NONTUBERCULOUS MYCOBACTERIA DIVERSITY IN KARST WATERS AND BIOFILMS IN BULGARIAN CAVES

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ABSTRACT

Background: Nontuberculous Mycobacteria (NTM) are emerging pathogens causing opportunistic infections in humans and animals. Their distribution in the waters and caves of Bulgaria is poorly studied. Climatic changes are associated with changes in the amplitudes of ambient and water temperature, as well as changes in the amount of precipitation which play an essential role in the creation of reservoirs of some types of NTM in the environment.

Material and Methods: We optimized the methods for successful isolation of environmental NTM and then used molecular genetic methods for identification.

Results: A total of 235 samples (karst water, sediments, soil, bat guano) were collected in some caves of the following karst regions: 203 in Vratsa Karst area, 204 in Ponor Karst area, 205 in Bezdenski area and 303 in Karst and caves of Bosnek region. Primary isolation of mycobacteria by Löwenstein–Jensen at room temperature was more successful than on liquid media at 37°C. We identified NTM in 10% (n=24) from these materials. Diverse NTM included: *M. chelonae* (n=3), *M. gordonae* (n=2), *M. intermedium* (n=3), *M. scrofulaceum* (n=1), *M. szulgai* (n=4), *M. fortuitum* group (n=4), NTM mix culture (n=5), *M. terrae* complex (n=1), *Mycobacterium sp*. (n=1). Rapidly growing NTM (*M. chelonae, M. fortuitum* group) were the most common. The isolates belonged to group of environmental saprophytes (Risk group 1) and potential pathogens (Risk group 2).

Conclusions: We successfully implemented a procedure for decontamination and isolation of NTM from the environment. For the first time in the country, NTM species were identified in biofilms, karst waters, soil and bat guano within caves. The presence of NTM in cave ecosystems represents a potential source for human infection.

Keywords: Nontuberculous Mycobacteria, geographical diversity, Bulgarian caves.

INTRODUCTION

Genus *Mycobacterium* includes *M. tuberculosis* complex, *M. leprae* and nontuberculous mycobacteria (NTM). NTM are represented by over 190 species and are the fastest growing group of the genus. In the recent years, an increased incidence of NTM infections has been reported worldwide. NTM cause most often chronic lung infections, but they are also involved in central nervous system diseases, and skin/soft tissue infections in children, adults, and especially in patients with immunocompromised conditions (1, 2). NTM are not transmitted from person to person, but rather from environmental sources. They have been found in various ecological niches worldwide. NTM can be isolated from soil, dust and water sources, including surface, recreational, ground, waste, tap water. Biofilms can also serve as a reservoir for these bacteria. *M. gordonae* is more commonly isolated from water sources using treated surface water, while *M. nonchromogenicum* predominates in water sources fed by chlorinated groundwater (3).

The presence of NTM in the environment of Bulgaria has been poorly investigated. A study conducted in 2015 did not detect NTM in the waters of the Iskar Dam and the Black Sea (4). Analysis of bat guano in eight European countries - the Czech Republic, France, Hungary, Italy, Romania, Slovakia, Slovenia, and Bulgaria, performed by a Czech team from Mendel

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Figure 1. A map of karst regions in Bulgaria. The borders of the karst areas from which materials were collected are colored in blue.

University in Brno (MENDELU) without Bulgarian participation, detected NTM representatives in eight Bulgarian samples (5).

These studies and the published data did not clarify the situation, and the question about the primary sources of NTM in the country's environment remained unsolved. The exploration of cave biofilms and karst waters for NTM is important because these habitats can determine the above-ground and subsurface environments, and water is the main source of NTMs distribution (6).

In Bulgaria, karst zones occupy 22.7% of the territory, and so far about 5100 caves have been discovered (6). They offer a unique living environment - a specific microclimate (almost constant temperature and humidity), limited or completely absent daylight (6). Caves are an independent and complete eco-geographic area in which a dynamic equilibrium is established under unique conditions. Caves and above-ground structures are interconnected and strongly influence each other. The stable conditions of deep dark cave areas reveal a wide variety of bacteria, algae and fungi living on the rock walls and speleothems, in the sediments and temporary pools. The adaptation mechanisms used by cave microorganisms are complex, with increasing potential to affect humans (7).

METHODS

A total of 235 samples from environmental materials (water, soil, sediment, biofilms, bat guano) were collected from caves. We used the cave zoning of the country's territory accepted by geologists (8). According to it, the collected materials were as follows: 203 Vratsa karst region (from 12 caves), 204 Ponor karst region (from 3 caves and 3 karst springs and rivers), and 303 Bosnek karst region (from 4 caves) **Fig. 1**. Collection was carried out in sterile containers, biofilms were collected using sterile swabs. Sampling requirements included: 5 g of soil from at least 3 cm depth from the surface; 50 ml of water. They were placed in clean transparent self-sealing envelopes, and labelled with the type of specimen, location and date. Samples were stored in refrigerator at +6° C for up to 48 hours. All of the samples were decontaminated in order to destroy

the accompanying bacterial and fungal microflora, homogenized and inoculated on a specific liquid and solid medium (MGIT - Mycobacteria Growth Indicator Tube (BACTEC MGIT 960, Becton Dickinson, USA) and egg-based medium – Löwenstein–Jensen (Becton Dickinson, USA)). The reagents used for decontamination, are described in detail in the Results and Discussion section as we feel that the successful isolation of NTM strains is one of the merits of our work. Samples were incubated for 76 days at room temperature and at 37°C, with continuous monitoring to detect fast-growing NTM. When growth was visible, a smear was made by the Ziehl-Nielsen technique to detect acid-fast bacteria (AFB). If the presence of AFB was confirmed, phenotypic, immunochromatographic (Capilia™ TB Neo) and molecular genetic determination of the isolates were performed. Geno Type® Mycobacterium CM and AS (Hain Lifescience GmbH, Nehren, Germany) tests (PCR tests known as LPA based on DNA strip technology) were used for identification of the most common and relevant to human pathology NTM. Genotypimg was performed after DNA isolation from a pure culture (GenoLyse® Hain Lifescience GmbH, Nehren, Germany) with subsequent amplification of the 23S rRNA gene and reverse hybridization to specific oligonucleotides immobilized on a membrane strip.

RESULTS AND DISCUSSION

Isolation of NTM from environmental materials is a serious challenge. Samples from natural habitats are rich in bacterial flora. The presence of contaminating spore-forming bacteria and moulds destroy the medium in a short time, and supress or mask the growth of NTM. It is necessary to destroy the other bacteria and fungi, in order to obtain visible colonies of NTM. Specific procedures to ensure homogenization and decontamination of the collected materials are used. The purpose of homogenization is to free the bacteria from the mucus, cells or tissues in which they are infiltrated. Decontamination is based on the high resistance of mycobacteria to acids and hydroxides. Relevant protocols have been successfully established for clinical material, however working with environmental samples requires the consideration of different variables depending on the conditions and type of specimen being processed. The procedure can cause a loss of up to 70% of the target NTMs from each sample, which would result to an underestimation of the amount and species diversity of NTM and possibly the inability to isolate strains because they could be sensitive to the decontaminating agents. (9) According to Kazda et al. 2009 (9) the conditions for successful cultivation of ecological NTM are not fully known. For this reason, for *in vitro* isolation of NTM it is imperative to select and use appropriate decontamination procedure, isolation medium and incubation temperature. The decontamination method we used was an adaptation of Parashar D. (10). Water samples were concentrated before decontamination, and soil samples were dissolved in 20 ml of sterile distilled water. The procedure of decontamination was two-step. First we are using 4% NaOH for 20 min followed by 5% oxalic acid for 30 min. The materials thus treated were inoculated into two tubes of liquid and solid medium (MGIT and Löwenstein–Jensen), which were cultured in parallel in the dark at room temperature and at 37°C. Once growth was observed, a Ziehl Neelsen microscope slide was made, and AFB-positive cultures were subcultivated on Löwenstein–Jensen and used for phenotypic, immunochromatographic and molecular genetic identification. Typically, rapidly-growing NTM took from one week to 10 days to grow, while slow-growing ones often formed visible colonies in a month (11). For this reason, it is also important to clearly and accurately distinguish specific growth from contaminants. There are very limited studies aimed at the detection of NTM in caves. (12, 13). According to literature data, the most frequently isolated NTM from cave waters and sediments are the species - *M. avium, M. mucogenicum, M. chelonae and M. fortuitum* (14). These data concurred with our established species diversity, according to which the fast-growing NTMs predominate among the isolates. From 235 samples that we collected in 19 caves and 3 karst springs from the above-mentioned Bulgarian karst regions, 10% (n=24) gave growth to NTM strains, that were isolated according to the described procedure. They are presented in Table 1. Most of NTMs were identified from bat guano (67%, n=16), the other materials positive for NTMs were: water and biofilm (13%, n=3 each), sediment and clay (3%, n=1 each). The mycobacterial species diversity

Table 1 Diversity of NTM isolates by site.

Name of Cave and Locality	Number of isolates	Type of specimen	Isolated NTM species
Vrazhite dupki 2431 ВД№9; Vratsa karst region	**2**	guano (n=1) sediment (n=1)	*M. chelonae* mix cilture
Dupkata pod asfalta Bosnek karst region	**1**	guano (n=1)	*M. fortuitum* group
Dushnika Iskrets, Ponor karst region	**2**	guano (n=2)	*Mycobacterium sp.* *M. terrae* complex
Zidanka Lakatnik, Vratsa karst region	**1**	biofilm (n=1)	mixed NTM culture
Opushenata nisha Lakatnik, Vratsa karst region	**1**	clay (n=1)	*M. szulgai*
Pepelyanka Bosnek karst region	**2**	biofilm (n=2)	*M. gordonae* *M. szulgai*
Svinska dupka Vratsa karst region	**13**	guano (n=12) water (n=1)	2 x *M. chelonae* 6 x *M. fortuitum* group 4 x *Mycobacterium sp.* from water -*M. scrofulaceum*
Proboinitza hut; Ponor karst region	**1**	water (n=1)	*M. gordonae*
Fountain in front town hall Buchin prohod; Ponor karst region	**1**	water (n=1)	mixed NTM culture

identified in the samples included: *M. chelonae* (n=3), *M. gordonae* (n=2), *M. intermedium* (n=3), *M. scrofulaceum* (n=1), *M. szulgai* (n=4), *M. fortuitum* group (n=4), mixed culture (n=4), *M. terrae* complex (n=1), one species was determined as belonging to the genus *Mycobacterium* without the possibility of a more precise identification because of the limitation of the test.

The distribution of NTM isolates by cave region was as follows: 73% (n=11) from 203 (Vratsa), 20% (n=3) from 204 (Ponor) and 7% (n=1) from area 303 (Bosnek). Interestingly, an isolate of NTM was detected far from the entrance of the cave and from the often visited area. The respective were samples collected approximately 104 meters from the cave entrance. This was a mixed culture of NTM species that remains to be precisely characterized.

CONCLUSION

Our study proved that NTM were spread in natural habitats in the country and that their species diversity was comparable to the most frequently isolated NTM species in Europe. The presence of NTM in cave ecosystems represents a potential source for human infection. We had successfully implemented a working protocol for decontamination and isolation of NTM species from the environment. For the first time in the country, NTM species were isolated from caves (biofilms and karst waters, sediments and bat guanos) and their geographical distribution were analyzed by habitat mapping of the most frequently isolated NTM. The species of the isolated NTM will be clarified by whole genome sequencing. The results obtained will form a database for tracking the trend of NTM distribution in the country and determining their clinical significance.

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DISCLOSURE OF CONFLICT OF INTEREST

There is no conflict of interest to declare.

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