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The drug susceptibility profile and inducible resistance to macrolides of *Mycobacterium abscessus* and *Mycobacterium massiliense* in Korea

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ABSTRACT

We conducted drug susceptibility testing (DST) against various antimicrobial agents, including new candidate drugs, and investigated the relationship between inducible resistance (IR) to macrolides and *erm*(41) gene in *Mycobacterium abscessus* complex. Sixty-two isolates of *M. abscessus* complex from 2 tertiary care hospitals in South Korea were tested against 10 antimicrobial agents. Thirty-five isolates were *M. abscessus*, and 27 were *Mycobacterium massiliense*. Amikacin, moxifloxacin, linezolid, clofazimine, and tigecycline were active against most isolates and cefoxitin and ciprofloxacin against moderate number of isolates. *M. massiliense* remained susceptible to macrolides; in contrast, *M. abscessus* became highly resistant on day 14 after incubation. DST pattern did not differ between clarithromycin and azithromycin. IR to clarithromycin was correlated with *erm*(41) genotype in *M. abscessus*. Variations in susceptibility to antimicrobial agents within species and the difference in DST patterns between *M. abscessus* and *M. massiliense* suggest that DST and identification of *M. abscessus* complex are significant before treatment.

1. Introduction

Rapidly growing mycobacteria (RGM) can cause various diseases in humans, including respiratory tract infections, abdominal infections, soft tissue infections, and localized skin infections (Griffith et al., 2007). *Mycobacterium abscessus* complex is the common mycobacteria in mycobacterial lung disease and extrapulmonary mycobacterial disease caused by RGM (Daley and Griffith, 2002; Griffith et al., 1993, 2007). *M. abscessus* complex is subclassified into 3 closely related subspecies of *M. abscessus*, *Mycobacterium massiliense*, and *Mycobacterium bolletii* (Howard, 2013). Identifying these related subspecies is important because the treatment response and susceptibility to drugs, particularly to macrolides, differ among subspecies.

Infections caused by *M. abscessus* complex are often difficult to treat, and use of multiple antimicrobial agents for an extended duration is usually required (Ballarino et al., 2009; Jeon et al., 2009) because these mycobacteria are intrinsically resistant to most currently available antimicrobial agents, including antituberculosis drugs (Brown-Elliott and Wallace, 2002). A poor correlation is observed between in vitro drug susceptibility testing (DST) results and in vivo treatment responses (Griffith et al., 2007); however, some studies have reported that in vitro DST results to several antimicrobial agents are correlated with the outcomes of treatment of RGM infections (Swenson et al., 1985; Wallace et al., 1985).

In addition, in vitro DST for challenged drugs such as linezolid (Wallace et al., 2001; Yoshida et al., 2013) and clofazimine (Shen et al., 2010), which are atypical agents, could suggest new treatment regimen. Therefore, DST remains valuable for searching for an appropriate treatment in *M. abscessus* complex infections.

Many pathogenic RGM species are susceptible to the newer macrolides (clarithromycin or azithromycin), which are considered important for treating RGM infections (Brown-Elliott and Wallace, 2002; Brown-Elliott et al., 2002; Griffith and Wallace, 1996; Griffith et al., 2007). However, *M. abscessus* complex lung diseases are often intractable to a macrolide-based regimen, although pretreatment isolates are usually susceptible to clarithromycin when previous Clinical and Laboratory Standards Institute (CLSI) guidelines are used (Griffith et al., 2007). Studies suggest that *M. abscessus* complex may have inducible resistance (IR) to macrolides, which would explain the decreased efficacy of a macrolide-based regimen against *M. abscessus* complex infections (Bastian et al., 2011; Koh et al., 2011). Nash et al. (2009) and Bastian et al. (2011) reported that IR to macrolides in *M. abscessus* complex is correlated with the presence of the novel *erm*(41) gene. Additionally, there are reports that the efficacies of macrolides differ. The reports suggested that MICs or inducing *erm*(41) could be different according to the type of macrolides (Bastian et al., 2011; Choi et al., 2012; Maurer et al., 2014).

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M. abscessus complex is the second most common pathogen of nontuberculous mycobacteria (NTM) lung disease in Korea, following the *Mycobacterium avium-intracellulare* complex (Koh et al., 2006; Ryoo et al., 2008). However, information about in vitro DST against various antimicrobial agents and IR to macrolides in *M. abscessus* complex is limited. The purpose of this study was to gather results of in vitro DST against various antimicrobial agents, including new candidate treatment drugs such as linezolid, tigecycline, and clofazimine and to examine the relationship between IR to the macrolides, clarithromycin and azithromycin, and the *erm*(41) gene within each subspecies of *M. abscessus* complex.

2. Materials and methods

2.1. Setting and bacterial strains

Eighty-nine clinical *M. abscessus* complex isolates were obtained from 71 in 2 tertiary care hospitals (Severance Hospital and Seoul National University Hospital) in Seoul, Republic of Korea, between January 2011 and August 2011. Duplicate isolates and isolates that had no final DST results because of contamination were excluded. As a result, 62 isolates were used for analysis. All clinical strains were isolated from respiratory specimens before initiation of antibiotic treatment. All patients from whom the clinical isolates were obtained met the diagnostic criteria recommended by American Thoracic Society guidelines for NTM lung disease (Griffith et al., 2007). Classification of *M. abscessus* complex was conducted by multilocus sequence analysis (MLSA), as reported previously (Kim et al., 2013).

2.2. Drug susceptibility testing

We tested 10 antimicrobial agents against *M. abscessus* complex isolates, including amikacin, cefoxitin, ciprofloxacin, doxycycline, moxifloxacin, linezolid, clofazimine, tigecycline, clarithromycin, and azithromycin. The MICs of all tested agents were determined by the broth microdilution method (CLSI, 2011). The MICs was read on days 3. The inocula were prepared from actively growing bacteria in 10 mL of cation-adjusted Mueller–Hinton broth. The strains were then adjusted with cation-adjusted Mueller–Hinton broth to a bacterial cell density of 10^6 CFU/mL and diluted to final inocula of approximately 5×10^4 CFU/well. The MIC breakpoints were interpreted according to CLSI recommendations and the modified values for tigecycline by Petrini (2006) and for clofazimine by Shen et al. (2010) (Table 1). Clarithromycin and azithromycin susceptibility tests were conducted using the broth microdilution method following preincubation with clarithromycin or azithromycin to test for IR. The MICs were determined on days 3 and 14 after incubation (Nash et al., 2009).

Table 1
Antimicrobial agents and MIC breakpoints.

	MIC (μg/mL)		
	S	I	R
Amikacin	≤16	32	≥64
Cefoxitin	≤16	32–64	≥128
Ciprofloxacin	≤1	2	≥4
Clarithromycin	≤2	4	≥8
Doxycycline	≤1	2–4	≥8
Moxifloxacin	≤1	2	≥4
Linezolid	≤8	16	≥32
Clofazimine	≤1	2	≥4
Tigecycline	≤4		>4

S = susceptible; I = intermediate; R = resistant.

2.3. DNA extraction

Cryopreservation beads were used for storage of bacterial strains at −70 °C, and Middlebrook 7H10 media (BD, Franklin Lakes, NJ, USA) was used for culturing. Bacterial strains were grown at 37 °C for 4 days before use. Genomic DNA was extracted using lysozyme, Tris-EDTA, and proteinase K, as described previously (Heym et al., 2009; Macheras et al., 2011).

2.4. erm(41) polymerase chain reaction (PCR) sequencing

erm(41) was amplified using primer sets: erm41-F (5′-ACC GTG CAG ATG GAG AAG TC-3′) and erm41-R (5′-CAG TCT GTC ACA GGG TCA GC-3′) (amplicon size, 822 bp; temperature, 59 °C). Amplification experiments were performed using 1 μL of each primer (10 pmol) and 25 μL of ReddyMix PCR master mix (Thermo Fisher Scientific, Epsom, UK). Deoxynucleotide sequencing of the amplified gene fragments was carried out on both strands using the Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) with the same primers as used for amplification. Sequencing products were purified, and an ABI 3700 DNA analyzer (International Equipment Trading, Vernon Hills, IL, USA) was used for analysis. The SeqMan II software system (DNASTAR, Madison, WI, USA) was used for trimming.

2.5. Ethics

This research protocol was approved by the Institutional Review Board (IRB) of Severance Hospital (IRB No. 4-2012-0174).

3. Results

3.1. Genetic distribution of isolates and patient characteristics

The MLSA analysis showed that 35 clinical isolates (56.5%) were classified as *M. abscessus* and 27 (43.5%) as *M. massiliense*. The patients' characteristics and the genetic distribution of the isolates are presented in Table 2. No significant differences were found between patients with *M. abscessus* and *M. massiliense* lung disease. Median age was 67 years (range, 20–83 years) in patients with *M. abscessus* and 60 years (range, 42–78 years) in those with *M. massiliense*. More than half patients (54.8%) showed the previous history of tuberculosis (TB) treatment (51.4% in *M. abscessus* infection and 59.3% in *M. massiliense* infection). There was no one who had positive result of HIV test. Radiological studies revealed that the nodular bronchiectatic form was the dominant pattern in patients with *M. abscessus* (n = 22, 62.9%) and *M. massiliense* (n = 21, 77.8%) infections, followed by the upper lobe cavitary form in those with *M. abscessus* (n = 7, 20.0%) and *M. massiliense* (n = 3, 11.1%) infections.

3.2. DST

DST was performed on *M. abscessus* from 35 patients and on *M. massiliense* from 27 patients (Table 3). A difference in the DST pattern between *M. abscessus* and *M. massiliense* was observed for cefoxitin. The proportion of susceptible isolates was higher in *M. abscessus* than in *M. massiliense* (60.6% versus 25.0%; $P = 0.015$). Amikacin (91.2% in *M. abscessus* versus 100% in *M. massiliense*), moxifloxacin (72.7% versus 66.7%), linezolid (97.0% versus 87.5%), clofazimine (87.9% versus 95.8%), and tigecycline (100% versus 100%) were active against most *M. abscessus* and *M. massiliense* isolates. Cefoxitin (60.6% versus 25.0%) and ciprofloxacin (14.3% versus 7.7%) had activity against a moderate number of *M. abscessus* and *M. massiliense* isolates. *M. abscessus* became highly resistant to clarithromycin on day 14 after incubation (proportion of resistance to clarithromycin on day 3, 2.9% versus on day 14, 88.6%). In contrast, susceptibility to clarithromycin in *M. massiliense* was relatively unchanged on day 14 after incubation (proportion of susceptibility to clarithromycin on day 3, 92.3% versus day 14, 92.3%). IR to clarithromycin was significantly different between *M. abscessus* and *M. massiliense* ($P < 0.001$). In comparison of 2 macrolides, clarithromycin and azithromycin, no significant difference in the DST pattern was observed. Fig. 1 shows distribution of MIC of *M. abscessus* (Fig. 1a and b) and *M. massiliense* to clarithromycin and azithromycin (Fig. 1c). *M. abscessus* showed clearly increased MIC level between day 3 and day 14 to both clarithromycin (Fig. 1a) and azithromycin (Fig. 1b). However, in *M. massiliense*, MIC level showed no change between day 3 and day 14 to both macrolides (Fig. 1c).

3.3. Susceptibility testing of clarithromycin and erm(41) genotyping in M. abscessus isolates

The *M. abscessus* isolates were classified into 2 groups according to the nucleotide at position 28 of the *erm*(41) gene, which is the 10th codon in the amino acid sequence. The first group was a thymine 28 (T28 *M. abscessus* sequevar), which corresponded to a