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RETROSPECTIVE ANALYSIS OF SLOVENIAN *MYCOBACTERIUM AVIUM* COMPLEX AND *MYCOBACTERIUM ABSCESSUS* COMPLEX ISOLATES AND MOLECULAR RESISTANCE PROFILE

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Abstract. Mycobacteria belonging to *Mycobacterium (M.) avium* complex (MAC) and *M. abscessus* complex (MABSC) are the most frequent causes of mycobacteriosis in the world. In the last few years MAC and MABSC taxonomy was rapidly changing due to new molecular methods conveying the possibility to differentiate between species. New techniques are able to identify *M. chimaera* that was previously recognized as *M. intracellulare* and also differentiate subspecies of MABSC. Due to their natural habitat, non-tuberculous mycobacteria (NTM) are constantly exposed to various concentrations of antimicrobial drugs and other chemicals and consequently they had developed different mechanisms of resistance. Macrolides and aminoglycosides are frequently used drugs to treat MAC and MABSC infections. The aim of our nation-wide survey was to obtain information about MABSC subspecies prevalence in Slovenia and to assess the percentage of misidentifications of *M. chimaera* isolates as *M. intracellulare* in the past. Moreover, the purpose of our study was to reveal, which of the two species *M. intracellulare* or *M. chimaera* is clinically more relevant in Slovenia. Further, the aim of the study was to detect mutations in *erm(41), rrl* and *rrs* genes, which are known to convey macrolide resistance (*erm(41)* and *rrl*) and aminoglycoside resistance *(rrs).* One hundred and thirty-two Slovenian mycobacterial isolates obtained from the National Mycobacterial Collection that belong to MAC and MABSC were analysed. GenoType NTM-DR was used to differentiate *M. intracellulare* from *M. chimaera* and subspecies of MABSC. Our results showed that 48% of previously identified *M. intracellulare* isolates were actually *M. chimaera* isolates and that *M. abscessus* subsp. *abscessus* was the most frequent subspecies of MABSC. Most of the MABSC isolates carried the inducible macrolide resistance genes (*erm(41)* and *rrl*), however none of the isolates of MAC and MABSC had mutations in *rrs* genes for aminoglycoside resistance.

Key words: *nontuberculous mycobacteria, Mycobacterium abscessus, Mycobacterium avium, macrolide resistance, aminoglycoside resistance, nation-wide study.*

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РЕТРОСПЕКТИВНЫЙ АНАЛИЗ СЛОВЕНСКИХ ИЗОЛЯТОВ *MYCOBACTERIUM AVIUM* COMPLEX И *MYCOBACTERIUM ABSCESSUS* COMPLEX И МОЛЕКУЛЯРНЫЙ ПРОФИЛЬ УСТОЙЧИВОСТИ

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Резюме. Микобактерии, принадлежащие к *Mycobacterium avium* complex (MAC) и *Mycobacterium abscessus* complex (MABSC), являются наиболее частыми причинами микобактериоза в мире. В последние несколько лет таксономия MAC и MABSC быстро менялась в результате появления новых молекулярно-генетических методов, позволяющих выявлять различия в пределах вида. Это позволило идентифицировать вид *M. chimaera*, который ранее относили к *M. intracellulare*, а также дифференцировать подвиды MABSC. Нетуберкулезные микобактерии являются типичными обитателями окружающей среды и в значительной мере подвержены воздействию различных концентраций противомикробных препаратов и других химических веществ, что привело к развитию различных механизмов природной резистентности. Макролиды и аминогликозиды наиболее часто используются для лечения инфекций, вызванных MAC и MABSC. Целью общенационального исследования являлась оценка распространенности подвидов MABSC в Словении, а также выявление случаев ошибочной идентификации изолятов *M. chimaera* как *M. intracellulare* ранее. Вместе с тем целью работы было выявить, какой из двух видов *M. intracellulare* или *M. chimaera* являлся клинически значимым на территории Словении, а также обнаружение мутаций в генах *erm(41), rrl* и *rrs*, которые, как известно, ассоциированы с развитием устойчивости к макролидам (*erm(41)* и *rrl*) и аминогликозиду *(rrs).* Нами были проанализированы 132 изолята MAC и MABSC, полученных из Национальной коллекции микобактерий Словении. GenoType NTM-DR использовался для дифференциации видов *M. intracellulare* и *M. chimaera*, а также подвидов MABSC. Результаты исследования показали, что 48% изолятов, ранее идентифицированных как *M. intracellulare*, относились к виду *M. chimaera*; наиболее распространенным подвидом MABSC являлся *M. abscessus* subsp. *abscessus.* Большинство изолятов MABSC обладали генами устойчивости к макролидам (*erm (41)* и *rrl*), однако ни один из изолятов MAC и MABSC не выявлено мутаций устойчивости к аминогликозиду в гене *rrs.*

Ключевые слова: *нетуберкулезные микобактерии, Mycobacterium abscessus, Mycobacterium avium, резистентность, макролиды, аминогликозиды, общенациональное исследование.*

Introduction

Non-tuberculous mycobacteria (NTM) are environmental microorganisms that colonise different surfaces and can be isolated from soil, natural waters, air, household plumbing systems, animals and human specimens too. They are resistant to many disinfectants and antibiotics and therefore many infections caused by NTM cannot be cured with commonly used antibiotics [1]. They are causing diseases especially among immunocompromised patients.

Among *Mycobacterium (M.) avium* complex (MAC) there are two well known species causing disease in humans, *M. avium* and *M. intracellulare.* In 2004, development of more specific molecular methods revealed a new species among MAC, *M. chimaera.* Previously *M. chimaera* was, due to similar phenotypic and genotypic characteristics, misidentified as *M. intracellulare.* When *M. chimaera* was first described in 2004 by Tortoli et al. [11] it was estimated that it is a highly virulent species. Afterwards results showed that *M. intracelullare* was more virulent than *M. chimaera* [9, 11]. In MAC, genes connected with macrolide and aminoglycoside resistance are *rrl* and *rrs*, respectively [6]. In 2012 *M. chimaera* caused two invasive infections after cardiac surgery [8]. Afterwards more than 100 cases of *M. chimaera* infections were revealed in European countries and around the world. This opportunistic pathogen became linked with heater-cooler units (HCUs) used during cardiac surgeries. *M. chimaera* has preferences to colonise warm, humid surfaces where it forms biofilms and has high potential to aerosolize. During surgeries, HCUs produce aerosols and *M. chimaera* is dispersed into the air and can colonize the patient. Due to *M. chimaera*'s slow-growth, it can take even several years after surgery to develop disease [4, 8].

Mycobacterium abscessus, belonging to *M. abscessus* complex (MABSC), is one of the most resistant pathogens as it possesses acquired and innate drug resistance. In the last years, MABSC was divided into three subspecies: *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *bolletii* in *M. abscessus* subsp. massiliense. It is known that the three subspecies have different resistance profiles, hence correct species identification is clinically important. Three genes are important for MABSC resistance: *erm(41), rrl* and *rrs.* Gene *erm(41)* encodes the inducible 23S rRNA methylase and contributes to inducible macrolide resistance. Two *erm(41)* sequevars depending on the T/C polymorphism at nucleotide 28, are present in the MABSC population. *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *bolletii* harbour gene *erm(41)* with a T at the nucleotide position 28 that leads to inducible

macrolide resistance. *M. abscessus* subsp. *massiliense* however, has due to a deletion in this region a non-functional gene and is therefore macrolide susceptible. Further, high level of macrolide resistance is caused by point mutations in the peptidyl-transferase-binding region of *rrl* gene, which can be present in all three subspecies. The macrolide antibiotic clarithromycin was the drug of choice in last decade, and still is for cystic fibrosis (CF) patients. Aminoglycoside resistance is caused by single point mutations in the *rrs* gene encoding 16S rRNA and is also present in all three MABSC subspecies [2, 5].

The aim of our study was to perform a retrospective analysis of all Slovenian MAC and MABSC isolates with new molecular test GenoType NTM-DR, which is known to successfully identify *M. chimaera* isolates and also enables mutation identification in *rrl, rrs* and *erm(41)* genes [2, 4]. Our purpose was therefore to identify how many *M. chimaera* isolates were misidentified as *M. intracellulare* and to estimate how many isolates are resistant to macrolides and aminoglycosides and which mutations are prevalent in Slovenia. Moreover, information about clinical relevance of isolates was obtained.

Materials and Methods

In total 133 clinical isolates (obtained from 126 patients in the period from January 2007 to September 2016) from the Slovenian National Mycobacterial Collection at the Clinic Golnik were included in our county-wide survey. Clinical isolates were retrieved from 70 male and 56 female patients. Fisher's exact test was used to statistically evaluate the data related to clinical relevance. The threshold for statistical significance was set at a P value of < 0.05. All isolates were previously identified with the diagnostic test GenoType CM/AS (Hain Lifescience, Nehren, Germany) as MABSC (n = 31) or *M. intracelullare* (n = 102). The previously used test cannot differentiate subspecies in MABSC and M. intracelullare from the closely related species *M. chimaera*. All investigated isolates were stored at –20°C on glass beads and subcultured on Löwenstein–Jensen medium or Middlebrook 7H10 agar plates. Total DNA was extracted from two loops of mycobacterial culture resuspended in 0,3 mL of sterile water. Cell lysis in the mycobacterial culture was done with incubation at 95°C for 20 minutes followed by sonication for 15 minutes. Samples were centrifuged at maximum speed 14 000 RPM for 5 minutes. Supernatant with the extracted DNA was used for GenoType NTM-DR. PCR protocol and DNA hybridisation, was done according to manufacturer instructions as previously described [6].

Results and Discussion

In Slovenia in the last decade, the number of NTM isolates is increasing [12]. In the period 2000–2016 MAC and MABSC isolates were second and seventh most frequently isolated NTM in Slovenia, respectively. A similar trend — increasing number of NTM's — was noticed in other countries around the world too [10].

Our nation-wide analysis of 102 MAC isolates showed that 53/102 (52%) isolates belonged to *M. intracellulare* and 49/102 (48%) isolates belonged to *M. chimaera*. We can therefore conclude that *M. chimaera* is nearly as common in our country as *M. intracellulare*. Schweickert et al. [9] reported that in Germany almost 86% of previously identified species as *M. intracelullare* are actually *M. chimaera*. Mok et al. [4] reported data from Ireland where 55% of *M. intracelullare* isolates were misidentified and are actually *M. chimaera*. Our study showed that *M. intracelullare* was more often clinically relevant than *M. chimaera* (29% vs. 6% of clinical isolates, respectively). Our obtained results are concordant with results of Schweickert et al. [9] and in contrary with Tortoli et al. [11] who proposed *M. chimaera* strains as more clinically relevant than other MAC species.

Retrospective analysis of Slovenian MABSC isolates from January 2007 to September 2016 showed that predominant species in our country was *M. abscessus* subsp. *abscessus* 24/31 (77.4%), followed by *M. abscessus* subsp. *bolletii* 4/31 (12.9%) and *M. abscessus* subsp. massiliense 3/31 (9.7%). Our results are comparable with other countries in Europe

Table. Isolates of *M. abscessus* complex, *M. intracellulare* and included in the study presented by patients status and clinical relevance

Mycobacterial species	All patients		Cystic fibrosis patients	
	No. of all isolates	No. (%) of CR isolates	No. (%) of all isolates	No. (%) of CR isolates
M. abscessus* subsp. *abscessus	24	7 (29.1)	4 (16.7)	4 (16.7)
M. abscessus* subsp. *massiliense	4	0	0	0
M. abscessus* subsp. *bolletii	3	1 (33.3)	0	0
M. intracellulare	53	15 (28.3)*	0	0
M. chimaera	49	3 (6.1)*	3 (6.1)	0
Total	133	26 (19.5)	7 (5.2)	4 (57.1)

P values obtained following Fisher exact test are indicated by asterisks as follows: * $P < 0.05$; CR: clinically relevant

and in US, where *M. abscessus* subsp. *abscessus* represents around 45–65% of all MABSC isolates [7, 13]. Meanwhile in East Asia, the percentage of MABSC isolates is much higher among all NTM isolates. Furthermore, Asian countries also report *M. abscessus* subsp. *abscessus* as frequently isolated as *M. abscessus* subsp. *massiliense* [3] Our hypothesis was also that *M. abscessus* subsp. *bolletii* is rarest subspecies among MABSC in Slovenia, which would be concordant with results yielded in other studies [3, 7, 13]. Our hypothesis failed, but the number of samples was relatively small so in future more isolates will be need to be tested to confirm it.

Molecular analysis of resistance genes in MABSC showed that all 4/4 (100%) *M. abscessus* subsp. *bolletii*i and 22/24 (92%) *M. abscessus* subsp. *abscessus* had the T polymorphism at position 28 in *erm(41)* gene, which leads to inducible resistance to macrolides. All 3/3 (100%) *M. abscessus* subsp. massiliense isolates also had the T polymorphism in *erm(41)* gene but due to deletion in this gene, isolates did not show inducible resistance to macrolides. No isolate of MABSC had a point mutation in *rrl* gene or in *rrs* gene. Thus, it can be concluded that high percentage of MABSC isolates can develop inducible macrolide resistance but high-level macrolide resistance is not present at the moment. None of Slovenian MABSC isolates from the study had aminoglycoside resistance.

Also in MAC isolates no mutation in *rrl* nor *rrs* gene was detected. Based on this observation it can be concluded that all analysed MAC isolates were sensitive to both, macrolides and aminoglycosides with molecular methods.

Slovenian *M. intracellulare* isolates were found to be statistically significantly more clinical relevant than *M. chimaera*. None of *M. intracellulare* isolates was obtained from CF patient specimens. On the other hand, three of *M. chimaera* isolates were isolated from CF patient specimens, but were not clinically relevant. Higher percentage of *M. abscessus* subsp. *abscessus* isolates were found as clinical relevant, but with no statistical significance ($P > 0,05$). Furthermore, *M. abscessus* subsp. *abscessus* was isolated from CF patients too (see Table).

To sum up, in Slovenia the number of MABSC isolates is slowly increasing, with *M. abscessus* subsp. *abscessus* being predominant subspecies. *M. abscessus* subsp. *abscessus* subspecies is the only subspecies isolated from specimens from patients with CF. Our MABSC isolates have high proportion of inducible resistance to macrolides. This fact needs to be considered when treating patients with MABSC infections, especially CF patients. *M. intracellulare* was a slightly more frequently isolated from human specimens than *M. chimaera*, and was more often clinically relevant in the last 10 years.

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