International Journal of Infectious Diseases 25 (2014) 170–174

Contents lists available at ScienceDirect

International Journal of Infectious Diseases

journal homepage: www.elsevier.com/locate/ijid

Species identification of *Mycobacterium abscessus* subsp. *abscessus* and *Mycobacterium abscessus* subsp. *bolletii* using *rpoB* and *hsp65*, and susceptibility testing to eight antibiotics

Wenjuan Nie [a], Hongfei Duan [a], Hairong Huang [b], Yu Lu [c], Dapeng Bi [d], Naihui Chu [a,*]

[a] Department of Tuberculosis, Beijing Chest Hospital, Capital Medical University, Tongzhou District, Beijing, PR China
[b] Reference Laboratory, Beijing Chest Hospital, Capital Medical University, Tongzhou District, Beijing, PR China
[c] Department of Pharmacology, Beijing Chest Hospital, Capital Medical University, Tongzhou District, Beijing, PR China
[d] Department of Internal Medicine, The Second People's Hospital of Jinan, Huaiyin District, Jinan City, Shandong Province, PR China

ARTICLE INFO

Article history:
Received 19 August 2013
Received in revised form 27 January 2014
Accepted 13 February 2014

Corresponding Editor: Eskild Petersen, Aarhus, Denmark

Keywords:
Mycobacterium abscessus subsp. *abscessus*
Mycobacterium abscessus subsp. *bolletii*
Clarithromycin
Susceptibility testing
Inducible resistance

SUMMARY

Objectives: To separate *Mycobacterium abscessus* subsp. *bolletii* from *Mycobacterium abscessus* subsp. *abscessus* using species identification, and to investigate the in vitro activity of amikacin, cefoxitin, imipenem, levofloxacin, moxifloxacin, clarithromycin, azithromycin, and linezolid against *Mycobacterium abscessus*.

Methods: Seventy *M. abscessus* isolates, previously identified by 16S rRNA sequencing, were further identified by comparative sequence analysis of *rpoB* and *hsp65*. Drug susceptibility testing was conducted using the microplate Alamar Blue assay in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines and interpreted using CLSI breakpoints.

Results: Of the 70 strains, 45 (64%) were *M. abscessus* subsp. *abscessus* and 25 (36%) were *M. abscessus* subsp. *bolletii*. The majority of *M. abscessus* isolates were susceptible to azithromycin, amikacin, linezolid, and imipenem (*M. abscessus* subsp. *abscessus*: 93%, 98%, 93%, and 73%, respectively; *M. abscessus* subsp. *bolletii*: 96%, 96%, 80%, and 68%, respectively). Approximately half of the *M. abscessus* isolates were moderately susceptible to cefoxitin and moxifloxacin (*M. abscessus* subsp. *abscessus* 53% and 49%; *M. abscessus* subsp. *bolletii* 72% and 68%). Nearly all the *M. abscessus* isolates were resistant to levofloxacin (*M. abscessus* subsp. *abscessus* 96%, *M. abscessus* subsp. *bolletii* 100%). Inducible clarithromycin resistance was found in *M. abscessus*. After 14 days of incubation, 83% *M. abscessus* subsp. *abscessus* and 36% *M. abscessus* subsp. *bolletii* were resistant to clarithromycin.

Conclusions: Using *rpoB* and *hsp65*, *M. abscessus* subsp. *bolletii* could be distinguished from *M. abscessus* subsp. *abscessus*. Amikacin and azithromycin showed excellent activity against *M. abscessus* in vitro. Imipenem, linezolid, cefoxitin, and moxifloxacin also showed good activity. Levofloxacin was inactive against *M. abscessus*. Although clarithromycin showed excellent activity against *M. abscessus* on day 3, inducible resistance occurred, and after 14 days clarithromycin showed little activity against *M. abscessus* subsp. *abscessus*, but still had good activity against *M. abscessus* subsp. *bolletii*.

1. Introduction

Non-tuberculous mycobacterial (NTM) disease in humans has been recognized as an emerging public health problem.[1,2] Drug therapy of NTM disease is long, costly, and often associated with drug-related toxicities, so the treatment of NTM disease, in particular pulmonary NTM disease, can be disappointing; clinical improvement and prolonged culture conversion is not achievable for all patients.[3] There are two kinds of NTM: rapidly growing mycobacteria and slowly growing mycobacteria. *Mycobacterium abscessus* is the most common etiological agent of lung disease caused by rapidly growing mycobacteria.[4–7] *M. abscessus* is resistant to many antibiotics in vitro,[8,9] so the cure rate of pulmonary disease is low, ranging from 30% to 50% in *M. abscessus* disease.[3]

In recent years, many novel NTM species have been discovered through the increased application of genetic investigation tools, and detailed genetic characterizations have helped define new taxonomic groupings.[10,11] Two new *M. abscessus*-related species,

* Corresponding author. Tel.: +86 13611326573; fax: +86 10 8950 9301.
E-mail address: dongchu1994@sina.com (N. Chu).

http://dx.doi.org/10.1016/j.ijid.2014.02.014

Mycobacterium massiliense and *Mycobacterium bolletii*, were identified, which had previously been grouped with *M. abscessus*.[12,13] Recently, it was proposed that *M. massiliense* and *M. bolletii* should be *Mycobacterium abscessus* subsp. *bolletii* comb. nov., and *M. abscessus* should be *Mycobacterium abscessus* subsp. *abscessus* subsp. nov. The species description of *M. abscessus* is also emended to cover both *M. abscessus* subsp. *bolletii* and *M. abscessus* subsp. *abscessus*.

It is reported that *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *bolletii* infections may have different drug sensitivity test (DST) results and clinical symptoms, and that their responses to antibiotics vary from each other.[14,15] Furthermore, a longer incubation can lead to clarithromycin resistance, which has been suggested as an explanation for the lack of efficacy of clarithromycin-based treatments. However, this inducible resistance has not been found in *M. abscessus* subsp. *bolletii*.[14] In China, isolate species are usually identified using 16S rRNA sequencing; however this method cannot distinguish *M. abscessus* subsp. *abscessus* from *M. abscessus* subsp. *bolletii*.

In this study, 70 clinical *M. abscessus* isolates (previously identified by 16S rRNA) were divided into *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *bolletii* using *rpoB* and *hsp65* sequence analysis. The sensitivities to eight drugs, including amikacin, cefoxitin, imipenem, levofloxacin, moxifloxacin, clarithromycin, azithromycin, and linezolid, were tested, and the development of inducible resistance was investigated.

2. Materials and methods

2.1. Isolates, drugs, and other materials

All of the 70 *M. abscessus* clinical strains were isolated from sputum samples of patients and had previously been identified as *M. abscessus* to the species level using 16S rRNA gene sequencing at the Reference Laboratory of Beijing Chest Hospital. The type strains of ATCC19977 and CIP108297 were used for comparison. All the strains were subcultured on Lowenstein–Jensen medium at 37 °C for approximately 4–6 days to observe colony morphology and were then used for species identification based on *rpoB* and *hsp65* sequence analysis. All the drugs, including amikacin, cefoxitin, imipenem, levofloxacin, moxifloxacin, clarithromycin, azithromycin, and linezolid, were purchased from Sigma–Aldrich Chemical Co. Alamar blue was purchased from Serotec. OADC was purchased from Becton Dickinson.

2.2. Genotypic identification

DNA was extracted from cultured colonies using the boiling method,[16] and used as templates for PCR. The following primer pairs were used: 5-GAC GAC ATC GAC CAC TTC GG-3 and 5-GGG GTC TCG ATC GGG CAC AT-3 for *rpoB* PCR,[17] and 5-ACC AAC GAT GGT GTG TCC AT-3 and 5-CTT GTC GAA CCG CAT ACC CT-3 for *hsp65* PCR.[18] Preparation of the PCR reaction mixture and amplification were done as described previously.[17–20] PCR products were purified and sequenced by the BGI Corporation using forward and reverse primers. Both strands were sequenced as a cross-check. Species identification of these strains was accomplished by the sequencing of *rpoB* and *hsp65*, using a BLAST search to measure the similarities.

2.3. Susceptibility testing

Susceptibility testing to the study drugs was carried out in accordance with the Clinical and Laboratory Standards Institute (CLSI) recommendations. The final drug concentrations tested were as follows: amikacin 0.5 to 256 μg/ml, cefoxitin 0.5 to 256 μg/ml, imipenem 0.25 to 128 μg/ml, levofloxacin 0.125 to 64 μg/ml, moxifloxacin 0.0625 to 32 μg/ml, clarithromycin 0.0625 to 32 μg/ml, azithromycin 0.5 to 256 μg/ml, and linezolid 0.25 to 128 μg/ml. Minimum inhibitory concentrations (MICs) of each drug were determined by broth microdilution method, as recommended by the CLSI, using 96-well plates[21]. For amikacin, cefoxitin, imipenem, levofloxacin, moxifloxacin, azithromycin, and linezolid, the MICs were determined after 3 days of incubation. For clarithromycin, however, plates were submitted to an extended incubation, with successive readings after 3, 5, 7, 9, and 14 days of incubation at 30 °C. After incubation, Alamar blue dye was added to each well and the plates were reincubated for 24 h. A color change from blue to pink indicates bacterial growth. The MIC was defined as the lowest concentration of the drug that showed no color change, which was the lowest concentration of drug capable of inhibiting the visible growth of tested isolates. MIC_{50} and MIC_{90} values were defined as drug concentrations that inhibited 50% and 90% of isolates, respectively. Susceptibility was evaluated according to CLSI breakpoint recommendations (Table 1). Finally, the proportions resistant were analyzed by Chi-square test or Fisher's exact test. A p-value of <0.05 indicates statistical significance.

Table 1
Breakpoints of each drug, according to the CLSI recommendations.

Antibiotic	MIC (μg/ml)		
	Susceptible breakpoint	Moderately susceptible breakpoint	Resistant breakpoint
Amikacin	≤16	32	≥64
Cefoxitin	≤16	32–64	≥128
Imipenem	≤4	8	≥16
Levofloxacin	≤1	2	≥4
Moxifloxacin	≤1	2	≥4
Clarithromycin	≤2	4	≥8
Azithromycin	≤16	32	≥64
Linezolid	≤8	16	≥32

CLSI, Clinical and Laboratory Standards Institute; MIC, minimum inhibitory concentration.

3. Results

The morphology of colonies was divided into two types, rough and smooth. The proportions of rough and smooth for *M. abscessus* subsp. *abscessus* were 47% and 53%, respectively, and for *M. abscessus* subsp. *bolletii* were 68% and 32%, respectively (Table 2). The proportions of rough and smooth were similar for *M. abscessus* subsp. *abscessus*. However, for *M. abscessus* subsp. *bolletii*, the proportion of smooth colonies was less than that of rough colonies. There were no mixed colonies observed, although it is reported that the morphology of colonies can be distinguished into three types: rough, smooth, and mixed.[19]

Species identification of these strains was accomplished by BLAST search to measure the similarities. All of the 70 *M. abscessus* isolates were divided into two subspecies: 45 (64%) were *M. abscessus* subsp. *abscessus* isolates and 25 (36%) were *M. abscessus* subsp. *bolletii* isolates.

Two methods were used in this test to identify subspecies: *rpoB* and *hsp65* gene sequencing. The results of these two methods were

Table 2
Morphology of colonies; No. (%) of strains.

Subspecies	Rough	Smooth	Total
M. abscessus subsp. *abscessus*	21 (47%)	24 (53%)	45 (100%)
M. abscessus subsp. *bolletii*	17 (68%)	8 (32%)	25 (100%)
Total	38 (54%)	32 (46%)	70 (100%)