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SPECIES OF *MYCOBACTERIUM TUBERCULOSIS* COMPLEX AND NONTUBERCULOUS MYCOBACTERIA IN RESPIRATORY SPECIMENS FROM SERBIA

IRENA ŽIVANOVIĆ[1], DRAGANA VUKOVIĆ[1], IVANA DAKIĆ[1] and BRANISLAVA SAVIĆ[1]

[1] *Institute of Microbiology and Immunology, Faculty of Medicine, University of Belgrade,* 11000 Belgrade, Serbia

Abstract - This study aimed to provide the first comprehensive report into the local pattern of mycobacterial isolation. We used the GenoType MTBC and CM/AS assays (Hain Lifescience) to perform speciation of 1 096 mycobacterial cultures isolated from respiratory specimens, one culture per patient, in Serbia over a 12-month period. The only species of the *Mycobacterium tuberculosis* complex (MTBC) identified in our study was *M. tuberculosis*, with an isolation rate of 88.8%. Ten different species of nontuberculous mycobacteria (NTM) were recognized, and the five most frequently isolated species were, in descending order, *M. xenopi*, *M. peregrinum*, *M. gordonae*, *M. avium* and *M. chelonae*. In total, NTM isolates accounted for 11.2% of all isolates of mycobacteria identified in pulmonary specimens. Our results suggest that routine differentiation among members of the MTBC is not necessary, while routine speciation of NTM is required.

Key words: Mycobacterium tuberculosis, nontuberculous mycobacteria, identification, GenoType MTBC, GenoType CM/AS

INTRODUCTION

Currently, the genus *Mycobacterium* encompasses 163 species and 13 subspecies described in the list of bacterial species with approved names (www.bacterio.cict.fr/m/mycobacterium.html). Strict pathogens are members of the *M. tuberculosis* complex (MTBC) and *M. leprae*, while all other species are collectively referred to as nontuberculous mycobacteria (NTM), and are considered opportunistic pathogens. The mycobacteria grouped in the MTBC are highly clonal, but differ significantly in terms of their host tropisms, phenotypic characteristics and pathogenicity. The complex includes obligate human pathogens *M. tuberculosis*, *M. africanum* and *M. canettii*, while species adapted to animals are *M. bovis* (cattle), *M. caprae* (sheep and goats), *M. microti* (rodents) and *M. pinnipedii* (seals and sea lions) (Gagneux and Small, 2007; Wirth et al., 2008). Novel MTBC pathogens such as *M. mungi* in banded mongooses (Alexander et al., 2010), and *M. orygis* in animals of the Bovidae family (van Ingen et al., 2012) have recently been described. Although all members of the complex are considered tubercle bacilli, the most important causative agent of tuberculosis (TB) in humans and the most frequently isolated species from clinical specimens is *M. tuberculosis*. With almost 9 million new TB cases and 1.4 million TB deaths in 2011, TB remains one of the major global health problems (WHO, 2012). NTM, though not as important as the MTBC, are being increasingly recognized as etiological agents of human diseases (Griffith et al., 2007; Kendall and Winthrop, 2013). Human disease resulting from NTM infection is classified into four major clinical syndromes: pulmonary disease, lymphadenitis, skin and soft tissue infections and disseminated disease. The isolation of NTM from clinical specimens increases in many countries, but the differences in geographical dis-

tribution of the species encountered are significant (Martin-Casabona et al., 2004; Griffith et al., 2007; Kendall and Winthrop, 2013).

The accurate identification of mycobacteria and, in particular, discrimination of MTBC isolates from NTM are essential for the appropriate treatment of patients, and have obvious epidemiological implications. Identification of mycobacteria to the species level based on the cultural and biochemical characteristics is often difficult and, in some instances, not possible (Springer et al., 1996). In addition, it is time consuming and error prone. The introduction of highly sensitive and specific molecular methods for the rapid identification of mycobacteria has changed the algorithm of laboratory diagnostics of mycobacteria and considerably increased the rapidity and accuracy of the process. Various techniques are used, such as DNA sequencing, PCR and real-time PCR assays, and reverse hybridization-based line probe assays (Harmsen et al., 2003; Richter et al., 2006; Pérez-Osorio et al., 2011). The main targets within the genome of mycobacteria are 16S rRNA, the *hsp65* gene, the internal transcribed spacer (ITS) and the *rpoB* gene (Harmsen et al., 2003; Pérez-Osorio et al., 2011). Commercially available tests for the molecular identification of mycobacteria, such as the GenoType assays (Hain Lifescience GmbH, Nehren, Germany), are of particular importance to routine laboratory practice. The GenoType assays are line probe assays based on reverse hybridization of multiplex PCR DNA amplicons with probes for different mycobacterial species immobilized to a nitrocellulose strip. The GenoType MTBC test identifies members of the MTBC based on detection of 23S rRNA gene sequences specific for members of the complex, species-specific sequences of the *gyrB* gene and the Region of Difference 1 (RD1). The GenoType CM assay (CM, common mycobacteria) targets 23S rRNA, and enables identification of the MTBC and the most frequently isolated NTM, while the GenoType AS assay (AS, additional species) also targets 23S rRNA and identifies uncommonly isolated NTM (Richter et al., 2003; Richter et al., 2006). The three tests were introduced into the TB laboratory network in Serbia in 2009.

The incidence rate of TB has significantly declined in the population in Serbia from 37 per 100 000 individuals in 2003, to 18 per 100 000 individuals in 2011. Thus, an increase in the prevalence of NTM isolation and disease may be expected, as was noted in other countries (Martin-Casabona et al., 2004; Griffith et al., 2007; Kendall and Winthrop, 2013). However, laboratory diagnostics of mycobacteria in Serbia is still primarily focused on *M. tuberculosis*. Prior to the introduction of molecular tests into the TB laboratory network in Serbia, the differentiation of MTBC species was not performed, apart from differentiation between *M. tuberculosis* and *M. bovis*. As far as NTM are concerned, identification of the most common NTM species was achievable only in four large laboratories, i.e. the majority of NTM isolates in the country were recognized as *Mycobacterium* sp. only. In this study, we used the GenoType MTBC and CM/AS assays to identify all mycobacterial cultures isolated from respiratory specimens, one culture per patient, in Serbia over a 12-month period, and provide the first comprehensive insight into the local pattern of mycobacterial isolation.

MATERIALS AND METHODS

Cultures analyzed

The number of patients with pulmonary specimens yielding a positive mycobacterial culture in Serbia over a 12-month period (December 1, 2010 through November 30, 2011) was 1 131. For the purposes of this study, one culture per patient was sent to the National Reference Laboratory for identification. The isolates of mycobacteria originated from 28 laboratories from all regions of the country. All cultures were grown on Löwenstein-Jensen (LJ) medium and identified as mycobacteria based on their phenotypic characteristics, i.e. microscopical and cultural characteristics. All strains were tested by the GenoType MTBC assay, and the isolates not recognized as MTBC were further tested by CM and AS assays.

GenoType assays

DNA isolation, PCR, hybridization and detection

Table 1. Identification of mycobacteria by the GenoType MTBC assay (interpretation chart).

Mycobacterium species	Band pattern
M. tuberculosis/"M. canettii"	1,2,3,4,5,6,7,8
M. africanum	1,2,3,4,5,6,7,10
M. microti	1,2,3,4,5,6,10,11
M. bovis ssp. bovis	1,2,3,4,7,9,10
BCG	1,2,3,4,7,9,10,13
M. bovis ssp. caprae	1,2,3,5,7,10,12
M. bovis ssp. caprae[1]	1,2,3,4,5,7,10

[1] Approximately 5% of the subspecies *M. bovis caprae* exhibit a banding pattern according to the right column.

Table 2. Identification of mycobacteria by the GenoType CM assay (interpretation chart).

Mycobacterium species	Band pattern
High GC Gram positive bacterium	1,2,(10)
Mycobacterium sp.[1]	1,2,3,(10)
M. avium ssp.	1,2,3,4
M. chelonae/M. immunogenum	1,2,3,5,10
M. abscessus/M. immunogenum	1,2,3,5,6,10
M. fortuitum 1[2]	1,2,3,7,14
M. fortuitum 2[2]/*M. mageritense*	1,2,3,7
M. gordonae	1,2,3,8,10
M. intracellulare	1,2,3,9
M. scrofulaceum[3]	1,2,3,9,10
M. interjectum	1,2,3,9,10,11
M. kansasii	1,2,3,(9),10,12
M. malmoense[4]/*M. haemophilum/M. palustre*	1,2,3,(9),10,13
M. marinum/M. ulcerans[5]	1,2,3,10,15
M. tuberculosis complex[6]	1,2,3,10,16
M. peregrinum/M. alvei/M. septicum	1,2,3,14
M. xenopi	1,2,3,17

[1] Species may possibly be further differentiated with the GenoType Mycobacterium AS kit.
[2] Due to variations in the probe region *M. fortuitum* is divided into two groups.
[3] *M. "paraffinicum"* and *M. parascrofulaceum* show the same banding pattern as *M. scrofulaceum*.
[4] *M. nebraskense* shows the same banding pattern. *M. haemophilum* can be identified by the GenoType Mycobacterium AS kit.
[5] *M. ulcerans* can be identified by the GenoType Mycobacterium AS kit.
[6] For further differentiation use GenoType MTBC kit.

were performed according to the manufacturer's instructions. The test results were interpreted using a template sheet and interpretation charts. The MTBC strip contains 13 probes: 1 – conjugate control which documents the efficiency of conjugate binding and substrate reaction; 2 – universal control which targets the 23S rRNA gene and detects Gram-positive bacteria with a high guanine (G) + cytosine (C) content; 3 – probe specific for the MTBC which targets 23S rRNA; 4-12 – species specific probes (*gyrB* gene);

Table 3. Identification of mycobacteria by the GenoType AS assay (interpretation chart).

Mycobacterium species	Band pattern
High GC Gram positive bacterium	1,2,(12)
Mycobacterium spec.	1,2,3,(12)
M. simiae	1,2,3,4,6
M. mucogenicum	1,2,3,5,12
M. goodii	1,2,3,6,7,14
M. celatum I+III	1,2,3,6,12,14
M. smegmatis	1,2,3,6,14
M. genavense/M. triplex	1,2,3,6,16,17
M. lentiflavum	1,2,3,6,16
M. heckeshornense	1,2,3,7,10
M. szulgai[2]*/M. intermedium*	1,2,3,8,12
M. phlei	1,2,3,8,16
M. haemophilum[1]	1,2,3,9,12
M. kansasii[3]	1,2,3,9,10,12 1,2,3,9,10,12,13 1,2,3,10,12 1,2,3,10,12,13
M. ulcerans	1,2,3,11,12
M. gastri	1,2,3,12,13
M. asiaticum	1,2,3,12,15
M. shimoidei	1,2,3,12,16

[1] *M. nebraskense* shows the same banding pattern as *M. haemophilum*.
[2] If this banding pattern is generated with the GenoType Mycobacterium AS kit, a differentiation of *M. szulgai* and *M. intermedium* can be performed using the GenoType Mycobacterium CM test. *M. szulgai* will display the banding pattern 1, 2, 3, 10 and 11; *M. intermedium* will display the pattern 1, 2, 3, 10.
[3] Due to sequence variations, 4 different *M. kansasii* banding patterns are possible.

and 13 – probe for *M. bovis* BCG (RD1). The MTBC species identified by the assay are listed in Table 1. The CM and AS strips contain 17 probes: 1 – conjugate control; 2 – universal control; 3 – genus control; and 4-17 – species specific probes. With the GenoType CM assay, 15 patterns can be obtained from 23 species (Table 2), while with the AS assay 16 patterns can be obtained from 18 species (Table 3).

RESULTS

Out of 1 131 cultures of mycobacteria recovered from pulmonary specimens, one culture per patient, over a 12-month period in Serbia, 35 were contaminated and therefore excluded from the study. In total, 1 096 cultures were tested by the GenoType assays and summarized results are shown in Chart 1. All 1 096 cultures were tested by using the GenoType MTBC assay. Membership of the MTBC was confirmed in 971 isolates, and all the isolates were identified as *M. tuberculosis* species. No other species of MTBC was detected in the sample analyzed. The remaining 125 isolates were tested by the CM assay and 123 were identified as NTM. Identification to the species level was achieved in 86 isolates; 37 cultures were identified as *Mycobacterium* sp., while for two strains identification as mycobacterial species failed. These two isolates were recognized as Gram-positive bacteria with a high G+C content in their DNA. Further identification of the 37 strains identified as *Mycobacterium* sp. by the CM test was performed using the AS assay, but none of them was identified to the species

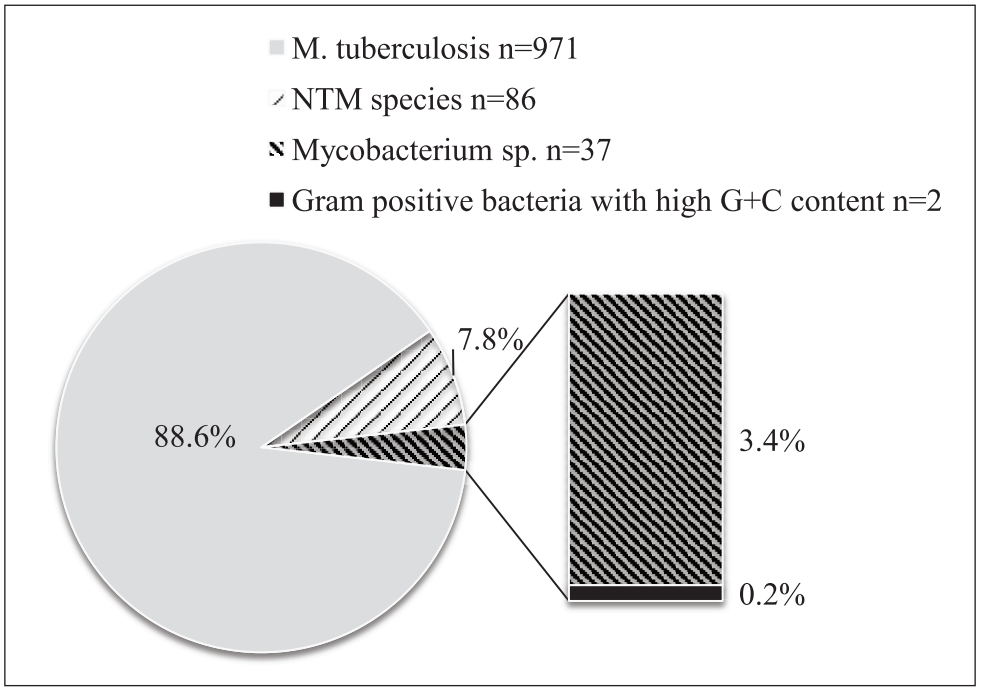

Chart 1. The identification of 1096 mycobacterial cultures by the GenoType MTBC, CM and AS assays.

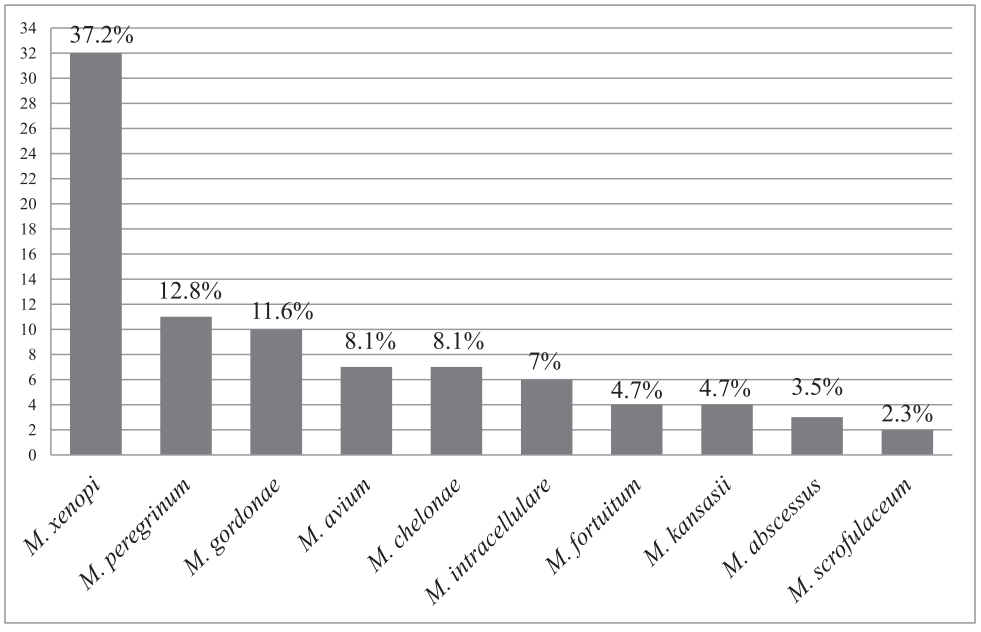

Chart 2. Frequency of NTM species among 86 isolates identified to the species level.

level. All 37 isolates were recognized as *Mycobacterium* sp. by the AS test as well. Two strains that were not recognized as mycobacterial species by the CM assay were also tested by the AS assay, and identification as Gram-positive bacteria with a high G+C content was confirmed. In summary, out of 1 094

cultures definitely identified as mycobacteria, 88.8% were identified as *M. tuberculosis*, while the fraction of cultures identified as NTM was 11.2%. The most frequently isolated NTM species was *M. xenopi* (32; 2.9%), while the isolation rates of the remaining 9 species were as follows: *M. peregrinum* (11; 1%), *M. gordonae* (10; 0.9%), *M. avium* (7; 0.6%), *M. chelonae* (7; 0.6%), *M. intracellulare* (6; 0.5%), *M. fortuitum* (4; 0.4%); *M. kansasii* (4; 0.4%); *M. abscessus* (3; 0.3%), and *M. scrofulaceum* (2; 0.2%). The fractions of differentiated NTM species out of the total number of NTM cultures identified to the species level are shown in Chart 2.

DISCUSSION

In this study, we used commercially available line probe assays to determine the species identities of clinical isolates of mycobacteria in Serbia. The sample of cultures analyzed was representative, since it included 97% of all mycobacterial cultures recovered from respiratory specimens, one culture per patient, over the study period in the country. Our study showed that the most predominant mycobacterial species in Serbia is *M. tuberculosis*. This was an expected result, since *M. tuberculosis* is, by far, the most frequently isolated mycobacterial species from respiratory specimens worldwide. We performed the first large-scale identification to the species level of local MTBC strains, and *M. tuberculosis* was the only MTBC species recognized among the isolates tested. Lack of occurrence of other members of the MTBC among local MTBC strains was also a somewhat expected result. *M. africanum* causes up to half of human TB in West Africa, and has infrequently been isolated in some European countries, such as Germany, England, France and Spain. The presence of *M. africanum* in areas outside the West African region was mostly detected in specimens from immigrants from this region (de Jong et al., 2010). In Serbia, however, the number of immigrants, including immigrants from Africa, is rather insignificant. Although *M. tuberculosis* and *M. canettii* produce identical patterns in the MTBC assay, and therefore cannot be differentiated by the test, it seems reasonable to assume that *M. canettii* is not present among local MTBC isolates. It is an infrequently isolated MTBC species, mostly from patients from, or with connection to Africa (Brosch et al., 2002). *M. microti* is also a rarely isolated MTBC species from human clinical specimens, and it typically causes TB in patients with immunologic deficits (Xavier et al., 2007). *M. bovis* used to be an important pathogen in Serbia decades ago, but the control programs focused on bovine TB, primarily pasteurization of milk and milk products, have significantly reduced the prevalence of the disease. Although a globally dispersed clonal complex of *M. bovis*, the *European 1* complex, has recently been described (Smith et al., 2011), no *M. bovis* isolates were recognized among MTBC strains tested in the present study. It is noteworthy, however, that a case of lymph node TB caused by *M. bovis* was registered in here two years ago (unpublished data). *M. caprae* has been recognized as a causative agent of human TB contracted from animals in different parts of Europe, and it is typically present in rural regions (Prodinger et al., 2002). Since Serbia is a largely rural country, we were particularly interested in the possible presence of this MTBC species among local isolates of mycobacteria, but none were detected among the strains tested. The MTBC assay does not enable identification of *M. pinnipedii*, *M. mungi* and *M. orygis*, the three most recently recognized members of the MTBC. Nevertheless, we do not believe that this is a limitation of our study, since *M. pinnipedii* and *M. orygis* are exceptionally rarely isolated from humans (Kiers et al., 2008; van Ingen et al., 2012), while transmission of *M. mungi* to humans has not been documented so far (Alexander et al., 2010).

As far as the pattern of isolation of NTM was concerned, out of 123 NTM isolates, 86 were identified to the species level. Our study showed that *M. xenopi* is the most frequently identified NTM in pulmonary specimens from Serbia. In several recent studies regarding isolation rates of NTM in the Netherlands (van Ingen et al., 2008), Poland (Safianowska et al., 2010) and Great Britain (Moore et al., 2010), *M. xenopi* has also been reported as one of the most frequently isolated NTM from respiratory specimens. The high isolation rate of *M. gordonae* noted among the NTM strains tested in the present study is not

surprising, since this species is typically encountered as a contaminant in respiratory specimens (Griffith et al., 2007). On the other hand, the high isolation rate of *M. peregrinum* was an unexpected result. A small number of cases of sporadic infections caused by *M. peregrinum* have been described (Brown-Elliot and Wallace, 2002), but frequent occurrence of this rapidly growing mycobacterium in respiratory specimens is unusual (Martin-Casabona et al., 2004; Griffith et al., 2007). One possible explanation is the presence of specific environmental sources of *M. peregrinum*, e.g. water distribution systems, but the environmental distribution of NTM has not been investigated in Serbia so far. Isolation rates of the *M. avium* complex (MAC), which includes *M. avium* and *M. intracellulare*, and *M. kansasii* established in our study were significantly lower than those reported in other studies (Martin-Casabona et al., 2004; Griffith et al., 2007; Kendall and Winthrop, 2013). The low frequency of MAC isolation, in particular, is a noteworthy finding, since it is the most frequently identified NTM in respiratory specimens from different parts of the world (Martin-Casabona et al., 2004; Griffith et al., 2007; van Ingen et al., 2008; Moore et al., 2010; Simons et al., 2011; Kendall and Winthrop, 2013). There is no apparent explanation for this finding, but it has already been perceived that the isolation of NTM species is constantly changing in most of the geographical areas studied (Martin-Casabona et al., 2004). It should be noted that in 37 NTM cultures, 30% of the 123 NTM isolates recognized in the present study were identified as *Mycobacterium* sp. only. Although determination of the clinical relevance of NTM isolates from pulmonary specimens was beyond the scope of this study, we retrospectively analyzed the laboratory records in order to establish the possible clinical significance of these isolates. We used the microbiological component of the American Thoracic Society (ATS) criteria (Griffith et al., 2007), and none of the 37 cases analyzed met the criteria. Namely, none of the patients had multiple (at least two) sputum specimens that yielded a positive culture of mycobacteria. In addition, 16 isolates identified as *Mycobacterium* sp. originated from the same regional laboratory, which may indicate a specific environmental exposure. These results suggest that other molecular methods for the further identification of isolates recognized as *Mycobacterium* sp. are not necessary in the routine diagnostic algorithm.

The results obtained should lead to a rational application of the GenoType assays in routine laboratory diagnostics of mycobacterial infections in Serbia, since we intend to increase the annual fractions of mycobacterial cultures identified by the molecular assays. As noted above, the GenoType tests were introduced into the country in 2009. Out of the total number of mycobacterial cultures isolated (one culture per patient was counted), the fractions of cultures identified by the assays were 23% and 42% in 2009 and 2010, respectively. Our results suggest that routine differentiation among members of the MTBC is not necessary, while routine speciation of NTM, on the other hand, is required. NTM isolates accounted for 11.2% of all isolates of mycobacteria identified in pulmonary specimens. Rapid speciation that distinguishes MTBC from NTM is an important prerequisite for the proper management of patients with mycobacterial infections, and will be introduced as a required standard into routine laboratory diagnostics of these infections in Serbia. The results of the study also indicate that assessment of the NTM disease burden in Serbia as well as a search for the environmental sources of these bacteria, are needed.

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