commonly used in the laboratory for routine diagnostics purposes (namely, the *hsp65* sequencing and genotype kits). Among the MABC subspecies, *M. abscessus* was predominantly detected within the PT dataset (16/30) (Table 3.3), which is consistent with previous studies (Redondo et al., 2020; Roux et al., 2009). With regard to the antimicrobial susceptibility tests, pDST confirmed the previously described high levels of resistance associated with the MABC isolates (Nessar et al., 2012). The antibiotics that were less effective against our isolates were DOX, CIP, and CLR, with resistance rates of 77.78%, 74.07% and 74.07% (Figure 3.4 and Supplementary Table S2), respectively, in line with previous observations. For example, a study performed in the United Kingdom considering 58 MABC isolates showed high resistance rates of 98% for DOX and 95% for CIP (Broda et al., 2013), and another performed in China using 20 MABC isolates of all three subspecies showed 70% and 95% resistance rates to DOX and CIP, respectively (Shen et al., 2018). Most of the PT isolates that showed phenotypic resistance to CLR were *M. abscessus* and *M. bolletii*. Concerning *M. abscessus*, these high resistance rates (i.e., more than 80% CLR resistance) were also reported in studies from China (Guo et al., 2021) and Japan (Kusuki et al., 2018). In contrast, a larger study evaluating 383 MABC clinical isolates, also from China, between 2014 and 2016 revealed much lower levels of CLR resistance (only 6.4% and 10.4% for *M. abscessus* and *M. massiliense*, respectively) (Wang et al., 2021). As expected, and also supported by previous studies (Broda et al., 2013; Cho et al., 2019; Guo et al., 2021), AMK seems to be effective for MABC species as 88.89% of the PT isolates were susceptible to this aminoglycoside (Figure 3.4), which is currently used for first-line treatment. However, it is clearly recognized that phenotypic in vitro testing has a poor correlation with the in vivo activity of antimicrobials (Bernut et al., 2014). In fact, our results seem to support this phenomenon, as, for example, for patient 3 (PTNTM_0010 and PTNTM_0030) for CLR and patient 4 (PTNTM_0014 and PT NTM_0028) for MXF, pDST showed acquisition of resistance, whilst the gDST results were

identical in both isolates (Supplementary Table S2). This observation supports either the lack of sensitivity of pDST or the presence of unknown genetic markers with an impact on resistance. Nevertheless, the use of WGS for the screening of specific resistance-associated genetic markers has the potential to be a powerful alternative approach for treatment regimen recommendation. In fact, in the present study, by using WGS, it was possible to analyze a wider panel of antimicrobials that could not otherwise be tested in vitro or via mDST (Supplementary Table S2). Although our findings indicate low levels of phenotypic resistance to AMK, all isolates presented aminoglycoside phosphotransferases (MAB_3637c, MAB_4910c, MAB_0951, and MAB_0327) and the *eis2* gene, all of which have been previously associated with resistance to this class of antimicrobial (Nessar et al., 2012; Ung et al., 2019). This highlights the need for additional large-scale phenotype/genotype studies either to identify novel resistance-associated markers or to understand the incongruence between resistance profiles, as the presence of the screened molecular markers may still be responsible for low-level resistance. Consequently, when using solely pDST, MABC isolates are likely to be classified as having a susceptible or intermediate resistance phenotype, prolonging potential inadequate treatment regimens that could be leading to the establishment of fully resistant strains. Furthermore, continued exposure to AMK has already been hypothesized to be a cause for the emergence of AMK resistance, highlighting the importance of the early detection of resistance markers (Zhang et al., 2022). As mentioned before, the successful macrolide-based treatment regimens for *M. massiliense* infections are related to its intrinsic susceptibility associated with an inactive *erm(41)* gene. This inactivation is due to a large deletion (H.-Y. Kim et al., 2010) that can only be detected through genomic sequencing, while mDST will only detect inactivation by a truncated C-terminal region (T28C) (Supplementary Table S2). In fact, all of our *M. massiliense* isolates showed this deletion, and just one (PTNTM_0030) exhibited a phenotypic resistance to CLR, probably due to another resistance mechanism that we could not predict. Due to the high CLR resistance observed in *M. bolletii* and *M. abscessus* species, we suggest that its use as a first-line treatment option may require revision. The introduction of intravenous-lactams, such as imipenem or FOX combined with AMK, was one of the approaches (Griffith et al., 2007; J. Philley & Griffith, 2013). Here, only two isolates were shown to be phenotypically resistant to FOX, one *M. massiliense* (PTNTM_0012) and one *M. abscessus* (PTNTM_0016) (Figure 3.4). Resistance to β-lactams was previously reported as being related to the presence of an endogenous-lactamase (*Blamab*) encoded by MAB_2875 that reduces the activity of FOX

and imipenem (Dubée et al., 2015; Luthra et al., 2018). WGS showed that all isolates carried a version of this protein, suggesting that pDST may lack sensitivity to FOX. Another example of a potential lack of pDST sensitivity was observed for the isolate PTNTM_0021, which showed only one resistance phenotype (to CIP), while presenting several additional resistance markers to FOX (*Blamab*) (Dubée et al., 2015), ethambutol (*embB*) (Alcaide et al., 1997), aminoglycosides (MAB_3637c, MAB_4910c, MAB_0951, and MAB_0327) (Chew et al., 2021; Nessar et al., 2012), and CLR (B. Li et al., 2020) (Supplementary Table S2). As this is a non-notifiable disease, changes in the public health policy are required in order to monitor MABC infections and better understand its epidemiology. By integrating the Portuguese MABC isolates into the dataset used by Diricks et al. (Diricks et al., 2022), we were able to clearly distinguish MABC subspecies and classify eleven isolates into DCCs (Table 3.3 and Figure 3.5). Although DCC isolates have been described to have multidrug resistance, causing infections with worse outcomes, in our dataset, regardless of the DCC, the majority of the isolates were resistant to more than two antimicrobials, suggesting the potential emergence of multidrug-resistant strains. Although we have to take into account the low number of Portuguese MABC isolates included in our study, the applied gene-by-gene analysis (Figure 3.6) showed that the Portuguese isolates have a high degree of genetic diversity, regardless of the subspecies, with isolates separated by more than 2000 ADs. In fact, several studies have already described high genetic diversity in MABC (Bryant et al., 2016; Diricks et al., 2022; Jin et al., 2022). For example, a multicountry study enrolling 1080 clinical MABC isolates revealed extensive genetic differences between isolates both within subspecies and different infected individuals, suggesting disease acquisition from diverse environmental niches (Bryant et al., 2016). Previous genomic studies have mainly analyzed MABC strains recovered from CF patients due to the high incidence of MABC infections in patients with this disease, showing that these clinical settings are less prone to yielding diversity and supporting human-to-human transmission (Bryant et al., 2013; Harris et al., 2014; Tortoli, Kohl, et al., 2017). Concordantly, in our dataset, only strains belonging to the same patient clustered at a low AD threshold (below ~20 ADs), suggesting that no human-to-human transmission could be detected (although patient clinical history was not available). Despite the fact that isolates belonging to DCCs have been associated with patients with CF (Bryant et al., 2016), our results, together with previous studies (Davidson et al., 2014; Everall et al., 2017; Ripoll et al., 2009), demonstrate the presence of these clones in non-CF patients. Furthermore, seven out of eleven Portuguese DCC isolates were clustered with

strains isolated in other countries at <50 ADs (Figure 3.7), suggesting the existence of MABC lineages with potential international circulation or potential cross-border transmission. Globally, we believe that studies focusing on comparative genomic analyses in non CF patients are still needed to fully comprehend the distribution and transmission of MABC. Additionally, considering the above-mentioned issues with pDST, gDST will likely be applied to predict resistance, although phenotype/genotype studies in MABC are still required to identify clear resistance-associated markers. Given the degree of genetic diversity observed for MABC circulating in Portugal, WGS-based surveillance of this pathogen may be key both for diagnostic purposes (i.e., subspecies identification and resistance prediction) and for disease monitoring (i.e., current circulation of environmental strains or early detection of possible human-to-human transmission). In this context, we also believe that environmentally sampled MABC isolates should likewise be surveyed from a One Health perspective. As such, this study constitutes the first step in the implementation of a robust surveillance system at the Portuguese NRL-TB.

4

MYCOBACTERIUM AVIUM COMPLEX

This chapter was adapted from the following documents

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Personal Contribution

SC contributed to the design of the study, experimental work, literature review and wrote the manuscript.

4.1 General introduction to the chapter

Members of *Mycobacterium avium* complex (MAC) are responsible for the majority of human disease cases (Dahl et al., 2022; Santos et al., 2024) and have also been isolated from environmental sources such as soil and water (Biet et al., 2005; Daley, 2017), increasing its potential as a global "one-health"-related health issue. This complex is composed by several species, being *M. avium*, *M. intracellulare* and *M. chimaera* the most commonly isolated (Van Ingen et al., 2018; Fernandez-Pittol et al., 2022). However, MAC taxonomy is not yet fully established (Daley, 2017; J.-I. Shin, Ha, et al., 2020; Van Ingen et al., 2018) and, for example, there is still no agreement as to whether we should consider *M. chimaera* as a species itself or a subspecies of *M. intracellulare* (Boyle et al., 2016; Nouioui et al., 2018; Van Ingen et al., 2018). Regarding pathogenicity, similar to MABC species, MAC members also present smooth and rough colonies. Nevertheless, in MAC, rough colonies are less virulent that smooth ones given to the lack of genes for the synthesis of glycopeptidolipids, which are key determinants of virulence, particularly on biofilm formation (Belisle et al., 1993; Bhatnagar & Schorey, 2006; Holt & Daley, 2019).

The number of cases of MAC infection/ disease are increasing in several countries (Akram & Attia, 2024; Dahl et al., 2022) and Portugal is no exception (Santos et al., 2024). MAC transmission occurs, as with other NTM, trough the exposure to contaminated environmental sources, with the inhalation of aerosols being a major route of infection (Falkinham, 2011, 2015). Treatment of MAC disease consists of a prolonged multi-drug approach and is associated with poor success rates and high toxicity side effects (Griffith et al., 2007; McGarvey & Bermudez, 2002; J. V. Philley & Griffith, 2015; Xu et al., 2014). Furthermore, as the disease caused by the different subspecies of MAC may differ in their potential to cause pulmonary infections, outcome, and prognosis, a correct speciation is mandatory to guarantee an adequate treatment of the patient. Therefore, a correct identification and prediction of resistance is of extreme importance, in order to optimize treatment regimens and, consequently, reduce the impact of NTM infections. Early diagnosis, along with effective surveillance and improved reporting systems, are essential for understanding the dynamics of MAC disease enabling the implementation of effective public health preventable measures.

4.2 Genome-scale analysis of *Mycobacterium avium* complex Isolates from Portugal reveals extensive genetic diversity

Abstract

Opportunist infections caused by nontuberculous mycobacteria (NTM) have emerged as a significant public health problem. Among these, species of the *Mycobacterium avium* complex (MAC) are the main responsible for the increase in the number of human disease cases. In order to address the current needs in the detection and surveillance of MAC disease cases, we evaluated different species classification methodologies (BLASTn-based marker-gene approach, Kraken v2, rMLST and MLST databases) and their congruence with a core-SNP phylogenetic approach, based on whole genome sequencing (WGS) data. For this purpose, we used a collection of 142 MAC isolates from Portuguese patients diagnosed between 2014 and 2022. The marker-gene approach (based on the *rpoB*, *hsp65* and *groEL* genes), showed the best results, allowing the identification of the 142 MAC isolates to the species/subspecies level (*M. avium* subsp. *hominissuis, M. intracellulare, M. intracellulare* subsp. *chimaera, M. intracellulare* subsp. *yongonense, M. marseillence* and *M. colombiense*). Additionally, we performed drug susceptibility testing that confirmed clarithromycin efficacy as a first-line treatment for MAC disease, as 93% of the Portuguese isolates were susceptible. Using a core-SNP approach we also performed an in-depth phylogenetic analysis within each identified species group, and despite the high genetic diversity within the MAC species, we were able to clearly distinguish all the species/subspecies and identify genetic clusters with epidemiological potential. We highlight not only the need for the standardization of an appropriate genotyping approach for species identification and management of MAC disease, but also a more robust large-scale WGS data analysis in a One Health perspective, in order to identify potential routes of transmission.

4.2.1 Introduction

Members of *Mycobacterium avium complex* (MAC) are the most common nontuberculous mycobacteria (NTM) causal agents of opportunistic human infections, representing a significant concern to human public health worldwide (Crilly et al., 2021; Van Ingen et al., 2018). These ubiquitous pathogens can be isolated from environmental sources such as soil and both natural and man-made water supply systems (Nishiuchi et al., 2017). MAC was initially composed of two species, *Mycobacterium avium* and *Mycobacterium intracellulare* (Holt & Daley, 2019). Breakthroughs in molecular taxonomy have allowed the identification of additional/new species and subspecies, although there is still no consensus in their nomenclature due to high genetic similarity between some of these species (Mizzi et al., 2022). In 2018, Jakko and colleagues proposed that MAC was composed of twelve species: *Mycobacterium avium, Mycobacterium intracellulare, Mycobacterium chimaera, Mycobacterium colombiense, Mycobacterium arosiense, Mycobacterium vulneris, Mycobacterium bouchedurhonense, Mycobacterium timonense, Mycobacterium marseillense, Mycobacterium yongonense, Mycobacterium paraintracellulare* and *Mycobacterium lepraemurium* (Van Ingen et al., 2018). Further studies, based on whole genome sequencing (WGS), have proposed that *M. yongonense* and *M. chimaera* are actually subspecies of *M. intracellulare* (Castejon et al., 2018), or even that *M. intracellulare* subsp. *yongonense* is a later heterotopic synonym *of M. intracellulare* subsp. *chimaera*, with the latter being the appropriate designation (Nouioui et al., 2018; Tortoli et al., 2019). Similar findings were observed in the case of *M. paraintracellulare*, suggesting that *M. intracellulare* consisted of only two subspecies: *M. intracellulare* subsp. *intracellulare* (absorbing *M. paraintracellulare*), and *M. intracellulare* subsp. *chimaera* (Tortoli et al., 2019). According to the literature, *M. avium* species are also divided into four subspecies: *M. avium* subsp. *avium*, *M. avium* subsp. *silvaticum*, *M. avium* subsp. *hominissuis* and *M. avium* subsp. *paratuberculosis* (Mijs et al., 2002; Thorel et al., 1990). In 2019, Tortoli and colleagues proposed that *M. avium* subsp. *hominissuis* should be considered a variant of *M. avium* subsp. *avium* and that *M. lapraemurium* should be included as a subspecies of *M. avium* (Tortoli et al., 2019).

Due to the high similarity between some MAC species, currently available techniques such as hybridization probe assays, sequencing of genes such as *hsp65* and *rpoB*, mycobacterial interspersed repetitive units - variable number of tandem repeats (MIRU-VNTR) analysis, and the more recent matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) are not able to accurately identify and type isolates (Bull et al., 2003; Dauchy

et al., 2010; S. H. Kim & Shin, 2018; Radomski et al., 2010; Rodriguez-Temporal et al., 2022). With an expansion of MAC genomes in public databases, WGS appears to be a better approach for species differentiation while simultaneously enabling us to understand transmission dynamics and/or identify pathogenic or specific genetic traits (e.g. resistance markers) of MAC species (Augusto et al., 2018; Keen et al., 2021; Mizzi et al., 2022). The management of the disease caused by MAC depends on the correct identification of subspecies since there is variability in the ability to cause lung infection, in the severity of the disease, and, consequently, in the prognosis (S.-Y. Kim, Shin, et al., 2017). For this reason, due to its ubiquitous character and the fact that there are no reports of person-to-person transmission of MAC infections, molecular genotyping becomes essential to understand the epidemiology of MAC and identify sources of infection (J.-I. Shin, Shin, et al., 2020). Therefore, to address the current needs in the identification and management of MAC subspecies, we evaluated different classification methodologies and their congruence with a WGS-based phylogenetic approach. We highlight the genetic diversity of MAC strains circulating in Portugal, within a period of nine years (2014-2022), aiming to contribute to a better understanding on the genomic characteristics of this NTM species and its phylogeny.

4.2.2 Materials and Methods

4.2.2.1 Sample Dataset Characterization

The current study enrols a dataset of 142 MAC strains isolated in Portugal (PT) between 2014 and 2022 at the National Reference Laboratory for Tuberculosis (NRL-TB) of the Portuguese National Institute of Health (NIH). Positive cultures were identified using *GenoType Mycobacterium* CM/NTM-DR® (Hain Lifescience, Germany) according to manufacturer's instructions, or *hsp65* DNA sequencing, as previously described (Hain Lifescience, n.d.-a, n.d.-b; Saifi et al., 2013). Social-demographic data were retrieved for epidemiological inferences and included age, gender, region from requesting hospital, and place of residence of the patients (defined according to Portuguese NUTS – Nomenclature of Territorial Units for Statistics). As clinical data was unavailable for most of the cases, case definition was based only on microbiological data. As such, three categories were defined: 1) Definite case – at least three positive NTM cultures, with identical identification, irrespective of sample anatomic source, or a single positive NTM culture isolated from naturally sterile anatomic sources (such as cerebrospinal fluid,

biopsies, lavages); 2) Possible NTM disease – positive NTM cultures isolated from unknown anatomic source (e.g., cultures received at the NRL-TB for identification and susceptibility testing without reference of site of disease/infection); 3) NTM colonization – only one positive NTM culture isolated from a respiratory sample (e.g., sputum).

4.2.2.2 DNA extraction

Total DNA was extracted from solid/liquid cultures using cetyltrimethylammonium bromide procedure, according to the previously described protocol from the National Institute for Public Health and the Environment (RIVM) (RIVM, n.d.) with slight adjustments. Namely, i) after lysozyme was added, suspensions were incubated overnight at 37ºC; and ii) after addition of SDS/proteinase K solution, suspensions were incubated at 65ºC until complete dissolution.

4.2.2.3 Drug Susceptibility Testing

Phenotypic drug susceptibility testing (pDST) was performed on 134 strains (eight isolates could not be recovered for further pDST), according to the Clinical and Laboratory Standards Institute, for clarithromycin (CLR), linezolid (LZD) and moxifloxacin (MXF) (Woods et al., 2011). Briefly, the inoculums were prepared in cation-adjusted Mueller-Hinton broth supplemented with 5% of oleic albumin dextrose catalase and incubated at 37ºC for seven days. At this point, minimum inhibitory concentrations (MIC) were registered. When growth in the control tube (without antibiotic) was not observed, cultures were re-incubated, and readings registered at days 10 and 14 of incubation. Molecular susceptibility testing (mDST) was performed by screening mutations in known genetic resistance markers, namely the*16SrRNA* (*rrs;* T1406A, A1408G, C1409T, G1491C, G1491T, and C1496T) (Brown-Elliott et al., 2013; S.-Y. Kim et al., 2021) and *23SrRNA* (*rrl;* A2058N and A2059N*)* (Moon et al., 2016) genes using the GenoType *Mycobacterium* NTM-DR® kit (Hain Lifescience, Germany) (Hain Lifescience, n.d.-b) and Sanger sequencing (Park et al., 2020).

4.2.2.4 MIRU-VNTR typing

MIRU-VNTR typing was performed for isolates that were previously identified as *M. avium, M. intracellulare,* and *M. chimaera* as described above. *M. avium* isolates were genotyped using eight MIRU-VNTR loci as described by Thibault and colleagues (Thibault et al., 2007), with slight modifications. Briefly, DMSO was used instead of betaine in all amplification reactions;

for VNTRs 10 and 32, PCR mixtures were performed in a final volume of 25 µL, using 1U of KAPA HotStart ReadyMix (KAPA Biosystems, USA), 1 µM of each primer and, 1 µL of DMSO; and annealing temperatures were increased by one degree. For the characterization of *M. intracellulare* and *M. chimaera* isolates, amplification of MIRU-VNTR loci was performed as described by Dauchy *et al* (Dauchy et al., 2010). Fragment sizing was determined automatically by the QiaXcel software (Qiagen; Germany), according to the manufacturers' instructions. The genetic relations between isolates were analysed by constructing a minimum spanning tree based on the obtained MIRU-VNTR allelic profiles using the *GrapeTree* software, with the MSTv2 algorithm to account for missing data (i.e., loci for which amplification was unsuccessful) (Zhou et al., 2018).

4.2.2.5 Whole genome sequencing and genome assembly

Total genomic DNA of 142 MAC isolates were subjected to NexteraXT library preparation (Illumina, USA) before paired-end sequencing (2x250 bp or 2x150 bp) on either a MiSeq, NextSeq 550 or NextSeq 2000 instrument (Illumina, USA), according to the manufacturer's instructions (Supplementary Table S5). All genome sequences were assembled using the INNUca v4.2.2 pipeline (https://github.com/B-UMMI/INNUca), an integrative bioinformatics pipeline for read quality analysis and de novo genome assembly (Llarena et al., 2018). Briefly, read quality analysis and improvement are performed using FastQC v0.11.5 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and Trimmomatic v0.38 (Bolger et al., 2014), respectively. Genomes were assembled with SPAdes v3.14.0 (Bankevich et al., 2012) and subsequently polished using Pilon v1.23 (Walker et al., 2014), with Quality Assurance/Quality Control (QA/QC) statistics being monitored and reported throughout the analysis (details in Supplementary Table S5).

4.2.2.6 Species' classification and phylogenetic analysis

A multi-step approach was applied in order to classify the PT isolates within the MAC. All publicly available complete genomes of MAC (n=103) were retrieved from Genbank (1st of February 2024; Supplementary Table S6). The nucleotide sequences of *hps65*, *rpoB* and *groEL1* were extracted from all genomes, individually BLASTn queried against the MAC and the reported consensus species at 100% identity and 100% query coverage were retrieved for each gene (Chawla et al., 2023). The consensus species classification (henceforth-named marker-

gene based consensus classification) across all three genes was then used as the baseline for taxonomic classification. The same strategy was then applied to each novel PT genome. Additionally, for comparison purposes, species classification was also performed on genome assemblies with: i) Kraken v2 (Wood & Salzberg, 2014) (database "Standard-16 10/09/2023", available at https://benlangmead.github.io/aws-indexes/k2); ii) the PubMLST's ribossomal Multilocus Sequencing Typing (rMLST) (Jolley et al., 2012) (https://pubmlst.org/species-id); iii) the PubMLST's *Mycobacteria spp.* MLST (https://pubmlst.org/organisms/mycobacteria-spp) (Jolley et al., 2018). Core-SNP alignment subsets were constructed using parsnp v2.0.5 (Kille et al., 2024), taking advantage of the 103 public genomes and the 142 PT genomes, with either GCA_002219285 (*M. intracellulare* subsp. *chimaera*), GCA_009741445 (*Mycobacterium avium*) or GCA_023278525 (*Mycobacterium intracellulare*) as reference genomes depending on the analysed subset (see Results Section). Phylogenetic analysis was performed by calculating pairwise distance matrices for each core-SNP alignment with snp-dists (https://github.com/tseemann/snp-dists), and performing single-linkage hierarchical clustering using Reportree v2 (Mixão et al., 2023). Simultaneously, using Reportree, cluster compositions were retrieved at all possible SNP thresholds and cluster stability regions (in SNP threshold ranges) were assessed to compare the obtained species classifications with each isolate's phylogenetic clustering.

Since the NRL-TB does not have access to patients' clinical data, making it difficult to associate the molecular relations between the strains with possible epidemiological links, for this study's purpose, we used a conservative threshold of 50 SNPs to define a close related genetic cluster for each subspecies. Additionally, for same-patient samples an in-depth SNP analysis was performed using Snippy (https://github.com/tseemann/snippy), by mapping quality-filtered reads (after Trimmomatic) to one of the assembled genome of the patient pair.

4.2.2.7 Data availability

All reads generated for the present study were deposited in the European Nucleotide Archive (ENA) under the study accession number PRJEB57933. Complete details of all isolates and individual ENA run accession numbers are provided in Supplementary Table S5.

4.2.3 Results

4.2.3.1 Sample dataset characterization

During the study period, the NRL-TB received 1,355 NTM-positive cultures, of which 616 were classified as MAC using the genotype kit (Hain Lifescience GmbH, Germany). These were retrieved from 476 patients (256 males and 220 females) with a median age of 67±15 years old. For the present study, we were able to regrow and further analyse 142 MAC strains from 132 patients (64 females and 68 males) with a median age of 68±14 years old. The majority of the cases were from the Lisbon Region (which includes the Lisbon Metropolitan Area and the Setúbal Peninsula), with 85 cases (59.86%), followed by the Centro region, with 20 cases (14.08%), North region with 19 cases (13.38%), Algarve with seven cases (4.93%), Alentejo with six cases (4.23%), and the Azores Autonomous Region with five cases (3.52%). Regarding "case definition", 61 (42.96%) of the cases were classified as "definite NTM disease", 60 (42.25%) as "possible NTM disease", and 21 (14.79%) as "NTM colonization" (see methods section). The strains enrolled in this study (n=142) were identified at the species level, by the molecular approaches used in the NRL-TB for routine diagnostics purposes (GenoType *Mycobacterium* CM and NTM-DR® kits, or *hsp*65 gene Sanger sequencing), as *M. intracellulare* (60 isolates), *M. avium* (35 isolates), *M. chimaera* (14 isolates) and MAC (33 isolates).

4.2.3.2 Drug Susceptibility Testing

For 134 strains out of the 142 (94%) we were able to perform pDST (Table 4.1). MAC strains were mostly susceptible to macrolides (i.e., CLR) with only four strains showing a resistant phenotype. In contrast, for LNZ and MFX, the majority of the isolates presented intermediate or resistant phenotypes, with 29.10% and 32.09% of the isolates exhibiting a resistant phenotype, respectively.

Table 4.1 - Phenotypic drug susceptibility testing (pDST) results for MAC isolates (n=134) in Portugal. *both drug selection and MIC values were retrieved from CLSI M24-A2 guidelines(Woods et al., 2011). CLR – Clarithromycin; LNZ – Linezolid; MXF – Moxifloxacin; S – Susceptible; I – Intermediate resistance; R – Resistant.

	pDST (n/%)			**MIC (µg/mL) Interpretation***		
Antimicrobial*	S	I	R	S	I	R
CLR	125 (93.28%)	5 (3.73%)	4 (2.99%)	≤8	16	≥32
LNZ	66 (49.25%)	29 (21.64%)	39 (29.10%)	≤8	16	≥32
MXF	56 (41.80%)	35 (26.12%)	43 (32.09%)	≤1	2	≥4

mDST revealed that 139 out of 142 isolates had no mutations in the *rrl* gene, and as such 97.89% of the isolates are presumably susceptible to macrolides (Supplementary Table S5). Still, two isolates (PTNTM_0047 and PTNTM_0107) presented the *rrl* A2059C alteration, suggesting resistance to macrolides. Curiously, one isolate (PTNTM_0037) carried a non-fixed mutation in *rrl* gene, A2058G, likely reflecting an infection with a mixed population. With regard to mutations in the *rrs* gene associated with resistance to aminoglycosides, none of the isolates revealed mutations and are therefore presumably susceptible to this class of antibiotics (Supplementary Table S5). Of note, strain PTNTM_0143 showed phenotypic resistance to CLR without the detection of mutations in the *rrs* gene by mDST. Additionally we observed, for LZD and MXF, higher rates of susceptibility in *M. intracellulare* subsp. *chimaera* (71.4% and 51.0% respectively) compared to *M. avium* (32.7% and 34.5% respectively) and *M. intracellulare* species (39.3% and 35.7% respectively).

4.2.3.3 Global genetic diversity and Isolate classification

In order to classify Portuguese MAC isolates at the subspecies level, we analysed 103 MAC publicly available reference genomes using three different genetic classifiers including Kraken v2, rMLST and the BLASTn consensus of the *hsp65*, *rpoB* and *groEL1* genes (see methods section). Single-linkage hierarchical clustering (SL-HC) of reference genomes was performed, enrolling 912787 sites, corresponding to a core-genome of 18.4% (Figure 4.1).

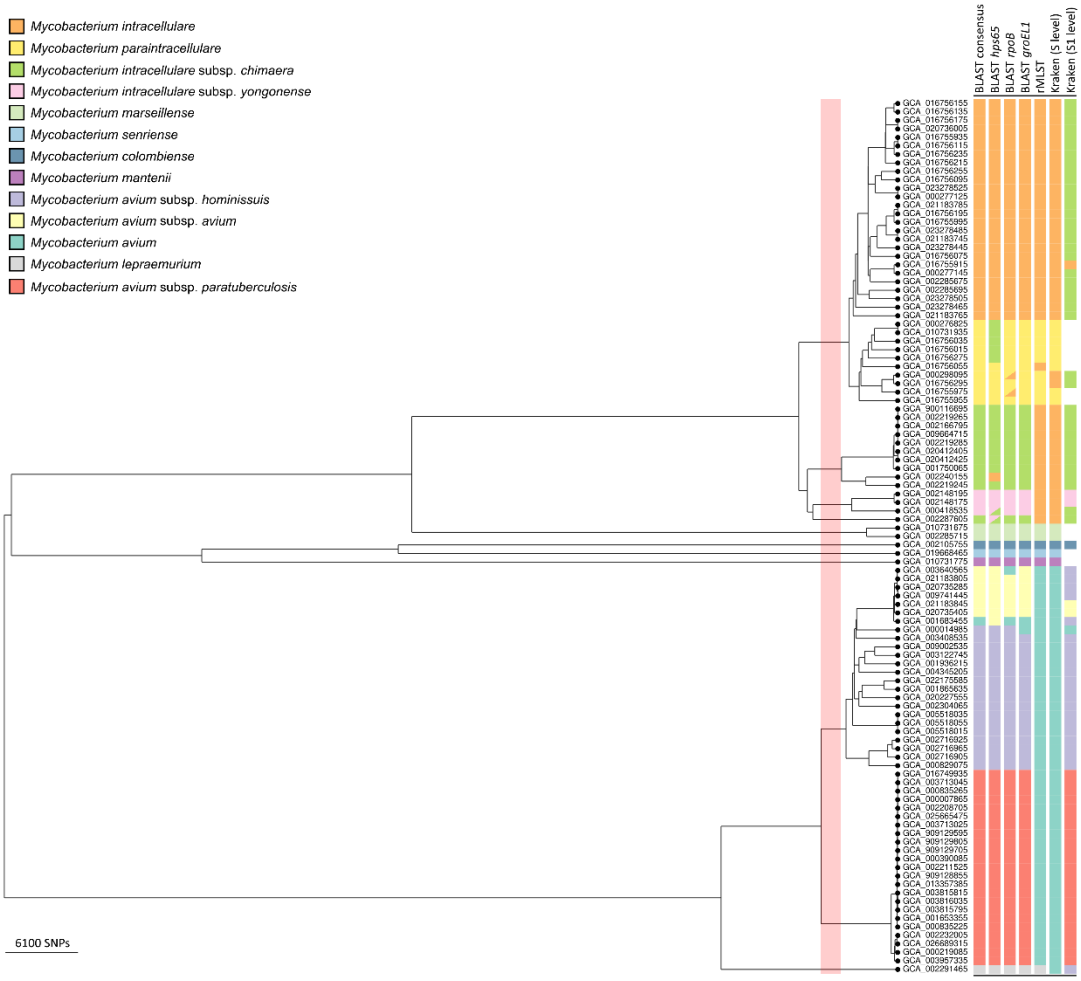

Figure 4.1 - Core-SNP-based single-linkage hierarchical clustering of different *Mycobacterium avium* complex reference genomes (N=103). Core-SNP alignment was reconstructed using GCA_009741445 as a reference. BLAST consensus, corresponds to the combined BLAST results for *hsp65*, *groEL1* and *rpoB* genes. Red box highlights the cluster congruence region with BLAST consensus species identification (corresponding to a SNP distance threshold range of 4626-6313.

A high genetic diversity [172637 single nucleotide variant sites (SNV), corresponding to 18.9% polymorphic sites] between MAC species was observed, however, the classifiers returned different results. Both the rMLST approach and the Kraken v2 approach proved to be insufficient in the taxonomic classification of MAC, since it was not possible to differentiate at the subspecies level (Figure 4.1). Indeed, only the marker-gene based consensus classification yielded taxonomic classification consistent with the phylogenetic structure, facilitating not only the differentiation of subspecies but also ensuring clear delineation between each of the groups. An extensive SNP distance threshold range was observed (from 4625 up to 6313 SNPs) where most genomes cluster congruently with the marker-gene consensus classification results, with

the exception of *M. intracellulare*/*M. paraintracellulare* and *M. avium* subsp. *hominisuis*/*M. avium* subsp. *avium*. Curiously, at this threshold range, reference genome GCA_002287605 does not cluster with other genomes identified as *M. intracellulare* subsp. *chimaera* (Figure 4.1). Integration of the PT MAC genomes into the global dataset (i.e., 103 reference genomes, plus 142 PT isolates) reduced the core-genome size from 18.4% to 9.9% (912878 to 492441 sites), due to the introduction of more genetic diversity from distinct species/subspecies (Figure 4.2).

Figure 4.2 - Core-SNP-based single-linkage hierarchical clustering of different *Mycobacterium avium* complex reference (N=103) and Portuguese (N=142) genomes. Core-SNP alignment was reconstructed using GCA_009741445 as a reference. Portuguese MAC genomes are highlighted as red nodes and reference genomes as black nodes. BLAST consensus, corresponds to the combined BLAST results for *hsp65*, *groEL1* and *rpoB* genes.

Still, this SL-HC analysis, together with the marker-gene consensus classification, allowed the potential classification of one isolate as *M. marseillence* (PTNTM_0033 / ~10780 SNPs from *M. marseillense* reference genomes) and two isolates as *M. colombiense* (PTNTM_0098 / 18963 SNPs and PTNTM_0106 / 2555 SNPs from *M. colombiense* reference genome), although they were very distinct from their respective reference genomes, suggesting, once again, high genetic diversity within species/subspecies of this complex.

In order to increase core-SNP resolution, underrepresent groups/members of MAC were removed from subsequent analysis, namely *M. columbiense*, *M. marseillense*, *M. senriense*, *M. mantenii* and *M. lepraemurium*. As such, core-genome size increased to 40.5%, with 2010210 sites under analysis (276082 SNV), and SL-HC revealed two major branches, separated by a mean pairwise distance of 191431±144 SNPs, one composed by isolates from the *M. avium* species and the other by isolates from *M. intracellulare* species (Figure 4.3).

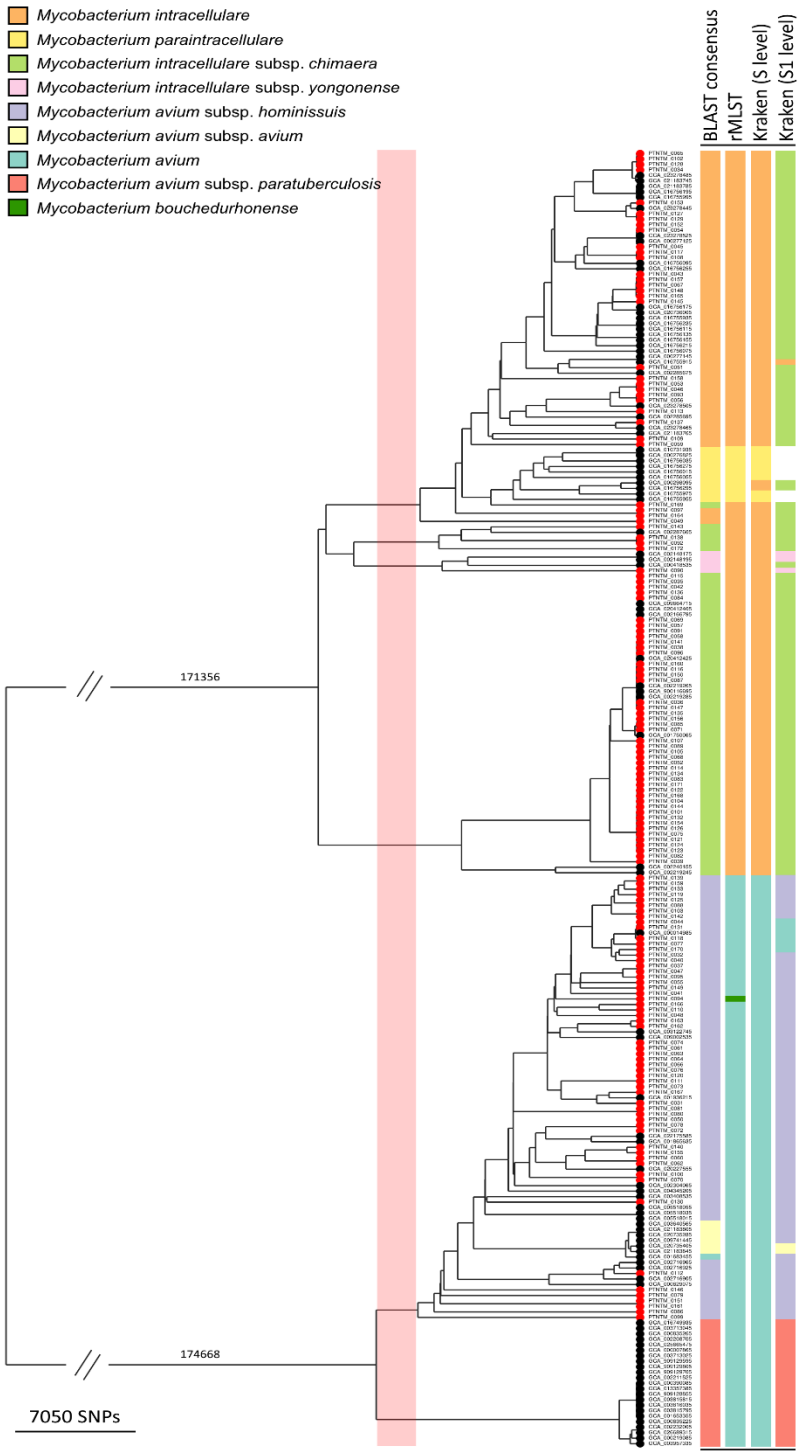

Figure 4.3 - Comparison of different *Mycobacterium avium* complex genomes classification methods with core-SNP-based single-linkage hierarchical clustering. Analysis enrols 97 reference genomes (black nodes) and 139 Portuguese MAC genomes (red nodes). Core-SNP alignment was reconstructed using GCA_009741445 as a reference. BLAST consensus, corresponds to the combined BLAST results for *hsp65*, *groEL1* and *rpoB* genes. Red box highlights the cluster congruence region with BLAST consensus species identification (corresponding to a SNP distance threshold of 12707-15090). Due to scaling cut, length values are presented for the first two branches.

As observed for the reference genome-based analysis (Figure 4.1), an extensive SNP distance threshold range was identified displaying high congruence between genome clustering and species classification (from 12707 up to 15090 SNPs). As such, this allowed the classification of 31 isolates as *M. intracellulare*, 45 as *M. intracellulare* subsp. *chimaera*, one as *M. intracellulare* subsp. *yongonense* and 57 as *M. avium* subsp. *hominisuis*. The only non-congruent classification was observed for isolate PTNTM_0169 that clusters together with the *M. intracellulare* and *M. paraintracellulare* genomes. Of note, at this threshold range, four PT isolates (PTNTM_0143, PTNTM_0138, PTNTM_0092 and PTNTM_0172) clustered with reference genome GCA_002287605 (Figure 4.3). Although these five genome were classified as *M. intracellulare* subps. *chimaera*, their highly divergent genetic background suggests that these might correspond to a potential distinct subspecies of *M. intracellulare* (hence denominated subsp. *chimaera-like*). Noteworthy, these genomes also had ambiguous classifications results for the *hps65* gene (Figure 4.2).

Aiming to further characterize the PT MAC species, the subsequent analysis focused on the two major phylogenetic groups, representing the two predominant species in the dataset: *M. intracellulare* (*M. intracellulare*, *M. intracellulare* subsp. *chimaera, M. intracellulare* subsp. *yongonense and M. intracellulare* subsp. *chimaera-like*) and *M. avium* (*M. avium* subsp. *hominissuis and M. avium* subsp. *avium*).

4.2.3.4 *Mycobacterium avium* species

A draft phylogenetic tree (SL-HC) was reconstructed using a dataset of 104 *M. avium* genomes (57 PT genomes and 47 reference genomes), which enrolled 3825777 sites corresponding to a core-genome of 77.2% (Figure 4.4), with 133716 SNV responsible for the observed genetic diversity. All of our isolates were previously identified as *M. avium subp. hominissuis* (Figure 4.3), and the increase of resolution still showed the clear early separation of the *M. avium* subsp. *paratuberculosis* from other subspecies (mean pairwise distance of 33918±66 between groups). As a means to potentiate the genome group identified as *M. avium* subsp. *avium*, we identified the earliest clustering region (from 16836 up to 19335 SNPs) that separated these from other species groups (Figure 4.4). As such, at this threshold range, four major groups were highlighted: Group A – exclusively composed by genomes classified as *M. avium* subsp. *hominissuis*; Group B – exclusively composed by *M. avium* subsp. *avium* genomes; Group C – composed by highly diverse *M. avium* subsp. *hominissuis* genomes (mostly unclustered at this

threshold range); and Group D – exclusively composed by *M. avium* subsp. *paratuberculosis* genomes.

For a better understanding of the characteristics of the *M. avium* subsp. *hominissuis* strains circulating in Portugal, the phylogeny was reconstructed using only PT genomes (N=57), which

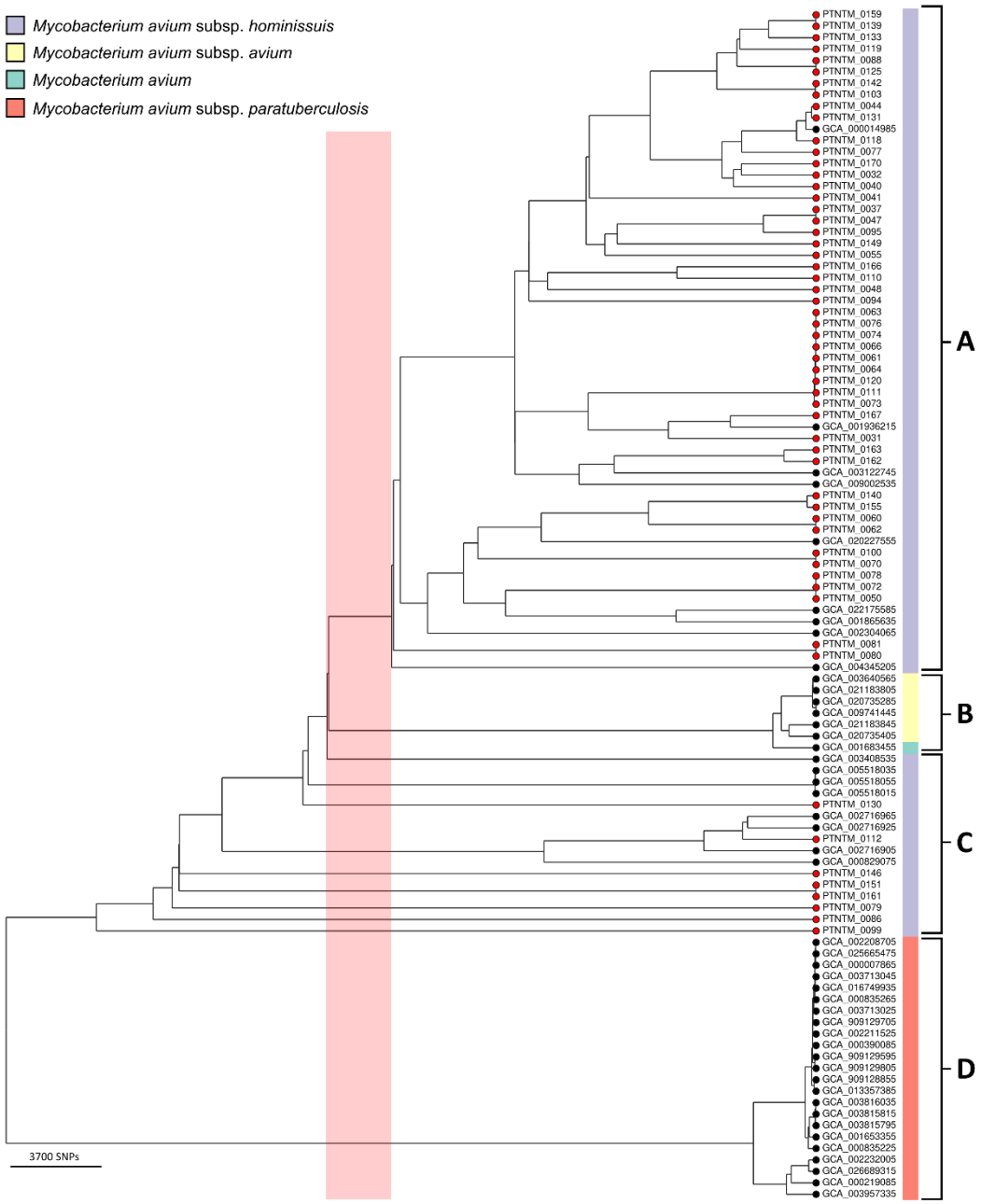

Figure 4.4 - Core-SNP-based single-linkage hierarchical clustering of different *Mycobacterium avium* spp. genomes. Core-SNP alignment was reconstructed using GCA_009741445 as a reference. Analysis enrols 47 reference genomes (black nodes) and 57 Portuguese genomes (red nodes). Red box highlights an extensive stable clustering region (corresponding to a SNP distance threshold of 16,836–19,335) corresponding to the earliest point at which *M. avium* subsp. *avium* genomes cluster together.

now enrolled 4069721 sites corresponding to a core-genome of 81.2% (Figure 4.5). Here, two different groups are included (indicated in Figure 4.5 as A and C) with most isolates presenting high SNP distances (overall mean pairwise distance of 23721±30, and a mean pairwise distance of 29676±36 between both groups), which emphasises the high genetic diversity within this subspecies. Still, although group A is composed by much more isolates than group C (49 *versus* 8, respectively) it displays less diversity within it (mean pairwise distance of 21256±32 versus 30852±35, respectively).

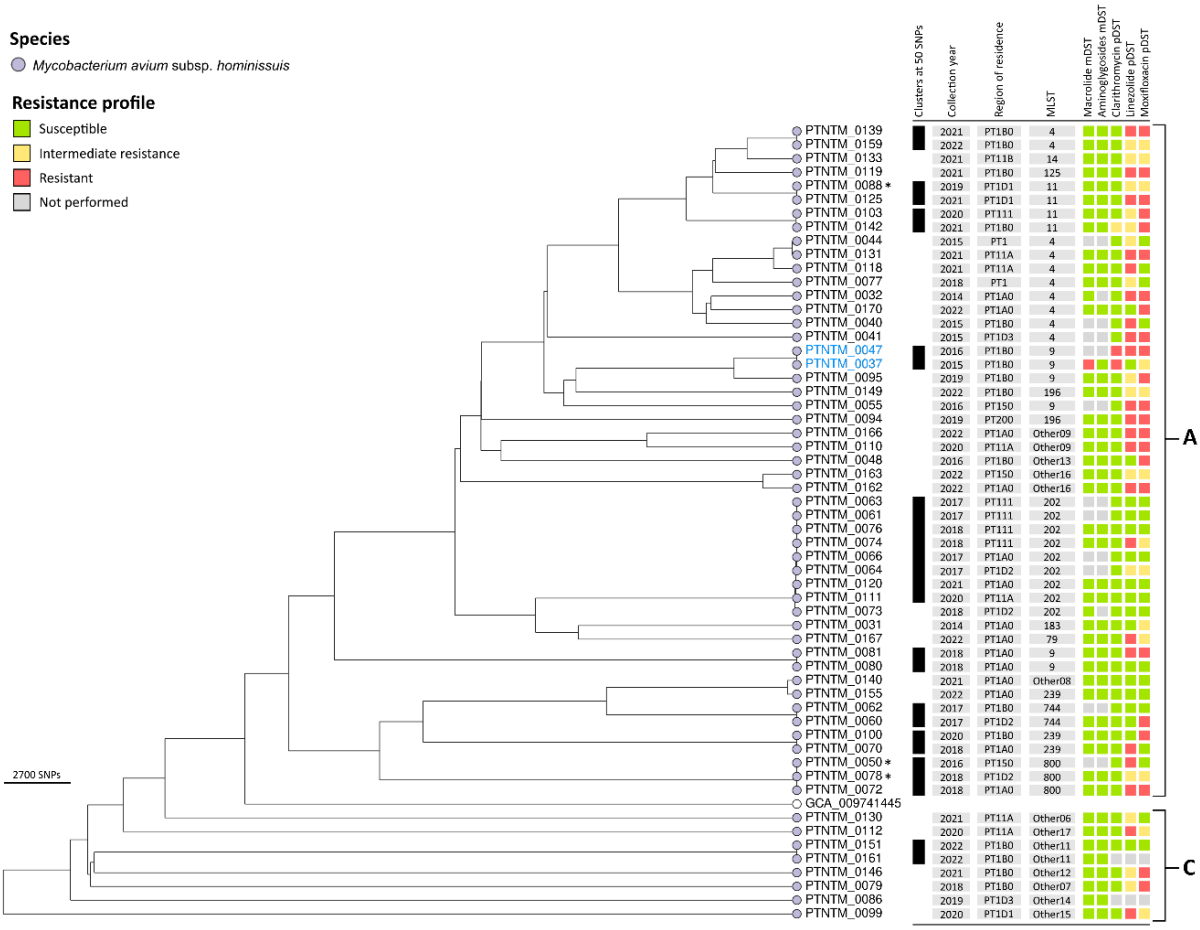

Figure 4.5 - Core-SNP-based single-linkage hierarchical clustering of Portuguese *Mycobacterium avium* species genomes (N=57). Core-SNP alignment was reconstructed using GCA_009741445 as a reference. Coloured sample labels correspond to same patient samples. *Patient with a MAC reinfection with another strain or species in the dataset. Nodes are coloured according to marker-gene consensus identification. Group A and C refer to groups identified in Figure S3. Regions of residence are presented according to the Portuguese NUTIII as: PT1B0 – Lisbon Metropolitan Area; PT11B – Alto Tamega; PT1D1 – Oeste; PT111 – Alto Minho; PT1 – Portugal Continent; PT11A – Porto Metropolitan Area; PT1D3 – Leziria do Tejo; PT1A0 – Setúbal Peninsula; PT150 – Algarve; PT200 – Azores Autonomous Region; PT1D2 – Medio Tejo.

This highlights the high diversity of *M. avium* subsp. *hominissuis* isolates circulating in PT and, as most cases originated in the Lisbon Region (52.6%, with 16 cases from the Setúbal Peninsula

and 14 from the Lisbon Metropolitan Area), the diversity in this geographical region. Within group A, nine close related genetic clusters were identified (≤ 50 SNPs), with the largest cluster enrolling eight isolates susceptible to most antimicrobials tested (only one isolate showed resistance to LNZ), from patients diagnosed between 2017 and 2022, mostly residing in the North region. Five other clusters were composed by isolates collected in the same geographic region, and separated by a mean of one year (ranging from zero up to two years). Within group C, only one genetic cluster was observed, and both cases were from the Lisbon Metropolitan Area and diagnosed in 2022.

Regarding MLST, within this dataset, although most close related isolates possessed the same ST, we observed cases where ST was associated with a polyphyletic structure, namely for ST4 and ST9 (Figure 4.5). Moreover, although 55 unique MIRU-VNTR profiles were generated for the 57 isolates (Figure 4.6), these were not concordant with WGS clustering analysis. In fact, even the isolates from the same patient (PTNTM_0037 and PTNTM_0047) that only differed by less than 40 SNPs did not group with the same MIRU-VNTR code, proving some inefficacy for this molecular typing technique.

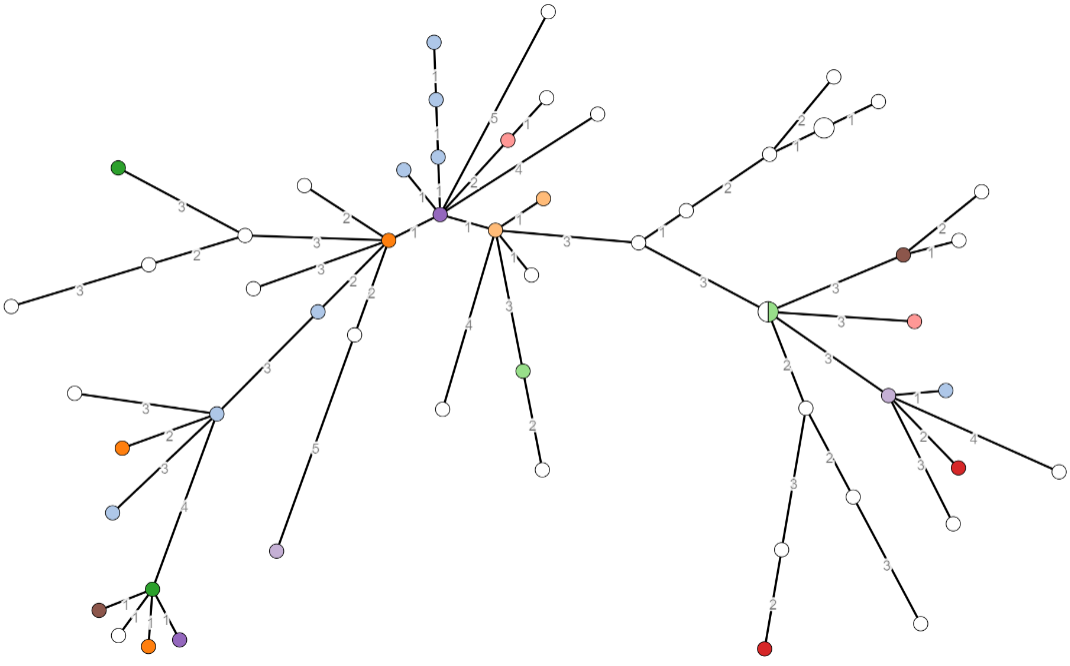

Figure 4.6 - Minimum spanning tree of the MIRU-VNTR allelic profiles for *Mycobacterium avium*. Nodes are coloured according to the identified genetic clusters at ≤ 50 SNP distance by single-linkage hierarchical clustering

4.2.3.5 *Mycobacterium Intracellulare* subspecies

As mentioned above, 82 PT isolates were classified as belonging to *M. intracellulare* species. These strains were isolated from 77 patients (41 males and 36 females), mostly from the Lisbon Region (37 cases from the Setúbal Peninsula and 14 from the Lisbon Metropolitan Area). A draft phylogenetic tree was reconstructed using 132 *M. intracellulare* genomes (82 PT genomes and 50 closed public genomes), which enrolled 4100630 sites corresponding to a core-genome of 75.9% (Figure 4.7). Results revealed an extensive stable clustering region from 26228 up to 39550 SNP distance thresholds where four major groups could be identified: Group E – enrolling *M. intracellulare* and *M. paraintracellulare* genomes; Group F – exclusively enrolling close related *M. intracellulare* subsp. *chimaera* genomes; Group G – enrolling highly divergent *M. intracellulare* subsp. *chimaera* genomes (subsp. *chimaera-like*); and Group H – exclusively enrolling *M. intracellulare* subsp. *yongonense* genomes. Of note, isolate PTNTM_0049 (*M. intracellulare)* is the only genome that segregates early within the phylogeny (Figure 4.7), suggesting a potential distinct *M. intracellulare* subspecies/lineage.

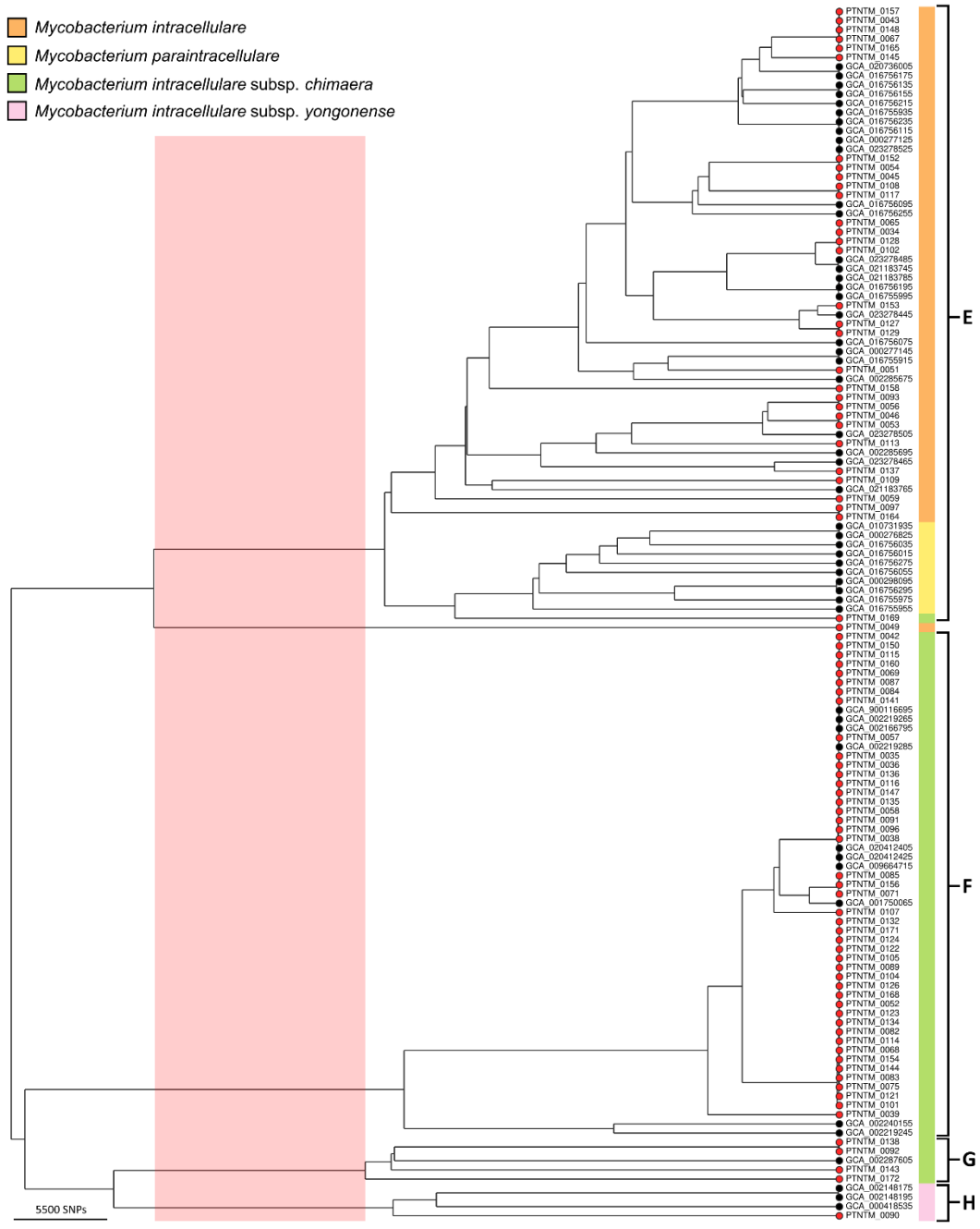

Figure 4.7 - Core-SNP-based single-linkage hierarchical clustering of different Mycobacterium intracellulare species genomes. Core-SNP alignment was reconstructed using GCA_023278525 as a reference. Analysis enrols 50 reference genomes (black nodes) and 82 Portuguese genomes (red nodes). Red box highlights an extensive stable clustering region (corresponding to a SNP distance threshold of 26228-39550).

To inspect PT genomes classified as *M. intracellulare*, a SL-HC phylogenetic tree based on the 31 isolates that composed the group E (identified in Figure 4.7) was reconstructed using GCA_023278525 as a reference genome, which enrolled 4628344 sites (corresponding to a core-

genome of 85.6%) and 113876 SNVs (Figure 4.8). Only one isolate of this group had been classified as *M. intracellulare* subsp. *chimaera* (PTNTM_0169) by the maker-based consensus method. Although this isolate seems to be genetically more related to the *M. intracellulare* group, and was therefore included in this analysis, it segregates in a more distant branch, being separated from the rest of the group by a mean of 41101±95 SNPs. Most isolates were collected between 2020 and 2022 (61.2.%) from the Lisbon Region (48.4%, which includes the Lisbon Metropolitan Area and the Setúbal Peninsula).

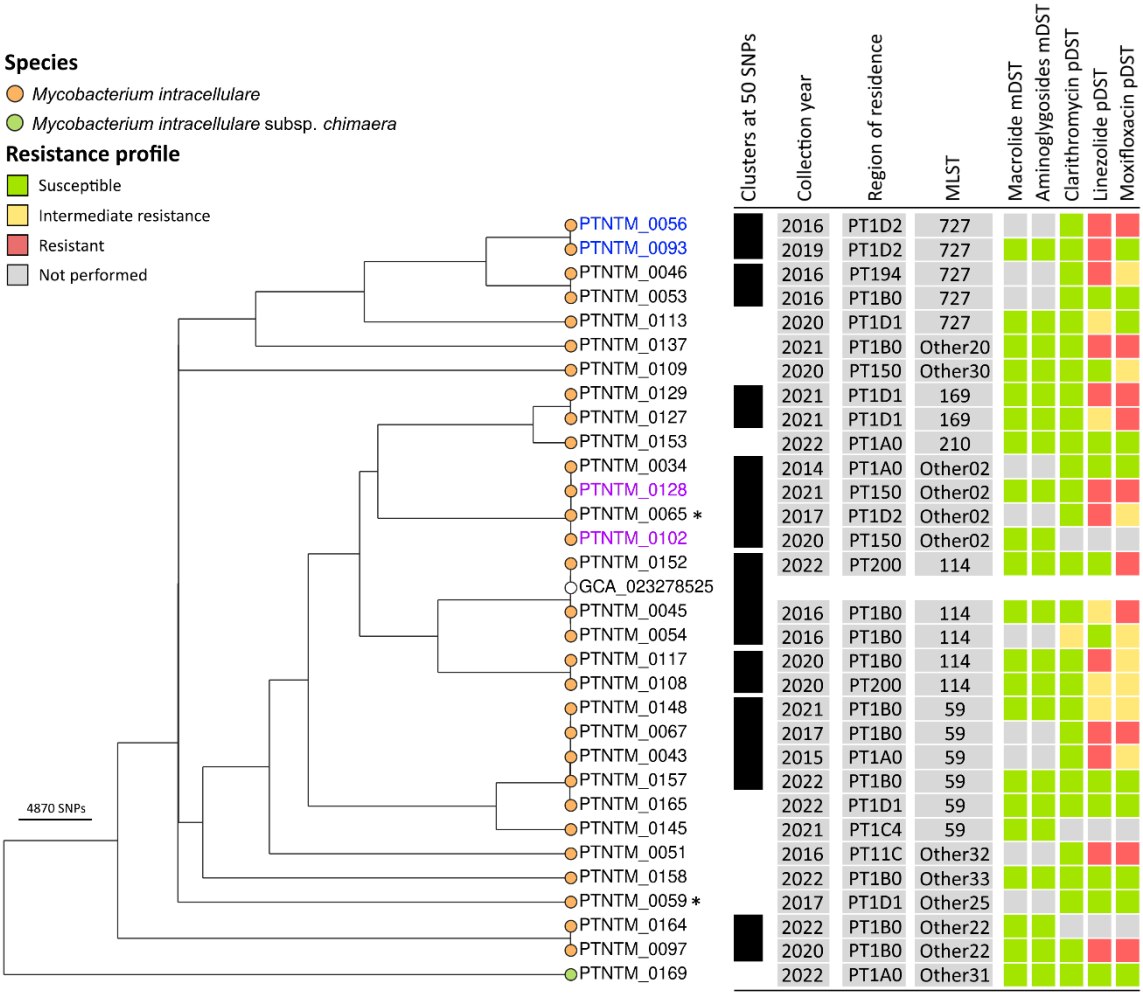

Figure 4.8 - Core-SNP-based single-linkage hierarchical clustering of Portuguese *Mycobacterium intracellulare* genomes (N=31). Core-SNP alignment was reconstructed using GCA_023278525 as a reference. Coloured sample labels correspond to same patient samples. *Patient with a MAC reinfection with another strain or species in the dataset. Nodes are coloured according to marker-gene consensus identification. Regions of residence are presented according to the Portuguese NUTIII as: PT1D2 – Medio Tejo; PT1B0 – Lisbon Metropolitan Area; PT1D1 – Oeste; PT150 – Algarve; PT200 – Azores Autonomous Region; PT1A0 – Setúbal Peninsula; PT11C – Tamega e Sousa; PT194 – Viseu Dao Lafoes; PT1C4 – Central Alentejo.

Within this subspecies, only one strain displayed an intermediate resistance phenotype to CLR, whereas twelve and ten strains exhibited resistant phenotypes to LNZ and MXF, respectively. Notably, none of the strains showed mutations within the *rrs* or *rrl* genes using mDST. Applying the conservative 50 SNP threshold defined above (see methods section) eight close related genetic clusters were revealed (Figure 4.8), two of these enrolling known same-patient samples. Still, in general, all clusters included isolates from distinct geographic regions and spanning different collection years. MLST typing for *M. intracellulare* showed a high congruence with the obtained phylogeny at a SNP threshold of 9226 up to 13390, highlighting its usefulness for long-term surveillance purposes. In contrast, MIRU-VNTR did not show good typing resolution, as isolates clustered by WGS were not grouped using MIRU-VNTR (Figure 4.9).

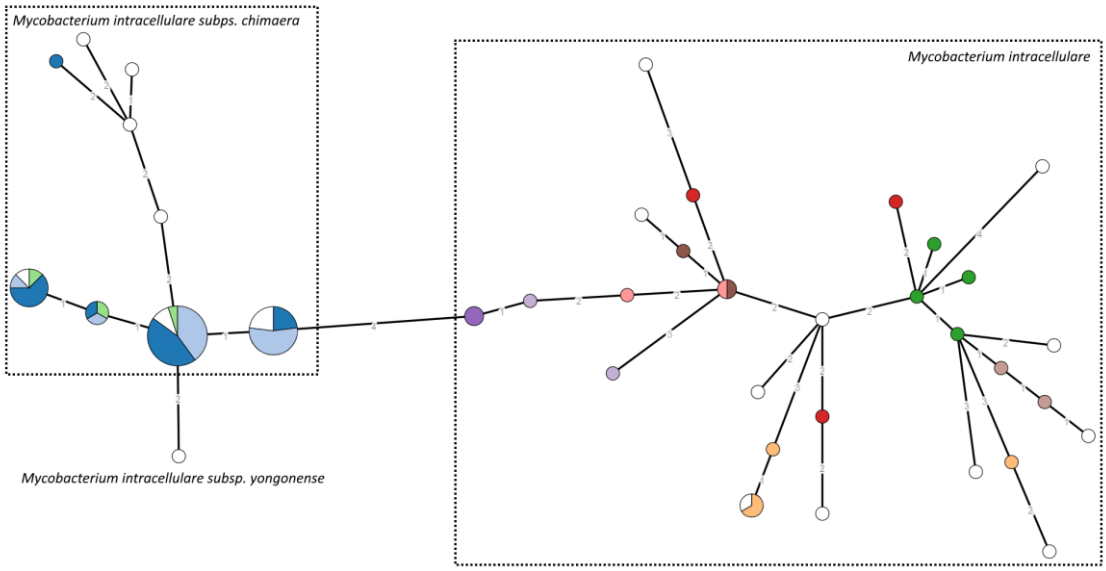

Figure 4.9 - Minimum spanning tree of the MIRU-VNTR allelic profiles for *Mycobacterium intracellulare* subsp. Nodes are coloured according to the identified genetic clusters at ≤ 50 SNP distance by single-linkage hierarchical clustering.

Regarding *M. intracellulare* subsp. *chimaera*, using 45 PT isolates (Figure 4.7; Group F) and the reference genome GCA_002219285, a draft phylogenetic tree was constructed, relying on 5038386 sites (corresponding to a core genome of 85.9%) and 29189 SNV (Figure 4.10). Two main genetic groups could be identified (F1 and F2 in Figure 4.10), with two additional isolates displaying a mean pairwise distance of 15687±19 to these groups (PTNTM_0039 and PTNTM_0107). Group F1 and F2 were separated by a mean pairwise distance of 8715±30 (mean within each group of 1333±10 and 31±2, respectively). Group F1 includes 22 isolates from 2014 up to 2022, mostly collected in the Setúbal Peninsula (54.5%), and exhibits two major

clusters separated by ~5000 SNPs. Close inspection of these shows a close related genetic cluster (< 50 SNPs), enrolling 17 and 3 isolates, respectively. None of the isolates in F1 showed resistance to CLR and only three and two isolates showed resistance to LNZ and MXF, respectively. As with F1, group F2 includes 21 isolates from 2015 up to 2022, mostly collected in the Setúbal Peninsula (85.7%), and exhibits a single large close-related genetic cluster composed of 19 isolates. All the isolates were sensitive to CLR and only two showed phenotypic resistance to LNZ. Still, 38% of isolates within F2 were resistant to MXF. Regarding aminoglyco-

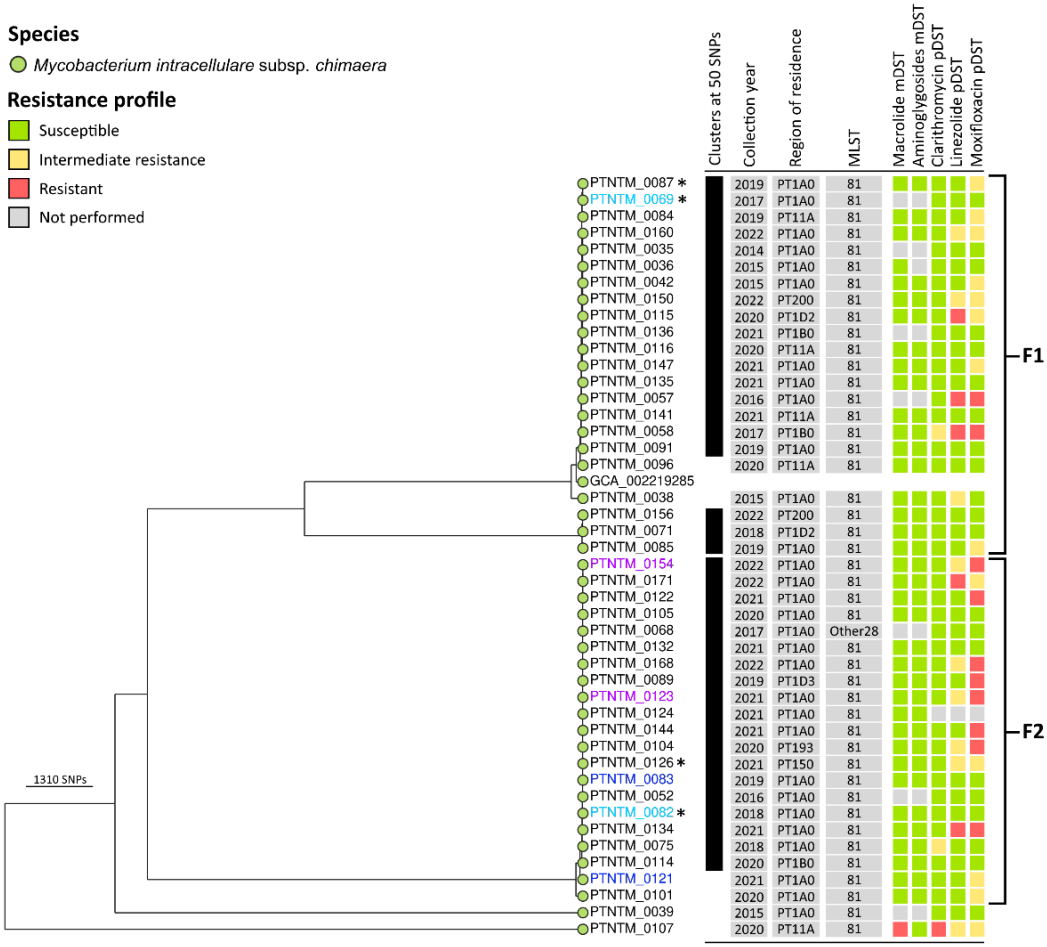

Figure 4.10 - Core-SNP-based single-linkage hierarchical clustering of Portuguese *Mycobacterium intracellulare subsp. chimaera* genomes (N=45). Core-SNP alignment was reconstructed using GCA_002219285 as a reference. Coloured sample labels correspond to same patient samples. *Patient with a MAC reinfection with another strain or species in the dataset. Nodes are coloured according to marker-gene consensus identification. Regions of residence are presented according to the Portuguese NUTIII as: PT1B0 – Lisbon Metropolitan Area; PT1D2 – Medio Tejo; PT200 – Azores Autonomous Region; PT1A0 – Setúbal Peninsula; PT11A – Porto Metropolitan Area; PT1D3 – Leziria do Tejo; PT193 – Leiria Region; PT150 – Algarve.

sides and macrolides mDST, none of the isolates from both groups harboured genetic alterations associated with resistance. In fact, only the divergent isolate PTNTM_0107 was presumed resistant to macrolides by both mDST and pDST. Of note, although three pairs of same-patient samples were identified in this dataset, only one (PTNTM_0154 / PTNTM_0123) clustered together at < 50 SNPs.

Additionally, all isolates shared the same ST81 (with the exception of PTNTM_0068) likely due to the close genetic relatedness within this subspecies. In fact, when analysing all isolates identified as *M. intracellulare* subsp. *chimaera* [i.e., including the additional four genetically divergent *chimaera-like* isolates (group G in Figure 4.7)], all subspecies *chimaera-like* presented distinct MLST profiles (Supplementary Table S5). Again, this highlights MLST typing adequacy for long-term surveillance of *M. intracellulare* species, and supports the suggestion that the *chimare-like* isolates are genetically different from the rest of the *M. intracellulare* subsp. *chimaera* strains circulating in Portugal, as well as most reference genomes similarly classified. Nevertheless, once again, MIRU-VNTR showed poor sensitivity, as it showed low congruence with the identified clusters by WGS (Figure 4.9).

4.2.3.6 Re-Infection or Persistence

Within our dataset, for ten patients we retrieved more than one sample, collected more than six months apart, in order to evaluate possible cases of persistence of infection. In four cases, re-infection was clear as each patient was infected with isolates belonging to different MAC species (highlighted in Figure 4.5, 21 and 23, with details in Supplementary Table S5), i.e., for patient 34 (PTNTM_0087 / PTNTM_0049), patient 37 (PTNTM_0059 / PTNTM_0088), patient 43 (PTNTM_0126 / PTNTM_0050) and patient 136 (PTNTM_0065 / PTNTM_0078). Taking advantage of the obtained complete genomes, for the other six cases, a fine-tuned paired analysis was conducted revealing, as observed above in the subspecies analysis, ≤ 40 genetic alterations (either SNPs or indels, ranging from one up to 40) between isolates from five patients, namely patient 13 (PTNTM_0121 / PTNTM_0083), 20 (PTNTM_0087 / PTNTM_0049), 30 (PTNTM_0154 / PTNTM_0123), 49 (PTNTM_0102 / PTNTM_0128), and 101 (PTNTM_0056 / PTNTM_0093). Considering that most paired isolates were collected within a one- or two-year interval, these data suggest that these cases may constitute persistent infections with strains displaying within-patient evolution. Of note, for patient 101, even though isolates were collected three years apart, only one SNP difference was observed between them. For patient 130

(PTNTM_0069 / PTNTM_0082) isolates showed > 7000 genetic differences between them, suggesting that, although both isolates belonged to *M. intracellulare subps. chimaera*, patient was, at some point, re-infected with a different strain (Figure 4.10).

4.2.4 Discussion

Currently, there is still a huge lack of knowledge not only on the global distribution of MAC subspecies but also on methodologies used for their accurate identification. This study aimed to enrich comprehension on MAC diversity and characterize the population of MAC isolates circulating in Portugal by integrating them within the available reference MAC genomes and applying different methodological approaches for species classification. To achieve this, we analysed 142 isolates collected in Portugal between 2014 and 2022, mainly from male patients (51.5%) from Lisbon region (59.86%). MAC isolates were mostly susceptible to CLR (3.0% of resistant isolates), reinforcing its use as a primary therapeutic option, whereas only about half of the isolates were susceptible to LNZ and MXF (49.3% and 41.8%, respectively).

As highlighted in Figure 4.3, MAC seems to be composed by two species (*Mycobacterium avium* and *Mycobacterium intracellulare groups*), however, by performing a deeper analysis of each of this species we clearly see the emergence of smaller groups of additional major lineages within each of these major branches. Using different classification approaches (Kraken, rMLST and marker-gene based results) we were able to classify our isolates at the subspecies level. The results obtained with the marker-gene based consensus approach were the most congruent with the SNP tree analysis of each species (Figure 4.2 and 16). Thus, we believe that, although there is still much to debate concerning taxonomic classification of MAC (Mizzi et al., 2022), this approach may be useful for quick classification of isolates at the species and subspecies level, proving its efficacy for identification purposes. Other studies have also reported the advantage of using the analysis of the *rpoB groEL-1 and groEL-2 (hsp65)* genes for NTM identification (Chawla et al., 2023; Rindi & Garzelli, 2014; Van Ingen et al., 2018). In fact, Chawla and colleagues, the first suggesting the use of a consensus analysis of these genetic markers, also reported sufficient discriminatory power for accurate MAC subspecies identification (Chawla et al., 2023). Still, the differences observed in classification for the distinct methodologies also highlights the need to have larger and more diverse genomic datasets, particularly relevant for underrepresented species (such as *M. lepraemurium* or *M. mantenii*). Additionally, powerful software such as Kraken, which relies on known genomic information, would benefit from

this to perform identification of MAC species (namely up to the subspecies level). Although we found a general agreement between the phylogenetic analysis and the taxonomic classification determined by the marker-gene consensus method, highly divergent strains were also observed. Thus, additional genome-scale data would strengthen our understanding on the population structure of MAC while helping in the establishment of a more robust taxonomic classification of the species of this complex.

Phylogenetic analysis of the PT *M. avium* isolates, revealed, not only that all analysed cases were caused by *M. avium* subsp. *homissuis*, but also a division into two potential highly genetically distinct groups (Figure 4.5, groups A and C). These results are in line with previous genomic studies that also showed great diversity among the isolates of this subspecies, which is probably related to the different environmental niches, different hosts and even different disease outcomes(Iwamoto et al., 2012; Mizzi et al., 2022; Uchiya et al., 2017). Noteworthy, we observed that PT *M. avium* isolates were more susceptible to LNZ than previous reported published data (32.7% vs 24.7%) (Cho et al., 2018). Although the majority of our cases of *M.avium* were collected in the Lisbon region and we observed at least 10 clusters of close related isolates (≤ 50 SNPs), no conclusions regarding their linkage can be drawn since the LNR-TB does not have access to clinical/epidemiological data, thereby hindering the establishment of relations between molecular and potential epidemiological links

It is still not established how to taxonomically classify *M. intracellulare* group and some authors even consider that *M. chimaera* is a subspecies of *M. intracellulare* (Nouioui et al., 2018). Our study revealed the existence of three phylogenetically distinct groups, separating the isolates of *M. intracellulare*, *M. intracellulare* subsp. *chimaera* and *M. intracellulare* subsp. *yongonense*, as well as a potential fourth group composed of *M. intracellulare* subsp. *chimaera-like* isolates (Figure 4.7, groups E, F, H and G, respectively). However, although *M. intracellulare* subsp. *yongonense* genomes clearly diverge from the remaining *M. intracellulare* genomes, we highlight the lack of both PT isolates and publicly available reference genomes, hindering the clarification whether *M. intracellulare* subsp. *yongonense* is really a subspecies or just a variant of *M. chimaera* (Castejon et al., 2018; Tortoli et al., 2019; Van Ingen et al., 2018). Still, even with limited data, the phylogenetic analysis conducted on the present study supports the former being more likely than the latter given their early divergence from the large *M. intracellulare* subp. *chimaera* (Figure 4.3). It has already been described that isolates of *M. chimaera* are linked to outbreaks associated with hospital heater-cooler units (Quintás Viqueira et al., 2021; Uchiya

et al., 2017), suggesting this subspecies is more likely to contaminate/inhabit these hospital niches than the other species of the complex, again suggesting well defined associations between origin and causative agent of infection. In fact, previous studies focusing on the genomic characterization of *M. intracellulare* subsp. *chimaera* isolates associated with the occasional contamination of heater-coolers, and subsequently cardiac surgery patients, revealed that those were caused by very genetically related strains (Lecorche et al., 2021; Schreiber et al., 2021) regardless of country of origin, and thus suggesting the existence of potential international highly fitted dominant lineages. This could lead to the assumption that, as already demonstrated for other NTM diseases, particularly, *M. abscessus* (Carneiro et al., 2023; Diricks et al., 2022), there may also exist Dominant Circulating Clones (DCC, terminology used for *M. abscessus*). In our dataset, we also observed low genetic diversity between *M. intracellulare* subsp. *chimaera* isolates, and only two major clusters (groups F1 and F2 in Figure 4.10) were identified suggesting the existence of potential DCC in Portugal. Nevertheless, more studies focused on the genomic comparison of isolates collected from environmental sources and isolates from patients worldwide are needed. Regarding antimicrobial resistance, contrary to previous reports (Fernandez-Pittol et al., 2022), PT *M. intracellulare* subsp. *chimaera* isolates were more susceptible to MXF (52.03%) compared to those of *M. avium* (34.5%) and *M. intracellulare* (35.7%). Once more, we believe that additional data are still needed to understand the differences in susceptibility patterns among MAC isolates across different geographical regions. It could be speculated that variations in antibiotic susceptibility patterns may be linked to delayed clinical diagnostics or to therapeutic schemes not guided by DST results, promoting the spread of more resistant strains.

WGS analysis allowed the identification of five cases of potential persistence of infection with the isolates showing less than 40 genetic alterations (including SNPs or indels) and five cases of clear reinfections, four of which with different species of MAC and one with the same subspecies. In fact, although poorly documented, cases of relapse with the same or different MAC species have also been reported (Boyle et al., 2016; B. S. Kwon et al., 2019; Wallace, Jr. et al., 2002). This high number of cases of reinfection (~4% of the total cases analysed) are also an evidence of the diversity of the MAC species in the environment and, consequently, high human exposure to potential sources of infection. Persistent cases, however, may be associated with treatment failure.

Regarding the limitations of this study, we highlight our lack of access to clinical data of the patients. We believe that it could have highly benefited from more detailed clinical and demographic relevant information in order to eventually relate the genomic data to distinct infection sources and/or disease outcomes of each patient, or to flag potential epidemiological links in the context of Public Health investigations. It is also of note that MAC disease is not mandatory notifiable, which also contributes to the lack of information, and there are no guidelines to the follow up on the diagnosed patients, leading, in some cases, to a potential chronic disease, that could otherwise be cured.

In order to achieve a global understanding of MAC in a One Health perspective, indiscriminate large-scale WGS data of MAC clinical and environmental isolates is needed both to establish associations between MAC species/subspecies and human disease outcomes and to reliably identify common sources of infection for epidemiological purposes. Moreover, establishing an appropriate genotyping approach for each MAC species is essential in managing disease progression, as it has been shown that identifying MAC subspecies is key for prioritizing patient treatment (Pan et al., 2021). We believe that by improving our understanding of the disease-subspecies dynamics in MAC infections, the application of WGS will have an impact on patient care that goes beyond laboratory diagnosis.

5

DEVELOPMENT OF A LABORATORY AND BIOINFORMATICS-BASED TOOL FOR RAPID DIAGNOSIS AND SURVEILLANCE OF NTM DISEASE

Ongoing work

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Personal Contribution

SC contributed to the design of the study, experimental work, literature review and wrote the manuscript.

5.1 Introduction

Nontuberculous mycobacteria (NTM) are increasing worldwide and are becoming a major public health problem (Zulu et al., 2021). Although NTM disease was initially related to immunocompromised patients, nowadays some NTM species have become clinically significant in both immunocompromised and immunocompetent patients (Bhanushali et al., 2023). Among the more than 180 NTM species identified to date, those belonging to *Mycobacterium avium* complex (MAC) and *Mycobacterium abscessus* complex (MABC) stand out as the most common cause of human disease (Hendrix et al., 2023; Parte, 2018; Santos et al., 2024).

Despite the growing importance of these infections, accurate data on the incidence and prevalence of NTM disease remains limited, mainly because it is not a notifiable disease in most countries. Nevertheless, recent data show that the number of cases has doubled in the last decade (Queensland Health, 2024; R. Thomson et al., 2017). This emphasises the urgent need to understand the challenges in diagnosing and treating NTM infections.

However, a definitive diagnosis of NTM disease is challenging. Not only NTM disease share some similarities with tuberculosis, which may delay and confuse the clinical diagnosis, but also a laboratory isolation of a NTM does not necessarily confirm the cause of infection. This Is due to the fact that NTM are ubiquitous in the environment and there is a strong probability that it may be a transient colonization of the patient or a contamination of the biological sample (Griffith et al., 2007). Thus, these challenges hinder the start of the treatment and even the clinical decision to continue to monitor the patient´s symptoms (Conyers & Saunders, 2024).

Treatment of NTM disease is equally challenging. NTM generally exhibit intrinsic and acquired resistance mechanisms to antibiotics and multi-drug regimens are associated with serious adverse effects. These regimens, which usually last at least 12 months with three different antibiotics (generally, ethambutol and rifampicin and a macrolide), have shown variable success rates. Indeed, it has been demonstrated that around 10 to 60% of patients experience a relapse or reinfection after completing the treatment (American lung Association, n.d.; Griffith et al., 2007; Koh, Moon, et al., 2017; B. S. Kwon et al., 2019; B. Y. Lee et al., 2015).

It is therefore clear that current approaches (empirical treatment) need to be revised, underlining the importance of adjusting the therapeutic regimens according to the resistance pattern of the infecting NTM in order to improve the patient´s outcomes. The gold standard drug susceptibility testing (DST) for NTM is based on broth microdilution, requiring a previous isolation of the bacteria in solid media which, in some cases, may result in delayed diagnostics

as some of the NTM species are slow growers (Calado Nogueira de Moura et al., 2023; Woods et al., 2011). Furthermore, phenotypic DST is not yet completely standardized and its interpretation may be inaccurate (Carneiro, n.d.; Kumar et al., 2022; J. V. Philley et al., 2016).

To overcome these limitations, and using the potential of technologies based on whole genome sequencing (WGS), studies on NTM are now increasing worldwide allowing a better understanding on the diversity of circulating strains, and the molecular mechanisms associated with resistance and virulence. (Carneiro et al., 2023; Dohál et al., 2021; T. Q. Nguyen et al., 2024; Solanki et al., 2022; Yoon et al., 2020). However, WGS-based methodologies still require the isolation of the NTM species in culture media to obtain sufficient quality and yield of genomic DNA for analysis.

Targeted Next Generation Sequencing (tNGS) has been suggested as an alternative, as it allows the detection of specific genetic regions directly from the patient´s biological sample without need for prior strain isolation (Huang et al., 2023; Liu et al., 2024; Schwab et al., 2024; Smith et al., 2020). This methodology relies on the simultaneous amplification of multiple target regions of interest, allowing both identification and resistance mutations, and minimizing the interference of contaminant flora that are often present in biological samples. In addition, using a tNGS approach with a MinION platform (a nanopore-based portable DNA/ RNA sequencing device for real-time sequencing), its application directly in the clinical laboratories is possible, making it a useful and accessible solution for the complete diagnosis of NTM (Jain et al., 2016).

In the present study, we aim to develop a tNGS methodology for MAC and MABC identification and resistance prediction, using an amplicon-based approach including 38 genomic regions, targeting 22 regions of interest both for the classification of species and subspecies and for the detection of genetic mutations associated with resistance.

5.2 Materials and methods

5.2.1 Bacterial strains and DNA extraction

We selected 31 strains from different species for developing the proposed methodology. They included 6 *M. avium* subsp. *hominissuis*, 4 *M. intracellulare* , 4 *M. chimaera*, 5 *M. abscessus*, 5 *M. bolletii*, 5 *M. massiliense*, 1 *M. marseiilense*, and 1 *M. colombiense,* which were previously identified to subspecies level using WGS. Phenotypic and genotypic resistance prediction to antibiotics had also already been performed in the previous studies.

We re-isolated the strains in solid media and performed DNA extraction using QIAamp DNA Mini Kit (Qiagen, Düsseldorf, Germany) according to manufacturer's Instructions after a previous heat inactivation step at 95ºC for 30 min. DNA concentration was determined using the Qubit Fluorometer with dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA).

5.2.2 Selection of the regions of interest

Using the International database PubMed (https://pubmed.ncbi.nlm.nih.gov/), we searched for relevant scientific peer-reviewed papers that described antimicrobial resistance (AMR) markers and MAC and MABC subspecies identification markers. Our review of 32 scientific papers led us to the definition of 27 regions of interest, nine of which were for speciation and 21 for resistance prediction to different classes of antibiotics, such as aminoglycosides, macrolides, fluoroquinolones and rifamycin (Table 5.1).

Table 5.1 - List of the genetic markers used for the amplicon-based tNGS approach.

REF* - scientific paper accessed.

Resistance markers

Locus_TAG	**Gene**	**NTM species**	**Antibiotic target**	**REF**
MAB_0185c	*embB*	*M. abscessus* complex	Ethambutol	(Alcaide et al., 1997)
MAB_0019	*gyrA*	*M. abscessus* complex	Fluroquinolones	(Guillemin et al., 1998)
MAB_0006	*gyrB*	*M. abscessus* complex	Fluroquinolones	(Guillemin et al., 1998)
MAB_4384	Transcriptional TetR repressor	*M. abscessus* complex	Thiacetazone	(Halloum et al., 2017; Richard et al., 2018)
MAB_2299c	Putative TetR transcriptional regulator	*M. abscessus* complex	Clofazimine and bedaquiline	(Gutiérrez et al., 2019; Richard et al., 2019)
MAB_2297	*erm41*	*M. abscessus* complex	Macrolides	(H.-Y. Kim et al., 2010; Nash et al., 2009)
MAB_0591	*ArrMab*	*M. abscessus* complex	Rifampicin	(Rominski, Roditscheff, et al., 2017)
MAB_2875	*BlaMab*	*M. abscessus* complex	Cefoxitin and Imipenem	(Dubée et al., 2015; Luthra et al., 2018)
MAB_4532c	*Eis2*	*M. abscessus* complex	Aminoglycoside	(Dubois et al., 2019; Ung et al., 2019)
MAB_2385	*MAB_APH*	*M. abscessus* complex	Streptomycin	(Dal Molin et al., 2018)

MAB_r5051	*rrs*	*M. abscessus* complex	Aminoglycoside	(Chew et al., 2021; H. Li et al., 2022; Nessar, Reyrat, Murray, et al., 2011; Prammananan et al., 1998)
MAB_r5052	*rrl*	*M. abscessus* complex	Macrolide	(Bastian et al., 2011; Bronson et al., 2021; Lipworth et al., 2019; Maurer et al., 2012; Wallace et al., 1996)
MAV_2512	Transcriptional regulator	*M. avium* complex	Bedaquiline	(Zweijpfenning et al., 2019)
MAV_1406	efflux pump	*M. avium* complex	Macrolide	(Rindi, 2020; Schmalstieg et al., 2012)
MAV_3306	efflux pump	*M. avium* complex	Macrolide	(Rindi, 2020; Schmalstieg et al., 2012)
23S rRNA	*rrl*	*M. avium* complex	Linezolid and macrolide	(S.-Y. Kim et al., 2019; Moon et al., 2016)
16S rRNA	*rrs*	*M. avium* complex	Aminoglycoside	(Brown-Elliott et al., 2013; S.-Y. Kim et al., 2021)
ND	*rplD*	*M. avium* complex	Linezolid	(S.-Y. Kim et al., 2019)
Species identification markers				
Locus_TAG	**Gene**	**NTM species**	**REF**	
ND	DT1	*M. intracellulare*	(S. J. Shin et al., 2010)	
ND	IS1311	*M. avium subsp. hominissuis*	(S. J. Shin et al., 2010)	
ND	SR1	*M. chimaera*	(Zozaya-Valdés et al., 2017)	
MAUC22_07270	MAUC22_07270	*M. abscessus*	(Akwani et al., 2022)	
MAUC_18635	MAUC_18635	*M. abscessus*	(Akwani et al., 2022)	
MASB_RS22045	MASB_RS22045	*M. bolletii*	(Akwani et al., 2022)	
MASB_RS03355	MASB_RS03355	*M. bolletii*	(Akwani et al., 2022)	
MMAS-JCM_0836	MMASJCM_0836	*M. massiliense*	(Akwani et al., 2022)	

MMAS-JCM_0834	MMASJCM_0834	*M. massiliense*	(Akwani et al., 2022)

5.2.3 Primer design

In order to design the primers for the previously selected regions of interest, we used two different bioinformatics tools: Primer-Blast (https:// www.ncbi.nlm.nih.gov/tools/primer-blast/) and PrimalScheme (https:// primalscheme.com/) softwares.

To guarantee sufficient genetic diversity, all the genomic MABC/MAC sequences for the selected target genes available in the NCBI Nucleotide collection (nt/nr) database were retrieved using the blastn suite (https://blast.ncbi.nlm.nih.gov/Blast.cgi; last accessed in January 2024). All redundant genomic sequences (i.e., genomes that shared the exact same sequences for the regions of interest under analysis) were discarded. As such, for identification of resistance markers, we used a total of 746 sequences from MABC and 269 sequences from MAC. For species/subspecies identification markers, we used 25 *M. intracellulare* sequences, 59 *M. avium* subsp. *hominissuis*, one *M. chimaera*, 47 *M. abscessus*, 28 *M. bolletii,* and 29 *M. massiliense*. Amplicon sizes ranged from 340-740 bp. Both the specificity of the primers and self-dimers and cross-dimers presence was checked using Primer-Blast (Table 5.2).

Table 5.2 - Detailed information on the selected regions of interest, the associated resistance determinant, **M. tuberculosis* H37Rv numbering; ** *E.coli* numbering; P/A - presence or absence

Locus_TAG	***Gene***	***Reference genome***	***Resistance determinant***	***Number of sequences used***
MAB_0185c	*embB*	*M. abscessus ATCC 19977*	I303Q; L304M*	84
MAB_0019	*gyrA*	*M. abscessus ATCC 19977*	NAla90; NAsp94*	100
MAB_0006	*gyrB*	*M. abscessus ATCC 19977*	D461N; R482; N499*	89
MAB_4384	Transcriptional TetR repressor	*M. abscessus ATCC 19977*	C32 insert; G40A; T169C ; T2C; A304 del, T305C; A334del	31
MAB_2299c	Putative TetR transcriptional regulator	*M. abscessus ATCC 19977*	T119G–L40W; C276del-P92fs; G541T-E181stop; Ins318A-D106fs; T452c–L151P; G643A-G215S	32

MAB_2297	*erm41*	*M. abscessus ATCC 19977*	T28; frameshit in *M. massiliense* (two nucleotes and/or 274 nucleotides	40
MAB_0591	*ArrMab*	*M. abscessus ATCC 19977*	P/A	39
MAB_2875	*BlaMab*	*M. abscessus ATCC 19977*	P/A	73
MAB_4532c	*Eis2*	*M. abscessus ATCC 19977*	P/A	58
MAB_2385	*MAB_APH*	*M. abscessus ATCC 19977*	P/A	59
MAB_r5051	*rrs*	*M. abscessus ATCC 19977*	A1375N; T1373N; C1376N; A1374N; G1458T	41
MAB_r5052	*rrl*	*M. abscessus ATCC 19977*	G795A, A2270N, A2271N, G2281N; A2293N	100
MAV_2512	Transcriptional regulator	*M. avium subsp. hominissuis* 104	173W>173R|2544950A>G	45
MAV_1406	efflux pump	*M. avium subsp. hominissuis* 104	P/A	37
MAV_3306	efflux pump	*M. avium subsp. hominissuis* 104	P/A	85
23S rRNA	*rrl*	*M. avium subsp. hominissuis 104 and M. intracellulare ATCC* 13949	Point mutations in 2279 and 2280 (*M. avium* strains)**; G2599A (*M. avium* strains)** and A2137T (*M. intracellulare* strains)**	32
16S rRNA	*rrs*	*M. avium subsp. hominissuis 104*	A1408G; C1496T; G1491C; G1491T; C1409T**	34
ND	*rplD*	*M. avium subsp. hominissuis 104 and M. intracellulare ATCC* 13949	A439G (*M. avium* strains)** and G443A (*M. intracellulare* strains)**	36
Identification				
Locus_TAG	**Gene**	**Reference genome**	**Identification Marker**	
ND	DT1	*M. intracellulare* ATCC 13950	P/A	25
ND	IS1311	*M. avium subsp. hominissuis* 104	P/A	59
ND	SR1	*M. chimaera* DSM 44623	P/A	1
MAUC22_07270	ND	*M. abscessus* UC22	P/A	30
MAUC_18635	ND	*M. abscessus* UC22	P/A	17
MAUC_RS22045	ND	*M. bolletii BD*	P/A	13
MASB_RS03355	ND	*M. bolletii BD*	P/A	15

MMASJCM_0836	ND	*M. massiliense* JCM15300	P/A	14
MMASJCM_0834	ND	*M. massiliense* JCM15300	P/A	15

5.3 Optimization of the multiplex PCR and subsequent NGS analysis

For genes encoding 16S and 23S regions (*rrl, rrs)* responsible for macrolide and aminoglycoside resistance, respectively, and *rplD* gene, responsible for macrolide resistance, we were unable to define sufficiently specific primers. These regions are highly conserved in most bacteria and, considering that the majority of our biological samples were respiratory and thus highly contaminated samples, we could not guarantee sufficient specificity.

Therefore, from the 27 selection regions of interest we screened 22 regions. We used 32 primers pairs, of which, 14 are for species/ subspecies identification and 18 for AMR prediction (Table 5.3). Of note, in order to have some redundancy aiming at increasing sensitivity, more than one primer pair was designed for some genes.

Table 5.3 - Detailed information regarding amplicons including, primer sequence and reference genome.

Reference Genome	*Region of interest*	*Primers*	*Primer sequence (5´-3')*	*Amplicon Size*
M. massiliense JCM15300	MMASJ_0834_1	For	CCRCGCCAACGGGTATATGC	~500bp
		Rev	TTTTCGGCGGGGCGATG	
M. massiliense JCM15300	MMASJ_0834_2	For	AGCAACTCGGCAAGAAGGC	~500bp
		Rev	AATCTCGTCGACGCGGC	
M. massiliense JCM15300	MMASJ_0836_1	For	TGACGGCCTGAACGACTTTG	~500bp
		Rev	CCTCAACACGTTCGATCCCG	
M. massiliense JCM15300	MMASJ_0836_2	For	TGGTGATCTCGGCAGGCTT	~500bp
		Rev	GTACCAGCTAGTGCATCCGC	
M. abscessus UC22	MAB_RS22045	For	GATCTCCTTCGGCCATGTCG	~500bp
		Rev	GGTTCGCGAGTTCGGTGTAA	
M. abscessus UC22	MAB_RS03355_1	For	AATTGTTTGACCGAACGCGC	~500bp
		Rev	GGGTACTCCGATTACCATTCCATG	
M. abscessus UC22	MAB_RS03355_2	For	TCGGTGGGCTCGCACAT	~500bp
		Rev	TGAGAGARTGGTCGATCCCG	
M. bolletii BD	MAUC_18635_1	For	CTACACATGCTCTCCAACCGA	~350bp
		Rev	TGACTGTGATAAATATCGGRCCTT	
M. bolletii BD	MAUC_07270_1	For	GGGTGGCTTATTCAGATCCGC	~500bp

		Rev	GCGATAAGCCGTCGGTGTC	
M. intracellulare subsp. chimera DSM 44623	SR1_1	For	TGAACTCGTCCCTCAGCGT	~340bp
		Rev	GTCTAGCCTGACACGCCTCT	
M. intracellulare subsp. chimera DSM 44623	SR1_2	For	AAAGAGAATAGAGCCTGCCGC	~360bp
		Rev	CAACGACTGCCGAACGACC	
M. intracellulare ATCC 13950	DT1	For	GGAACRGTTGTCGGGCA	~350bp
		Rev	CTAGRTGRATCGCGCCGAAC	
M. avium subsp. hominissuis 104	IS1311_1	For	TCGCGARARTCTCTGTGGT	~350bp
		Rev	AACAAGCACTGATCGACCCG	
M. avium subsp. hominissuis 104	IS1311_2	For	CCARRTRCCCAAGGTCGAAA	~350bp
		Rev	GAGGCTCTGCGRGTTTCCAC	
M. abscessus ATCC 19977	MAB_0591	For	TTCRAGGCGCACGAATCG	~400bp
		Rev	TTTCCCTTGCGCTTGAGGTC	
M. abscessus ATCC 19977	MAB_2299c_1	For	TGCGTTATCCTCGGCAAGTG	~470bp
		Rev	GACTCGGCGATCGACATGA	
M. abscessus ATCC 19977	MAB_2299c_2	For	GAAGTGTTGTTGCATCTGGCTG	~580bp
		Rev	AAGGAGCCTTGGTTCGCCC	
M. abscessus ATCC 19977	MAB_4532c_1	For	AARTCGTCGGGCGCRCT	~500bp
		Rev	GGACGAATCCGCGGTCCTA	
M. abscessus ATCC 19977	MAB_4532c_2	For	GCCGATGCCAGGAAGGTCT	~500bp
		Rev	TGTCTGAGCTGACCCTACGC	
M. abscessus ATCC 19977	MAB_2875	For	TGATCTCTCGTCGCRCACTTC	~500bp
		Rev	GTCCATGCGAGRGACGTTGT	
M. abscessus ATCC 19977	MAB_0185	For	CCGATGGCCACCGCAT	~540bp
		Rev	AAGGCAGCGGTGATGGTC	
M. abscessus ATCC 19977	MAB_4384_1	For	CGGCCCTTTCTGAGTTGGAT	~400bp
		Rev	AAGTGCCGCTTCCGCC	
M. abscessus ATCC 19977	MAB_4384_2	For	ACGTAACCGGAAGCGTGTAAT	~500bp
		Rev	CGTCTTCGCATGCCCTCGAT	
M. abscessus ATCC 19977	MAB_0019	For	CCCGGCGGTGATGACG	~500bp
		Rev	ATACCGCCCGAACCGTTG	
M. abscessus ATCC 19977	MAB_2297	For	TCGCTCGCCAACGACGA	~500bp
		Rev	GTGCGCTGGTGACRTGGTAG	
M. abscessus ATCC 19977	MAB_0006	For	ACRGTTGTGAATAAGGCGGTT	~450bp
		Rev	GCTGCGCCAAGAAGATGTGG	
M. abscessus ATCC 19977	MAB_2385	For	CGATCTGTCGGCRTGGGAG	~500bp
		Rev	CCCCATGRCACACCACRAG	
M. avium subsp. hominissuis 104	MAV_2512	For	CCCCACGACGGGAACAC	~402bp
		Rev	GGATCGCCTTGTTGCTGAAG	
M. avium subsp. hominissuis 104	MAV_3306_1	For	GTARGACTTGCTCAGCCCR	~740bp
		Rev	AAAAGCAGCAGGCGRTGATG	

M. avium subsp. hominissuis 104	MAV_3306_2	For	CAGGCGAGCTCGATGAGA	~420bp
		Rev	CTARTTCGCGCAGGARCACG	
M. avium subsp. hominissuis 104	MAV_1406_1	For	CGACACCGATCGCCAGGAT	~480bp
		Rev	TGATCGACCTGGCCGTGA	
M. avium subsp. hominissuis 104	MAV_1406_2	For	AGGACAAGGCGAAACAACCC	~521bp
		Rev	GATCTTGTTCGCAACGCTGC	

To guarantee the accuracy of the selected primers, we successfully tested all individual PCR reactions using DNA isolated from grown cultures from each subspecies. All amplification reactions were performed with the same annealing temperature (61ºC) and, after checking for competition of primers pairs and guaranteeing that overlapping amplicons were not included in the same pool, five multiplex PCR reactions were established (Table 5.4).

Table 5.4 - Distribution of primers in the five multiplex reactions. The table shows the composition of each pool, specifying the primers included in each multiplex, according to the experimental design of the study

Multiplex 1	***Multiplex 2***	***Multiplex 3***	***Multiplex 4***	***Multiplex 5***
MMASJ_0834_1	MAB_RS22045_1	MMASJ_0836_1	MAV_IS1311_2	MAV_3306_1
MAV_SR1_1	MAUC_07270_1	MAV_DT1_1	MAB_0019	MAB_RS03355_1
MAB_0591	MMASJ_0834_2	MAV_SR1_2	MAB_4384_2	MAB_RS03355_2
MAB_2299c_2	MMASJ_0836_2	MAB_0185c	MAB_2297_1	MAUC_18635_1
MAB_4532c_1	MAV_IS1311_1	MAB_4384_1	MAV_2512_1	MAB_2299c_1
	MAB_2875_f	MAB_2385	MAV_1406_1	MAB_0006
	MAB_4532c_2	MAV_3306_2		
	MAV_1406_2			

After amplification, and for each sample tested, the five multiplex PCR products were pooled together and then subjected to library preparation using the Oxford Nanopore Technologies (ONT) Ligation Sequencing kit (SQK-LSK109) with the Native Barcoding Kit (EXP-NBD104), for multiplexing. The libraries were run on a MinION Mk1B device with R9.4.1 flow cells (FLO-MIN106). Basecalling was performed with Guppy v6.4.6 using the high accuracy base-calling model. Sequencing coverage of each amplicon/sample was monitored throughout the

runs using the Artic Network RAMPART tool (https://artic-network.github.io/rampart/) with a custom protocol. To assess the targeted sequencing success, the generated reads were mapped against the target genes sequences (extracted from the reference genomes) using Minimap2 (https://github.com/lh3/minimap2) (H. Li, 2018) and visually inspected using the Integrative Genomics Viewer (IGV). The depth and breadth of coverage were assessed with samtools (https://github.com/samtools/samtools) (Danecek et al., 2021).

5.4 Preliminary results and future perspectives

Although this is an ongoing study, our preliminary results show that the targeted sequencing approach constitute a robust tool for the identification of both species/ subspecies and resistance associated mutations, with the majority of species/resistance profiles being correctly identified. In fact, from the multiplex PCR analysed, we were able to select 16 primer pairs that allowed the correct identification of the species to the subspecies level, and 14 that allowed the detection of all of the resistance mutations. However, it is still necessary to test this approach directly on biological samples, collected from patients with confirmed NTM disease, in order to validate its use for clinical diagnosis.

The continued development and optimization of diagnostic methodologies for NTM disease represent a critical step in addressing a growing public health challenge. NTM species are emerging as responsible for severe disease, particularly among vulnerable populations. As such, the ability to accurately and quickly identify both species and resistance profiles is crucial for effective patient monitoring and treatment, as well as further informing about the epidemiology of these pathogens. The application of this methodology into the clinical practice will be key to adapt future diagnostic and therapeutic strategies, contributing to improving patient outcomes and better public health management in the fight against these persistent environmental pathogens.

6

FINAL OVERVIEW, CONCLUDING REMARKS AND FUTURE DIRECTIONS

The main goal of this PhD dissertation was to understand the dynamics of NTM species circulating in Portugal and improve their monitoring. As such, our first approach, as described in Chapter II, was to estimate incidence and prevalence rates and to identify the different NTM species circulating over the last ten years. We started by conducting a nationwide retrospective study of all positive NTM cultures received at the National Reference Laboratory for Tuberculosis (NRL-TB) from 2014 to December 2020. For discussion purposes of this PhD thesis, we further included data from January 2021 to August 2024. Our findings revealed no significant increase in the number of NTM disease cases over the last 10 years. As expected, and since this is a disease that mainly affects older people, in Portugal the most affected age group was 60 - 65. One of the major difficulties throughout our study was to understand whether a positive isolation for NTM was sufficient to confirm NTM disease, as the clinical and radiological criteria were not available. As such, during our research, we only used laboratory data to classify the cases as a definite case of NTM disease, a possible case or colonization. We believe that the access to clinical data would allow a more precise classification of the NTM cases, and thus a better knowledge on the real number of NTM disease in Portugal, highlighting the importance of clinical and laboratory notification, as is already happening in other European countries. These environmental bacteria are able to survive in different environments, such as soil and treated water systems that supply homes, offices, hospitals and irrigation systems which results in unspecific geographical distribution. In our study, we found that Lisbon Metropolitan Area is the most affected Portuguese region, followed by the North region, that, despite having a higher population density than LMA, recorded a significantly lower number of cases ($p < .05$). This suggests that, in Portugal, other factors than population density, such as climate conditions and/ or genetic factors, may influence the distribution of NTM cases.

After recognizing the need for a standard and mandatory notification system of NTM disease, we aimed to understand the genetic variability of circulating strains and to identify resistance markers. As we had previously identified that species from MAC and MABC were the most responsible for NTM disease in Portugal, we conducted further in-depth studies of the isolates of these complexes, which are described in chapters III and IV.

In the study described in chapter III, we aimed to assess the genomic diversity and the resistance profiles of MABC strains circulating in Portugal, emphasizing the importance of WGS data analysis for diagnostics improvement, resistance prediction, and epidemiological data of NTM. We started by studying a case of a patient chronically infected with *M. abscessus subsp.*

bolletii, highlighting the treatment challenges due to its ability to develop multidrug resistance over time. Over a period of 8 years, the patient was subjected to several incorrect therapeutic regimens, and treatment failures, which likely contributed to the persistence and adaptation of the *M. bolletii* strain. In fact, pDST results during the treatment period revealed acquisition of resistance to AMK. gDST allowed us to search for a more broad resistance-associated mutations, however, none of the already described genetic alterations responsible for AMK resistance were detected, suggesting the acquisition of a novel resistance mechanism. The use of WGS analysis also provided information on antibiotics that are not normally tested phenotypically, such as ethambutol (*embB* gene), streptomycin (MAB_ApH gene) and tetracycline (MabTetX gene). Furthermore, it was possible to observe the fixation of some mutations known to be associated with bacterial evolution within the host, namely, affecting the MAB_0364c gene, which encodes an ATPase linked to host adaptation. This highpoints the importance of using genomic approaches to better understand host adaptation and antibiotic resistance, assisting in the definition of specific antibiotic therapeutic schemes for each patient. Our results support that untreated or inadequately treated infections create conditions for bacterial adaptation, hindering future treatment efforts. Given the complexity of MABC resistance, this study highlights the importance of genomic surveillance in the management of MABC infections and the need for continued research to understand/ predict the genomic mechanisms behind bacterial persistence and resistance.

In chapter III we also describe another study where we aimed to understand the genomic diversity of the MABC strains circulating in Portugal. This study provided significant information on the epidemiology, resistance profiles and genetic diversity of a collection of MABC strains isolated in Portugal from 2014 to 2022. pDST revealed high levels of resistance to the antibiotics most commonly used to treat MABC infections, mainly DOX, CIP and CLR. Although high levels of resistance to AMK were not confirmed in pDST, using WGS we were able to detect that all strains carried genetic markers associated with aminoglycosides resistance, suggesting the need for pDST re-evaluation. For phylogeny purposes, we used a gene-by-gene approach (wgMLST) to analyse the diversity of circulating MABC strains. We observed a high degree of genetic diversity, with no evidence of person-to-person transmission, which suggests, as expected, that infection occurs via contamination from environmental sources. By integrating our genomes into a collection of public genomes from a previous published work (Diricks *et. al*. 2022), this wgMLST approach enabled us to classify the Portuguese isolates into

DCC, i.e. dominant circulating strains of MABC that are associated with higher disease prevalence, higher levels of resistance and worse clinical outcomes. In order to increase the resolution of our analysis, we used reportree, a flexible pipeline that allowed us to maximize the shared genome between strains in a dynamic way enabling the detection of genetic clusters. It was possible to cluster Portuguese isolates with international strains, which suggests potential cross-border transmission or the circulation of common environmental strains. Once again, WGS proved to be a more suitable approach for both identification of resistance markers and the taxonomic classification of isolates, offering higher resolution than the traditional molecular techniques used in routine diagnostics. This study was the first in Portugal to support the implementation of a surveillance WGS-based analysis to monitor MABC infections. Our results suggest that phenotypic testing alone may not be sufficient and that genomic approaches should be integrated into routine surveillance and clinical decision-making in order to control the increase of multidrug-resistance MABC strains. Nevertheless, more phenotypic/ genotypic studies are needed to identify new resistance markers. We emphasize the need for a "One health" approach, promoting the inclusion of environmental isolates in future studies to better understand the epidemiology of MABC infections.

In chapter IV, our main goal was to address the complex taxonomy of the members of MAC. With this purpose, we used a marker-gene consensus approach (*rpoB, GroE-1* and *hsp65*) and compared this taxonomical classification to a phylogenetic analysis using WGS data. Although the majority of the cases were identified as being caused by the *M. avium* and *M. intracellulare* species, during our analysis different lineages were identified within each group. Among the *M. avium* species, all the isolates were classified as *M. avium* subsp. *hominissuis,* but two main phylogenetic groups were found. Whitin *M. intracellulare* group, four main genetically distinct groups were found with isolates being classified as *M. intracellulare, M. intracellulare* subsp. *chimaera, M. intracellulare* subsp. *chimaera*-like and, *M. intracellulare subsp. yongonense* illustrating the taxonomic complexity of *M. intracellulare* species. The detection of genetically distinct groups, such as the subsp. *chimaera*-like, suggests that *M. intracellulare* subsp. *chimaera* may actually comprise several distinct lineages or variants. Although our phylogenetic analysis supports the distinction between *M. intracellulare* and *M. intracellulare* subsp. *chimaera*, these results are not sufficient to clarify whether *M. intracellulare* subsp. *chimaera* should be considered a subspecies of *M. intracellulare* or recognized as a distinct species. This also highlights the importance of an accurate identification of these NTM ensuring appropriate surveillance

and understanding the dynamics of this disease. Additionally, pDST results revealed differences in antibiotic susceptibility between species, with *M. intracellulare* subsp. *chimaera* being more susceptible to LNZ and MOX than *M. avium* and *M. intracellulare,* once again reinforcing the need for an accurate identification for a proper initial diagnosis. Throughout our study it was also possible to identify cases of persistence (in which infections continue despite treatment) and cases of reinfection (patients that were infected again, sometimes, with different species), underlining the environmental diversity of MAC strains and the high exposure-level to contaminated sources. This study allowed us to understand the importance of MAC species distribution and resistance patterns, the main NTM responsible for human disease. However, we emphasize that studies integrating genomic data from clinical and environmental isolates are needed to better understand the epidemiology of MAC disease and its true impact on human health. Furthermore, we emphasize the importance of consistent genotyping methods to improve patient care and infection management.

Nowadays, there is still a significant lack of knowledge on NTM disease epidemiology, transmission and resistance mechanisms. In addition to being widely recognized as environmental species, which may hinder a clinical diagnosis, in Portugal there is still no uniform and mandatory reporting of this disease. Moreover, NTM are often resistant to commonly used antibiotics, requiring complex multi-drug regimens that differ depending on the specific species and resistance profile. Currently, pDST is of limited use, as it depends on the growth of the NTM in culture media, sometimes requiring months until visible growth, and there is still need for standardization of the laboratory procedures. To address these challenges, in Chapter V we describe an ongoing work where we aimed to develop a tNGS approach for species/subspecies identification and detection of resistance-associated mutations, in particular for isolates of the MAC and MABC. For this purpose we analysed 27 regions of interest (for both complexes) aiming for a more rapid and sensitive methodology that could be used directly on the clinical samples of the patients. The preliminary results suggest a high potential of this approach, as it was possible to optimize, in multiplex-PCR methods 16 primer pairs that allowed the correct identification of the species to the subspecies level, and 14 that allowed the detection of all resistance mutations. The next experimental step will consist on testing this approach directly on samples collected from patients with confirmed NTM disease, in order to validate its use for clinical diagnosis.

Globally, our findings clearly advise for the establishment of a national mandatory notification system for NTM disease. In addition to improving surveillance, this system would enable more robust collaborations between health institutions, a more accurate understanding number of cases and allowing targeted interventions. Thus, the establishment of mandatory reporting will allow Portugal to align with public health practices already established by other European countries, increasing the capacity to address the potential emergency caused by these opportunistic bacteria and improving the overall patient´s outcomes. In addition, as can be highlighted from our results, the use of genomic analyses, such as WGS and tNGS, into routine diagnostics and surveillance brings unequivocal benefits that may be used for individual and public health purposes. In fact, the development of NGS-based pipelines, applied to the laboratory daily routine, would provide high-resolution information on circulating NTM species and antibiotic resistance profiles, allowing for adapted treatment regimens and reducing the risks of multidrug-resistant NTM strains. It is also crucial to adopt a 'One Health' strategy that integrates animal and environmental isolates into future genomic analyses in order to significantly advance the understanding of NTM transmission routes and niches. All these approaches will enable health authorities to proactively identify, monitor and control NTM disease, opening the way for a more effective and targeted public health interventions.

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LIST SUPPLEMENTARY MATERIALS

Supplementary Table S1

https://figshare.com/articles/dataset/Supplementary_Table_S1/28376243?file=52596335

Supplementary Table S2

https://figshare.com/articles/dataset/Supplementary_table_S2/28484501?file=52596431

Supplementary Table S3

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Supplementary Table S4

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Supplementary Table S5

https://figshare.com/articles/dataset/Supplementary_Table_S5/28484567?file=52596617

Supplementary Table S6

https://figshare.com/articles/dataset/Supplementary_table_S6/28484573?file=52596662

2025

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GENOMICS OF NONTUBERCULOUS MYCOBACTERIA (NTM): DEVELOPMENT OF A LABORATORY TOOL FOR RAPID AND PRECISE DIAGNOSIS AND SURVEILLANCE OF NTM INFECTIONS