Original Article

Prevalence and Antimicrobial Susceptibility of *Mycobacterium abscessus* in a General Hospital, China*

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Abstract

Objective To gain greater insight into the prevalence drug resistant profiles of *M. abscessus* from a general hospital in Beijing, China.

Methods Partial gene sequencing of *16S*, *hsp65*, and *rpoB* were used to distinguish the species of NTM isolates. All strains identified as *M. abscessus* were further enrolled in the drug susceptibility testing by using broth microdilution method.

Results We found that *M. avium* complex was the most frequent NTM organism, accounting for 54.1% (33/61) of all isolates. Behind MAC, the second most common organisms were *M. abscessus* (22 out of 61, 36.1%). Average rates of resistance were 4.5% for AMK, 9.1% for LZD, and 13.6% for CLA, respectively. In contrast, resistance to LEV (17/22, 77.3%), IMI (9/22, 40.9%), and SMX (10/22, 45.5%) was noted in more than 40% of *M. abscessus* isolates. DNA sequencing revealed that all the CLA-resistant isolates harbored nucleotide substitutions in position 2058 (1/3, 33.3%) or 2059 (2/3, 66.7%) of 23S rRNA.

Conclusion In conclusion, our data demonstrated that *M. intracellulare* and *M. abscessus* were the most common NTM species in the general hospital of Beijing. CLA, AMK, LZD showed promising activity, where as LEV, IMI, and SMX exhibited poor activity against *M. abscessus in vitro*.

Key words: *Nontuberculous mycobacteria*; *Mycobacterium abscessus*; China

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INTRODUCTION

Although *Mycobacterium tuberculosis* is one of the most important mycobacterial species threatening the public health all over the world, other species, known as *nontuberculous mycobacteria* (NTM), are being responsible for increasing emergency of NTM disease in human[1-3]. NTM are commonly isolated from different environmental sources, including soil, treated and untreated water, animals and food, divided into slow growing mycobacteria (SGM) and rapid growing mycobacteria (RGM) according to their behavior in the culture[2].

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Of the rapid growing mycobacteria relevant to human disease (including *M. fortuitum*, *M. abscessus*, and *M. chelonae*), *M. abscessus* is the most common type to cause lung disease, and is also the most difficult to treat in the clinical practice[4-5], the mobility rate of which have be nearly 20% in susceptible individuals in the past decades[5]. The major problem during treatment is that *M. abscessus* is not responsive to 'standard' antituberculosis agents, but susceptible to other common antibiotics, such as macrolides, beta-lactams or tetracyclines[6]. The drug susceptibility of the isolates is variable, which make it essential to obtain *in vitro* drug susceptibility profile of individual *M. abscessus* strains to generate an effective therapeutic regimen for the patients[7].

In China, *M. abscessus* is one of the most common nontuberculous mycobacetria causing lung disease[8]. To gain greater insight into the prevalence drug resistant profiles of *M. abscessus*, we firstly identified the *in vitro* susceptibility of *M. abscessus* isolates from patient's respiratory specimens from a general hospital in Beijing, China. Thirteen antibiotics, which were extensively used in the clinical practice for the treatment of NTM infections, were selected to perform the minimum inhibitory concentration (MIC) of *M. abscessus*.

MATERIALS AND METHODS

Bacterial Strains

Data were obtained from patients at Beijing Hospital during the 36-month period between April 1, 2012, and March 31, 2015. During this period, sputum samples from all TB suspect patients were collected, and the sputum was digested with NALC-NaOH (4%) for 15 min, and inoculated onto Löwenstein-Jensen (L-J) medium according to the previous report[9]. The NTM isolates were firstly distinguished from *M. tuberculosis* isolates with PNB and TCH modified L-J medium. Partial gene sequencing of *16S*, *hsp65*, and *rpoB* were used to distinguish the species of NTM isolates[8]. All strains identified as *M. abscessus* were further enrolled in the drug susceptibility testing. In addition to clinical isolates, one reference strain of *M. abscessus*, ATCC35761 were obtained from National Tuberculosis Reference Laboratory of China. The protocols applied in this study were approved by the Ethics Committee of Beijing Hospital, and informed consent was obtained from all patients whose sputum specimens were used in scientific studies.

Species Identification

All the clones growing on the L-J medium were scraped and genomic DNA was extracted by a rapid-boiling method[10]. The genomic DNA was used for the sequencing of multiple genes, including 16S rRNA, *hsp65*, and 16S-23S rRNA internal transcribed spacer (ITS) sequence, to perform molecular species identification[8]. The 50 μL PCR mixtures were prepared as follows: 5 μL 10×PCR buffer, 200 μmol/L of each dNTP, 0.2 μmol/L of each primer set, 5 μL crude genomic DNA, 1 U HotStar Taq polymerase (Qiagen). The amplification was performed in athemocycler (Bioer, Hangzhou, China) as follows: 5 min at 94 °C for initial denaturation, and then 35 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min, and extension at 72 °C for 2 min, followed by a final extension at 72 °C for 10 min. The PCR products were sent to Sango Company (Beijing, China) for sequencing. All the sequencing results were aligned to the GenBank database over the Internet by using the NCBI BLAST server (www.ncbi.nlm.nih.gov).

Minimal Inhibition Concentration (MIC)

Antimicrobial susceptibility testing was performed using broth microdilution method according to the guidelines by the National Committee for Clinical Laboratory Standards (NCCLS)[11]. Briefly, organisms scraped from the culture media was transferred to saline with 0.02% Tween 80. The suspension was mixed vigorously on a votex for 1 min until the bacterial colonies were dispersed homogeneously. Then the suspension was diluted to the density of a 0.5 McFarland standard. Followed by further dilution two hundred times with cation-adjusted Mueller-Hinton broth media (CAMHB), the diluted bacterial suspension was inoculated at a final concentration of 3.75×10^5 CFU/mL. MICs were determined for 13 antimicrobial agents, which were used in the treatment regimen against *M. abscessus* infection, were selected for drug susceptibility profile analysis, including clarithromycin (CLA), azithromycin (AZM), amikacin (AMK), cefoxitin (CFX), imipenem (IMI), linezolid (LZD), moxifloxacin (MOX), levofloxacin (LEV), tigecycline (TGC), capreomycin (CAP), tobramycin (TOB), sulfamethoxazole (SMX), and clofazimine (CLO). All agents mentioned above were purchased from Sigma-Aldrich. The breakpoints used to

determine the resistance of *M. abscessus* were referenced from the the guidelines by NCCLS, which were shown in Table 1.

DNA Sequencing

The fragments of 23S rRNA and 16S rRNA were amplified by PCR, respectively. The primer pairs were synthesized as previously reported. PCR products were sent to Qingke Company (Beijing, China) for sequencing service. The resulting sequences were aligned to the homologous sequences of the reference *M. abscessus* strain (ATCC35761) using BLASTn in the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov/BLAST).

RESULTS

Identification of NTM Species

A total of 61 NTM isolates identified by conventional biochemical method were enrolled in further molecular identification. By the use of multilocus sequence analysis, the NTM strains could be divided into the species level. As shown in Table 1, *M. avium* complex was the most frequent NTM organism, accounting for 54.1% (33/61) of all isolates. Of 33 isolates classified as *M. avium* complex, there were 25 (36.1%) *M. intracellulare* and 8 (13.1%) *M. avium* isolates, respectively. Behind MAC, the second most common organisms were *M. abscessus* (22 out of 61, 36.1%); other organisms (6 out of 61, 9.8%) included *M. Kansasii* (*n*=3), *M. fortuitum* (*n*=2), *and M. chelonae* (*n*=1).

Table 1. Breakpoint Values of Different Antimicrobial Agents

Antimicrobial Agent	MIC Value (μg/mL)		
	Susceptible	Intermediate	Resistant
Clarithromycin	≤2	4	≥8
Azithromycin	≤16	-	≥32
Amikacin	≤16	32	≥64
Cefoxitin	≤16	32-64	≥128
Imipenem	≤4	8-16	≥32
Linezolid	≤8	16	≥32
Moxifloxacin	≤1	2	≥4
Levofloxacin	≤1	2	≥4
Tigecycline	≤1	2-4	≥8
Capreomycin	-	-	-
Tobramycin	≤2	4	≥8
Sulfamethoxazole	≤32	-	≥64
Clofazimine	-	-	-

Drug Susceptibility Profiles

The distribution of MICs of each antimicrobial agent for *M. abscessus* isolates was shown in Table 2. Overall, CLA was highly active against *M. abscessus* strains, with MIC_{50} of 0.06 μg/mL and MIC_{90} of 8 μg/mL, respectively. Similarly, AMK, LZD, MOX, TGC, and CLO also showed activity against *M. abscessus,* the MIC_{50}s and MIC_{90}s of which were lower than 2 and 16 μg/mL, respectively. When using the breakpoint listed in Table 1, resistance to AMK (4.5%, 1/22) was rare, and only one AMK-resistant isolate was encountered in this study. Average rates of resistance were 9.1% for LZD and 13.6% for CLA, respectively. For fluoroquinolones, MOX and LEV exhibited different drug resistant profiles, and statistical analysis revealed that the percentages of MOX-resistance (6/22, 27.3%) isolates were significantly lower than LEV-resistant (17/22, 77.3%) among *M. abscessus* (P<0.01). In addition to LEV, resistance to IMI (9/22, 40.9%) and SMX (10/22, 45.5%) was noted in more than 40% of *M. abscessus* isolates (Table 3).

Mutations Conferring CLA and AMK Resistance

We further analyzed the genomic mutation conferring antimicrobial agent resistance. Mutations in 23S rRNA, which are associated with CLA resistance in mycobacteria, were firstly detected by DNA sequencing in all CLA-resistant *M. abscessus* isolates. As shown in Table 4, all the CLA-resistant isolates harbored nucleotide substitutions in position 2058 (1/3, 33.3%) or 2059 (2/3, 66.7%) of 23S rRNA. For AMK resistance, we found no genetic mutation within *rrs* gene, indicating that other drug-resistant mechanism may play an essential role in the AMK resistance in *M. abscessus*.

DISCUSSION

Lung diseases caused by NTM have been increasing worldwide, while the distribution of mycobacteria species varies significantly by geographic region[8]. In United States, *M. avium* complex is the most frequent pathogen associated with NTM lung diseases, followed by *M. kansasii*[12]. In England and Welsh, *M. kansasii* was the most common[13]. According to previous literatures from different regions of China, *M. avium* complex and *M. abscessus* accounted for the majority of isolated NTM species[8]. In line with previous findings, our data demonstrated that these two species were also

Table 2. *In vitro* Susceptibility of 22 *M. abscessus* Strains

Antimicrobial Agent	MIC Range (μg/mL)	MIC_{50} (μg/mL)	MIC_{90} (μg/mL)	No. of Strains with Different MIC (μg/mL)												
				0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256
Clarithromycin	0.06-16	0.06	8	13	2	1	1	1	0	1	2	1	0	0	0	0
Azithromycin	0.06-64	1	64	2	1	3	4	3	2	1	1	0	2	3	0	0
Amikacin	0.06-64	2	16	0	0	0	1	3	12	2	1	1	1	1	0	0
Cefoxitin	8-256	64	128	0	0	0	0	0	0	0	1	2	6	6	5	2
Imipenem	0.5-256	16	128	0	0	0	1	0	1	1	4	6	3	3	2	1
Linezolid	0.5-64	2	16	0	0	0	2	4	6	6	1	1	1	1	0	0
Moxifloxacin	0.06-4	2	8	2	0	1	1	6	6	3	2	1	0	0	0	0
Levofloxacin	0.12-32	4	16	0	1	1	0	1	2	6	7	3	1	0	0	0
Tigecycline	0.06-16	1	8	2	2	1	2	3	4	4	3	1	0	0	0	0
Capreomycin	0.25-64	8	16	0	0	2	1	0	1	2	5	9	1	1	0	0
Tobramycin	0.25-128	4	16	0	0	1	0	1	3	9	5	1	0	1	1	0
Sulfamethoxazole	2-256	32	64	0	0	0	0	0	1	1	1	2	7	8	1	1
Clofazimine	0.06-16	0.25	4	3	1	12	2	0	1	1	1	1	0	0	0	0

Table 3. Percentage of *M. abscessus* Isolates Against Different Antimicrobial Agents

Antimicrobial Agent	No. of Isolates (%)		
	Susceptible	Intermediate	Resistant
Clarithromycin	18 (81.8)	1 (4.5)	3 (13.6)
Azithromycin	17 (77.3)	-	5 (22.7)
Amikacin	20 (90.9)	1 (4.5)	1 (4.5)
Cefoxitin	3 (13.6)	12 (54.5)	7 (31.8)
Imipenem	3 (13.6)	10 (45.5)	9 (40.9)
Linezolid	19 (86.4)	1 (4.5)	2 (9.1)
Moxifloxacin	10 (45.5)	6 (27.3)	6 (27.3)
Levofloxacin	3 (13.6)	2 (9.1)	17 (77.3)
Tigecycline	10 (45.5)	8 (36.4)	4 (18.2)
Tobramycin	5 (22.7)	9 (40.9)	8 (36.4)
Sulfamethoxazole	12 (54.5)	-	10 (45.5)

Table 4. Mutations Conferring CLA and AZM Resistance in 3 Clinical *M. abscessus* Isolates

Isolate	CLA MIC (μg/mL)	AZM MIC (μg/mL)	23S rRNA Mutation
MA-3	16	64	A2058G
MA-8	8	32	A2059G
MI-17	8	64	A2059G

the most predominant NTM species in the general hospital of Beijing. In China, most of patients with TB suspects primarily seek health care in the general hospital rather than TB specialized hospital or TB dispensary[14]. Different from pulmonary TB patients, patients caused by NTM infection can receive treatment in the general hospitals, which may avoid the nosocomial infection with tuberculosis during in-patient period. Hence, the high prevalence of NTM in China highlights the urgent need to perform rapid species identification among TB suspects in the general hospitals.

Due to the broad spectrum of drug resistance against many antibiotics, *M. abscessus* has been considered as the most resistant organisms to chemotherapeutic agents[6]. Before anti-infection treatment, *in vitro* susceptibilities to antimicrobial agents for clinical *M. abscessus* isolates are recommended to generate the effective chemotherapy regimens. According to the ATS/IDSA guidelines, CLA, CFX, and AMK are recommended for the treatment of patients infected with *M. abscessus*[1]. In the present study, we observed that CLA and AMK were *in vitro* active against *M. abscessus*, which was in consistent to previous report[15]. In contrast, 31.8% of *M. abscessus* in the present study showed resistant against CFX, indicating the addition of CFX in the treatment regimen might not improve the prognosis among one third of *M. abscessus* diseases. Compared with

CFX, LZD, a member of the oxazolidinone class of antibiotics[16], showed better *in vitro* activity against *M. abscessus* isolated from China. The frequency of LZD-resistant *M. abscessus*isolates observed in this study (9.1%) is similar to that in India (7%)[17], although it is lower than that in Britain (63%)[18] and that in South Korea (20%)[19]. Several explanations may be attributed to this difference. On one hand, LZD is a new antimicrobial agent introduced in developing countries, such as China and India. The relatively short period used for the antimicrobial treatment in these regions may be the most important reason for the low proportion of LZD-resistance among *M. abscessus* isolates. On the other hand, the *M. abscessus* strains from Britain were mainly isolated from individuals with cystic fibrosis (CF), an indicator associated with poor clinical outcome[18]. The higher resistant prevalence aganist LZD may be due to their constant exposure to antibiotics. Our findings have demonstrated that LZD serves as an alternative for clinical treatment of *M. abscessus*, whereas the high price of LZD poses the biggest obstacle to its routine use against *M. abscessus* diseases in China.

CLA resistance in mycobacterium has been proved to be associated with mutations located in the 23S rRNA gene[20-22]. In agreement with previous studies, all CLA-resistant *M. abscessus* isolates harbored nucleotide substitutions at position 2058 and 2059 of the 23S rRNA gene, suggesting that this gene may used as an promising target for the prediction of CLA resistance in *M. abscessus*[6,23]. We also found an AMK-resistant *M. abscessus* isolate in this study. Although numerous literatures have mutations affecting the 16S rRNA gene confer high-level resistance to AMK[6,24], no nucleotide substitution has been detected in this AMK-resistant strain of this study. Further molecular analysis will provide new insight onthe functions of other additional resistance mechanisms conferring AMK resistance in *M. abscessus*, including efflux pump and cellular permeability.

In conclusion, our data demonstrated that *M. intracellulare* and *M. abscessus* were the most common NTM species in the general hospital of Beijing. CLA, AMK, LZD showed promising activity against *M. abscessus in vitro*, whereas LEV, IMI, and SMX exhibited poor activity against *M. abscessus*. In addition, mutations located in 23S rRNA could be used as a promising target for the prediction of CLA resistance in *M. abscessus*.

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