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ORIGINAL ARTICLE

Nontuberculous Mycobacteria in Portugal: Trends from the last decade

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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. 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

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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.

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*.[1-3] 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.[4,5] 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.[6] Outbreaks due to NTM are also reported in the literature.[7-9]

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.[10,11] However, it is difficult to assess the true clinical impact of NTM infections due to the difficulty in discriminating between 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,[10,12-15] 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 [12,16,17] 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.

Material and methods

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.[18]

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

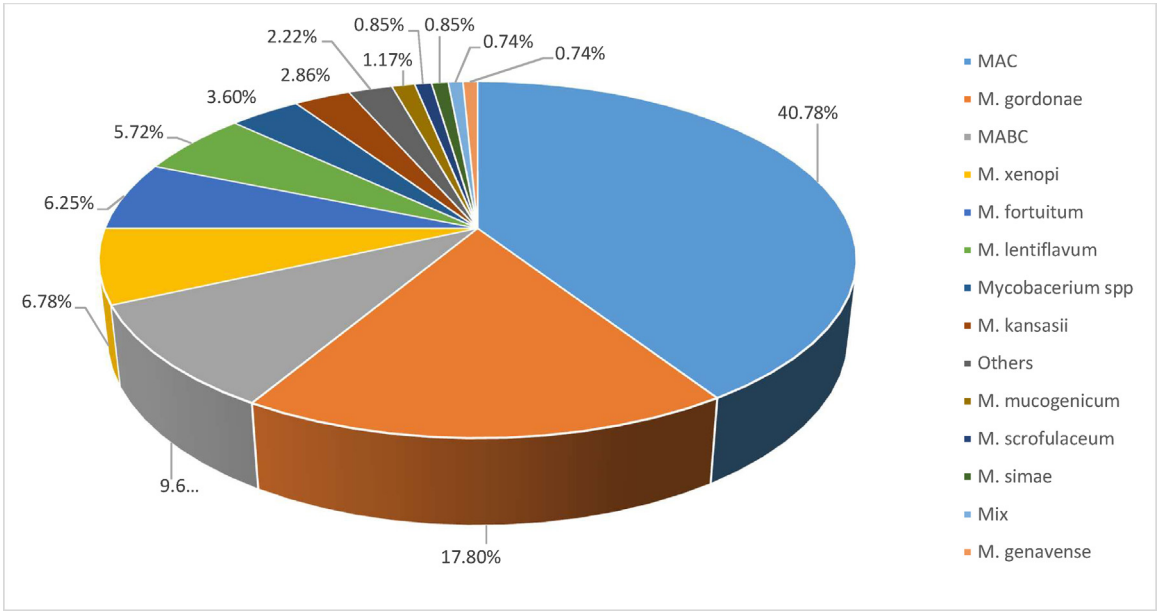

Fig. 1 Distribution of NTM species isolated from patients in Portugal from 2014-2020. *Others refer to the following species: *M. asiaticum, M. goodii, M. interjectum, M. malmoense, M. paraffinicum, M. peregrinum, M. shimoidei, M. terrae complex, M. triplex, M. triviale, and M. marinum.*

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".[10,19,20]

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 χ^2 tests, respectively. The p values $<.05$ were considered significant.

Ethical approval

This study was approved by the Ethical Commission of the National Institute of Health Dr. Ricardo Jorge.

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 (supplementary Figure 1).

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. Fig. 1 present the frequency of NTM species isolated. For seven patients (0.74%) more than one NTM species was isolated in same sample. 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 nontuberculous mycobacteria 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 1). 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 (Fig. 1, Table 1).

The median patient age was 64.1±15.9 years old. Age-standardized rates did not reveal significant differences between age groups (supplementary table 1). 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 (supplementary table 1) 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.

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 (Fig. 2, supplementary figure 2). Algarve and

Table 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.

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 Setúbal (21.3%) and Lisbon (10.6%) (both from LMA), Viseu (9.6%) (Centre), and Vila Real (9.2%) (North) (Fig. 2).

Given that we observed that the regions with the highest number 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 (supplementary figure 2). 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 1; Fig. 1) MAC (40.8%), MABC (9.6%) and *M. fortuitum* (6.3%) were the NTMs that showed more association with disease. To better understand the

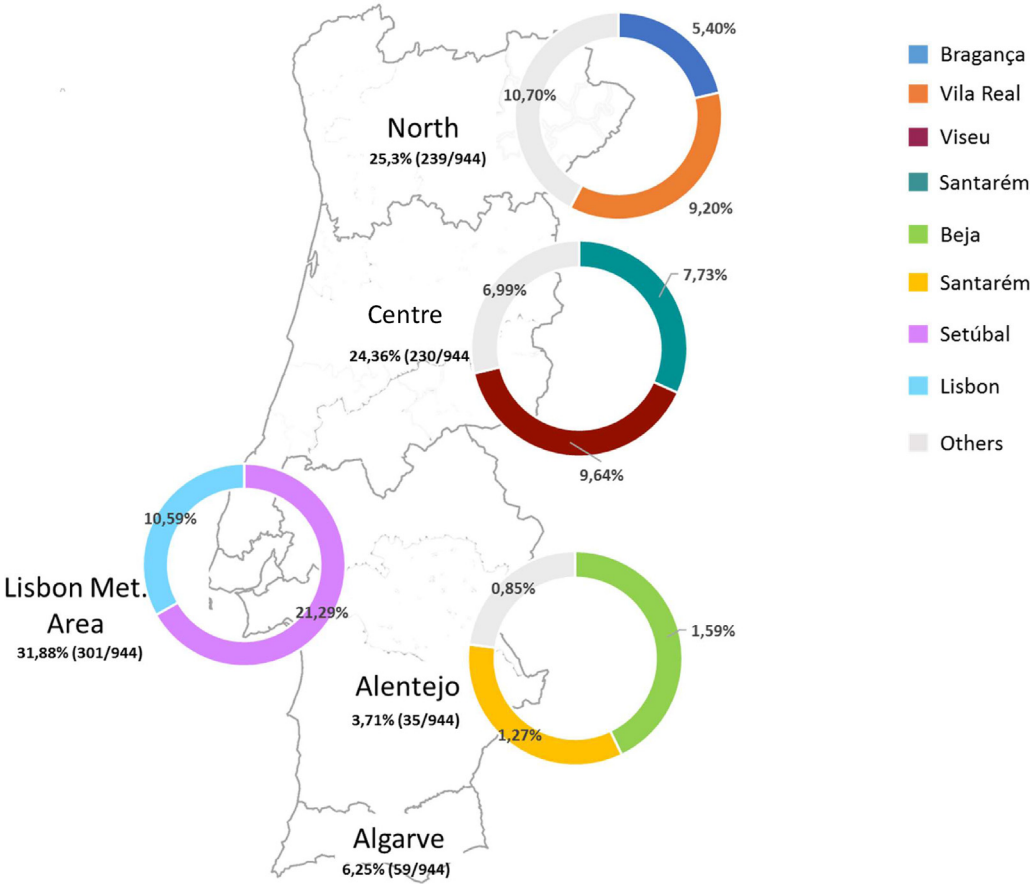

Fig. 2 Distribution of NTM cases in Portugal mainland between 2014-2020.

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 (supplementary table 2). North region showed the highest number of "definite NTM cases" (n = 33) (supplementary table 3).

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 (supplementary figure 1), which is expected for countries with a low TB incidence.[10,21] In Portugal, TB incidence has been steadily declining since 2000.[22] Nevertheless, the increasing number of NTM described worldwide [13,15,23] is justified by an aging population, comorbidities, and enhanced clinical and laboratory capabilities [4,10,12-14].

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%).[12]

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",[3,24,25] 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.[26] To fully understand the dynamics of these infections in our population, clinical based studies of NTM infections are crucial.

As described in other studies,[10,19,27] 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. Setúbal, 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,[3,28] 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.[3] 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).[4,10,19,29] 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.[9,30] 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,[31] 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.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.pulmoe.2022.01.011.

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