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Original Article

Mycobacterium Intracellulare among TB Suspected Patients in Bulgaria – Microbiological Aspects

Elizabeta V. Bachiyska[1], Yuliana A. Atanasova[1], Ana I. Baykova[1], Stanislava P. Yordanova[1]

[1] *National Center of Infectious and Parasitic Diseases (NCIPD), Sofia, Bulgaria*

Corresponding author: Elizabeta Bachiyska, National Reference Laboratory of Tuberculosis, National Center of Infectious and Parasitic Diseases, Department of Microbiology, 44A Gen. Nikolai Stoletov Blvd., 1233 Sofia, Bulgaria; Email: elizabetbatchiiska@abv.bg; Tel.: +359 2 9446445

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Abstract

Introduction: Nontuberculous mycobacteria (NTM) are representatives of the genus *Mycobacterium* with a worldwide distribution, associated mainly with water, soil, and biofilms. Some of NTMs, such as *Mycobacterium avium* complex (MAC), are etiological agents of human diseases – disseminated or with different localization, most often pulmonary.

Aim: In the present study, we analyzed *Mycobacterium intracellulare* isolates recovered from clinical specimens of tuberculosis (TB) suspected patients in Bulgaria, 2018-2020.

Materials and methods: The cultures were grown on solid and liquid media. For species identification, we used immune chromatographic (TB Ag MPT64) test and Line Probe Assay (LPA) from the positive cultures.

Results: *M. intracellulare* was identified in 32 patients from 82,780 specimens. It was predominantly isolated in females – 62.5% vs. 37.5% in males. The most affected age group was 65 years and over (38%). The distribution of the isolates in Bulgaria was uneven. Most of them (65.6%) were concentrated in two districts of the country: Plovdiv and Sofia-city. All strains were sensitive to macrolides and aminoglycosides except one with macrolide resistance. NTM pulmonary disease was confirmed in 16 patients with *M. intracellulare* isolate.

Conclusions: Analysing the 32 *M. intracellulare* isolates identified among TB suspected patients in Bulgaria between 2018 and 2020, we found that only half of them met the American Thoracic Society (ATS) diagnostic criteria for NTM pulmonary disease. For the remaining patients with *M. intracellulare* isolates we did not have sufficient data to support this diagnosis. Efforts by Bulgarian respiratory and microbiological societies are needed for adherence to the international guidelines.

Keywords

M. intracellulare pulmonary disease, nontuberculous mycobacteria

INTRODUCTION

Nontuberculous mycobacteria (NTM) are representatives of the *Mycobacterium* genus other than *Mycobacterium tuberculosis* complex and *Mycobacterium leprae*. NTM are widely spread in the environment, and associated mainly with water, soil, and biofilms. To date, more than 190 NTM species have been described.[1] *M. intracellulare* is a slow growing NTM member of the *Mycobacterium avium* complex (MAC) group. A lot of studies from multiple countries indicate the global increasing of NTM infections and the MAC infections are the main driver of

this increase.[1-6] While *M. avium* is usually described in the literature as an acquired immunodeficiency syndrome (AIDS) disseminated infection associated agent, *M. intracellulare* causes very often pulmonary infections.[7,8] NTM disease and detection is not mandatory notifiable globally and therefore high quality information is not readily available.[6,9] The reported data show that *M. intracellulare* is one of the most frequently isolated NTM species in European Union (EU) and European Economic Area (EEA): in Finland – 636 isolates for a 17-year period (1995-2011), in The Netherlands – 359 for a 6-year period (2006-2011), in Germany – 272 for 2011, Italy – 104 for a 10-year period (2001-2010), Denmark – 15 for 2011, Estonia – 52 for 8-year period (2004-2011) and Luxemburg – 3 for 3-year period (2009-2011).[9] Some Balkan countries neighboring Bulgaria have published data about *M. intracellulare*. In Greece for a 7-year period (2005–2011), there were reported 49 isolates of *M. intracellulare,* in Slovenia for an 11-year period (2000-2010) – 156 isolates and in Croatia for a 3-year period (2008-2010) – 23.[9]

AIM

The present study aimed to perform an analysis of *M. intracellulare* isolates from clinical specimens among TB suspected patients in Bulgaria over a 3-year period between 2018 and 2020.

MATERIALS AND METHODS

In the TB laboratory network of Bulgaria (consisting of 30 TB cultural laboratories) for the period from 2018 to 2020 there were processed 82,780 clinical samples of 44,247 patients suspected of having tuberculosis (or in the differential diagnostic plan of other pulmonary diseases): sputum, bronchoalveolar lavage (BAL), gastric washes (in children), and biopsy materials. They were collected in the regional pulmonary hospitals or wards performing the diagnosis, treatment and control of tuberculosis in Bulgaria. A smear microscopy for AFB (Ziel-Neelsen) from the specimens was performed. The strains of *M. intracellulare* were isolated using solid (Lowenstein-Jensen) and liquid media (MGIT, BACTEC 960 System) according to the relevant standard operating procedures of the national and international guidelines.[10,11] The species identification of NTM in the country was performed only in National Reference Laboratory of Tuberculosis (NRL TB), National Center of Infectious and Parasitic Diseases (NCIPD) from pure culture. After a negative immunochromatographic test (SDTB Ag MPT64 Rapid, Standard Diagnostics, Republic of Korea), LPA were used (Geno Type Mycobacterium CM and NTM-DR, HAIN Lifescience, Nehren, Germany) based on DNA strip technology: DNA amplification of 23S rRNA gene, reverse hybridization and immobilization on a membrane strip. Macrolide (clarithromycin, azithromycin) resistance was identified by examining the most common resistance-associated mutations of the *rrl* gene (coding for the 23S rRNA). For detection of aminoglycoside (kanamycin, amikacin, gentamicin) resistance, the most significant resistance-associated mutations of the *rrs* gene (coding for the 16S rRNA) were examined. The tests were performed according to the recommendations of the manufacturers.[12,13] A PCR diagnosis was supported by phenotypic analysis, including monitoring of grow characteristics, temperature tolerance, and colony pigmentation. The quality of the microbiological diagnosis of NRLTB, NCIPD was ensured by an External Quality Assessment for TB/NTM scheme from INSTAND e.V., Germany once a year. In order to summarize the data, we used descriptive statistical analysis.

RESULTS

M. intracellulare was identified in 0.07% (32 patients with 52 isolates) from 82,780 clinical specimens of 44,247 patients suspected of having tuberculosis over a 3-year period (2018–2020). The strains of these 32 patients were isolated mainly from sputum – 75% (n=24), followed by BAL – 12.5% (n=4) and 12.5% (n=4) from BAL and sputum. Positive for acid-fast bacilli microscopy were 34.4% (n=11). During the same period of time, 183 patients were isolated with NTM, and these 32 patients with *M. intracellulare* accounted for 17.5% of them. *M. intracellulare* was the most frequently isolated NTM species in Bulgaria after *M. gordonae* – 25.2% (n=46), followed by *M. avium* – 13.4% (n=25), *M. fortuitum* – 9.3% (n=17), *M. chelonae* – 8.7% (n=16), *Mycobacterium* spp. – 8.2% (n=15), *M. abscessus* – 4.4% (n=8), *M. mucogenicum* – 4.4% (n=8), *M. xenopi* – 3.3% (n=6), *M. lentiflavum* – 2.8% (n=5), and the species: *M. malmoense, M. scrofulaceum, M. shimoidei, M. simiae* and *M. interjectum* – 0.5% (n=1) each **(Fig. 1)**. Using NTM-DR test one isolate of *M. intracellulare* turned out to be *M. chimaera*. It was identified in only one patient with bronchiectasis, female, in the age group over 65 years, non-smoker, without reporting data on cardiac surgery.

M. intracellulare was isolated predominantly in females – 62.5% vs. 37.5% in males. Regarding the age structure, the most affected were the age group over 65 years – 38% (n=12), followed by the 55-64-year group – 34% (n=11), 45-54-year group – 22% (n=7) and 25-34-year group – 6% (n=2) **(Fig. 2)**.

The distribution of *M. intracellulare* isolates on the territory of Bulgaria was uneven. They were concentrated in two districts of Bulgaria – 65.6% (n=23): Plovdiv (n=17) and Sofia-city (n=4). In the district of Dobrich, we found two isolates. In the districts of Burgas, Varna, Gabrovo, Kyustendil, Pazardzhik, Pernik, Pleven, Silistra, and Haskovo, we identified one case in each; no strains were isolated in the other 16 districts for the 3-year period **(Fig. 3)**.

In our study, only half of the patients (n=16) with *M. intracellulare* isolates can be classified as *M. intracellulare*

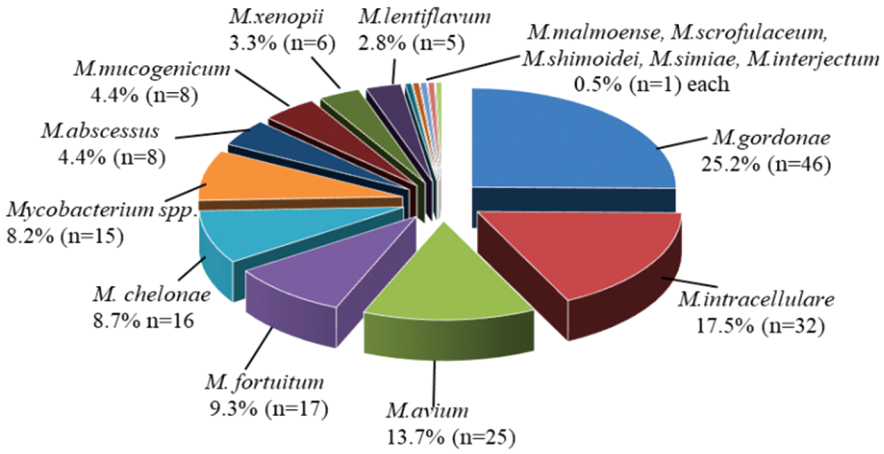

Figure 1. Species diversity of NTM in Bulgaria, 2018-2020.

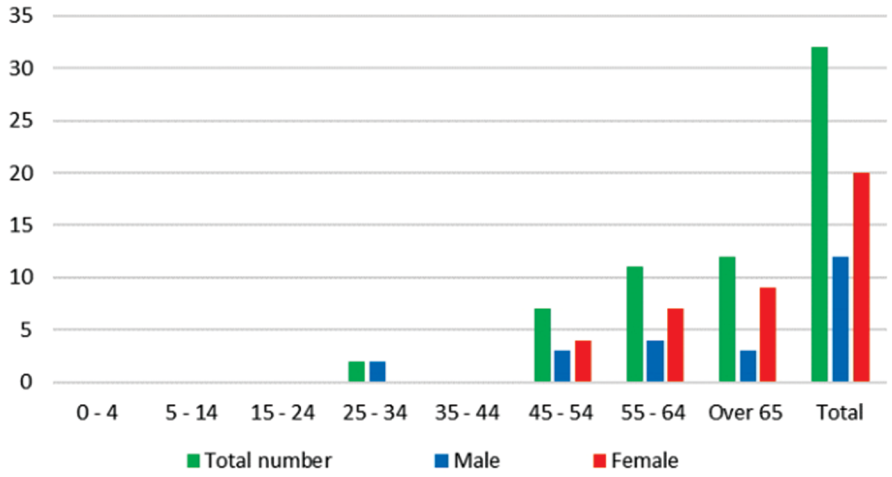

Figure 2. Age structure of patients with isolated *M. intracellulare* in Bulgaria, 2018-2020.

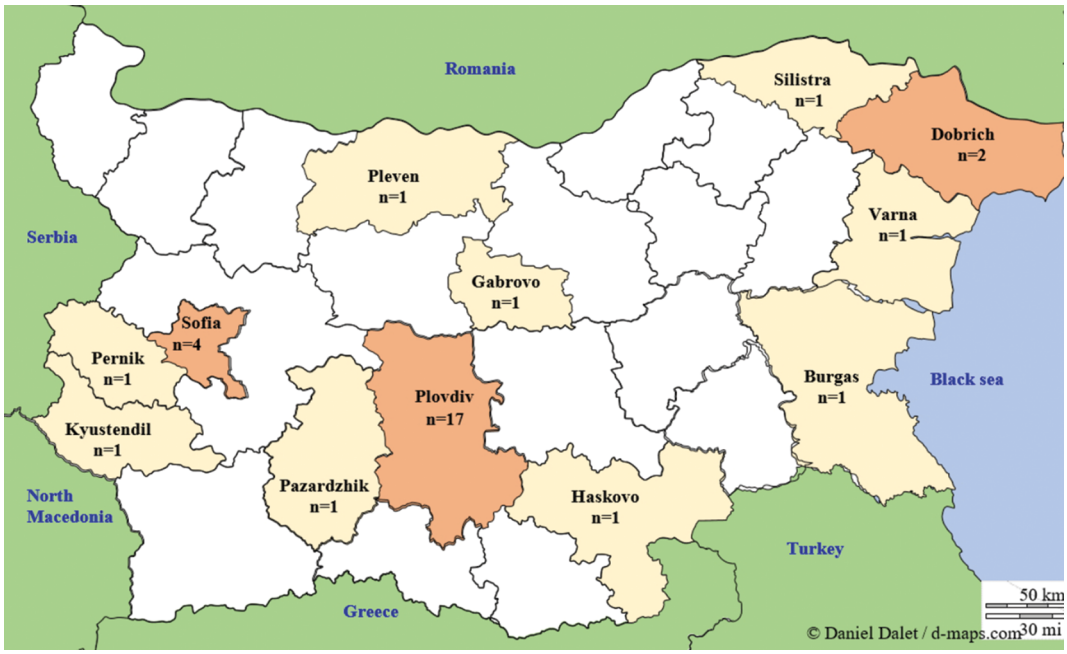

Figure 3. Distribution of *M. intracellulare* isolates on Bulgarian territory, 2018-2020.

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pulmonary diseases in the context of the microbiological criteria of the three official NTM guidelines of American Thoracic Society (ATS), British Thoracic Society (BTS), European Respiratory Society (ERS), European Society of Clinical Microbiology and Infectious Diseases (ESCMID) and Infectious Diseases Society of America (IDSA) for the management of nontuberculous mycobacterial pulmonary disease.[14-16] Four of these 16 patients were with positive culture results from BAL; another four – with positive culture results from BAL plus one sputum. Eight patients had positive culture results from at least two separate expectorated sputum samples: four of them with two sputa, three with three sputa and one patient with four sputa. Nine of the patients with *M. intracellulare* pulmonary disease also had positive Ziehl-Neelsen microscopy for AFB. Molecular drug susceptibility testing was performed for all patients with *M. intracellulare* pulmonary disease, but only for antibiotics with documented good correlation between in vitro activity and microbiological response, i.e. clarithromycin/azithromycin and amikacin/gentamicin.[16,17] Except for one isolate, which was resistant to macrolides and sensitive to aminoglycosides, the other 15 strains were sensitive to both of them. According to the limited clinical and radiographic information provided to the TB laboratories along with the samples: nine of the patients had bronchiectasis (one of them with the rare Alport's syndrome – polycystic kidney disease, deafness and kidney failure); three – with thick-walled cavities in the right lung apically; one – with chronic obstructive pulmonary disease (COPD) and for the other three patents, there was no detailed information, except "obs. tuberculosis".

DISCUSSION

Correct species identification of NTM is crucial as it can predict the clinical significance of an isolate as well as aid in the choice of treatment regimen by clinicians.[16,17] In our previous study over an 8-year period (2010-2017), *M. intracellulare* was isolated in 56 patients, i.e. the number of patients has already increased by more than 52%.[18] An increase in NTM has been observed with a decrease in the incidence of tuberculosis, as is the situation in Bulgaria in last decade.[19] *M. intracellulare* was the most common isolated NTM species of NTM in the country after *M. gordonae* – 32 isolates for a 3-year period. The average annual isolation of *M. intracellulare* was similar in other southern European countries with similar climatic conditions, such as Italy, Croatia and Greece.[9] The predominance of *M. intracellulare* among the isolates of MAC group in Bulgaria could be explained by the fact that the species is more virulent than M. *avium* and leads to a more severe pulmonary disease, as well as that *M. avium* is more often associated with coinfection in HIV-positive patients, and for the country the incidence of HIV remains low.[19,20] There was no data on HIV positivity among *M. intracellulare* patients in our study, while for the same period of time we isolated *M. avium* in five HIV-positive patients. Regarding the primary immune deficiency – the background of often mycobacteria isolation, we had data for one patient with Alport's syndrome.[21,22] NTM pulmonary disease is an emerging condition that affects not only immunocompromised patient, but also immunocompetent subjects, usually with structural pulmonary disease.[21,22] In our study we had data on nine patients with bronchiectasis, three with thick-walled cavities in the right lung apically, one – with COPD and for other three patients, there was no detailed information except "obs. tuberculosis".

In 62.5%, *M. intracellulare* was isolated in females and those over 55 years of age were most affected, which is consistent with the literature data.[2,21] The concentration of most isolates in the two Bulgarian cities – Sofia and Plovdiv, is probably due to a number of reasons. Firstly, they are the two largest cities in the country and are natural gravitational centers for the population. Secondly, two types of media are used there simultaneously for cultivation – both solid and liquid media, which leads to an increase in yield. In addition, the southern climate in Plovdiv contributes to the higher number of isolates.

Isolation of *M. intracellulare* from clinical samples does not always mean NTM pulmonary disease. The diagnostic criteria of NTM pulmonary disease – clinical, radiographic, and microbiologic criteria are equally important, according to the official guidelines of ATS, BTS, ERS, ESCMID and IDSA.[14-16] The microbiologic criteria include positive culture results from at least two separate expectorated sputum samples or positive culture results from at least one bronchial wash or lavage, or transbronchial or other lung biopsy.[14] Insufficient clinical data for all 32 patients with isolated *M. intracellulare* allowed only 16 of them to be classified with *M. intracellulare* pulmonary disease. Better collaboration between microbiologists and clinicians would be of help to resolve this problem.

NTM species identification is significant for both microbiologists and clinicians. The differential diagnosis between TB and NTM pulmonary disease should always be taken into consideration given that the treatment of the two diseases is different. For instance, isoniazide and pyrazinamide – the two potent drugs used in the intensive TB treatment phase are not applicable to the *M. intracellulare* infection due to innate resistance.[14-16] The official guidelines recommend susceptibility-based treatment for macrolides and amikacin over empiric therapy.[16] The studied *M. intracellulare* isolates of patients with *M. intracellulare* pulmonary disease showed sensitivity to macrolides and aminoglycosides, except one macrolide resistant isolate, which will make it easier for clinicians to choose the appropriate therapy. We need to gather more data on the resistance of *M. intracellulare* isolates in Bulgaria, as these are still insufficient for more in-depth sensitivity study in the country.

CONCLUSIONS

Analysing the *M.intracellulare* isolates identified among TB suspected patients in Bulgaria we found that their number has increased during the last 3 years (2018-2020) and only half of them met the ATS diagnostic criteria for NTM pulmonary disease. For the remaining patients with *M. intracellulare* isolates we did not have sufficient data to support this diagnosis. Better collaboration is needed between microbiologists identifying the etiological agent and clinicians having all the clinical information for NTM patients. Efforts by Bulgarian respiratory and microbiological societies are needed for adherence to the international guidelines.

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Mycobacterium Intracellulare среди пациентов с подозрением на туберкулёз в Болгарии – микробиологические аспекты

Елизабета В. Бачийска[1], Юлияна А. Атанасова[1], Ана И. Байкова[1], Станислава П. Йорданова[1]

[1] *Национальный центр инфекционных и паразитарных заболеваний (НЦИПЗ), София, Болгария*

Адрес для корреспонденции: Елизабета В. Бачийска, Национальная референс-лаборатория туберкулёза, Национальный центр инфекционных и паразитарных заболеваний (НЦИПЗ), Отделение микробиологии, бул. „Ген. Столетов“ № 44А, 1233 София, Болгария; E-mail: elizabetbatchiiska@abv.bg; Тел.: +359 2 9446445

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Резюме

Введение: Нетуберкулёзные микобактерии (НТМ) – представители рода *Mycobacterium* с мировым распространением, связанные в основном с водой, почвой и биоплёнками. Некоторые из НТМ, такие как комплекс *Mycobacterium avium* (MAC), являются этиологическими агентами заболеваний человека - диссеминированных или с различной локализацией, чаще всего лёгочных.

Цель: В настоящем исследовании мы проанализировали изоляты *Mycobacterium intracellulare*, выделенные из клинических образцов пациентов с подозрением на туберкулёз (ТБ) в Болгарии, 2018-2020 гг.

Материалы и методы: Культуры выращивали на твёрдых и жидких средах. Для идентификации видов мы использовали иммунный хроматографический (TB Ag MPT64) тест и анализ линейного зонда (LPA) из положительных культур.

Результаты: *M.intracellulare* был идентифицирован у 32 пациентов из 82 780 образцов. Преимущественно он был изолирован у женщин - 62.5% против 37.5% у мужчин. Наиболее пострадавшей возрастной группой была группа 65 лет и старше (38%). Распределение изолятов в Болгарии было неравномерным. Большинство из них (65.6%) были сосредоточены в двух районах страны: Пловдив и София. Все штаммы были чувствительны к макролидам и аминогликозидам, кроме одного с устойчивостью к макролидам. НТМ лёгочное заболевание было подтверждено у 16 пациентов с изолятом *M.intracellulare*.

Заключение: Анализируя 32 изолята *M.intracellulare*, выявленных среди пациентов с подозрением на туберкулёз в Болгарии в период с 2018 по 2020 год, мы обнаружили, что только половина из них соответствовала диагностическим критериям Американского торакального общества (ATS) для НТМ лёгочной болезни. Для остальных пациентов с изолятами *M.intracellulare* у нас не было достаточных данных для подтверждения этого диагноза. Необходимы усилия болгарских респираторных и микробиологических обществ для соблюдения международных рекомендаций.

Ключевые слова

M.intracellulare болезнь лёгких, нетуберкулезные микобактерии