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The Drug Resistance Profile of *Mycobacterium abscessus* Group Strains from Korea

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Background: Bacteria of the *Mycobacterium abscessus* group are the second most common pathogens responsible for lung disease caused by nontuberculous mycobacteria in Korea. There is still a lack of studies investigating the genetic mechanisms involved in *M. abscessus* resistance to antibiotics other than clarithromycin. This study investigated the characteristics of drug resistance exhibited by *M. abscessus* clinical isolates from Korea.

Methods: We performed drug susceptibility testing for a total of 404 *M. abscessus* clinical strains. Subspecies were differentiated by molecular biological methods and examined for mutations in drug resistance-related genes.

Results: Of the 404 strains examined, 202 (50.00%), 199 (49.26%), and 3 (0.74%) strains were identified as *M. abscessus, M. massiliense*, and *M. bolletii*, respectively. Of the 152 clarithromycin-resistant strains, 6 possessed *rrl* mutations, while 4 of the 30 amikacin-resistant strains contained *rrs* mutations, and 5 of the 114 quinolone-resistant strains had *gyr* mutations. All mutant strains had high minimal inhibitory concentration values for the antibiotics.

Conclusions: Our results showed the distribution of the strains with mutations in drug resistance-related genes was low in the *M. abscessus* group. Furthermore, we performed drug susceptibility testing and sequence analyses to determine the characteristics of these genes in the *M. abscessus* group.

Key Words: *Mycobacterium abscessus* group, Drug resistance, Mutation

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INTRODUCTION

Mycobacterium abscessus-induced pulmonary disease accounts for approximately 65%-80% of the pulmonary diseases caused by rapidly growing mycobacteria [1-3]. On account of the resistance of *M. abscessus* against various antibiotics, pulmonary diseases are very difficult to treat [4-6]. A combination treatment (determined by *M. abscessus in vitro* drug susceptibility testing) using specific antibiotics, such as amikacin, cefoxitin, imipenem, and macrolides, has been recommended by the American Thoracic Society and Infectious Disease Society of America [7]. However, the appropriate treatment duration has not yet been clearly established, and the cure rate is currently low. In Korea, *M. abscessus* pulmonary disease is the second most common pulmonary disease induced by nontuberculous mycobacteria [8, 9].

Recently, it was found that the *M. abscessus* group consists of *M. abscessus* (group I), *M. massiliense*, and *M. bolletii* (group II) strains [10]. A study also confirmed inducible resistance to clarithromycin in clinical strains of *M. abscessus*, in which the susceptibility to clarithromycin changed to resistance during *in vitro* drug susceptibility testing as the culture period progressed [11].

Furthermore, researchers have observed that the erythromycin ribosome methyltransferase (*erm*) gene is involved in the

generation of inducible resistance to clarithromycin and that gene sequence variations between *M. abscessus* and *M. massiliense* strains are useful for bacterial identification in such cases [11, 12]. Additionally, inducible resistance has not been observed in *M. massiliense*, and the treatment outcome of *M. massiliense* infections with clarithromycin is better than that of *M. abscessus* [13]. On the basis of the inducible resistance of *M. abscessus*, the CLSI recently released its recommendations for analyzing the susceptibility test results of clarithromycin after a maximum incubation period of 14 days [14].

In contrast to earlier studies that have been performed with a limited number of strains, we investigated the distribution of *M. massiliense* and *M. abscessus* among the *M. abscessus* group clinical strains that the Korean Institute of Tuberculosis had received for nontuberculous mycobacteria identification and drug susceptibility testing and performed differential identification of the strains.

Because the mechanism of inducible resistance to clarithromycin in *M. abscessus* plays a role in the clarithromycin-based clinical outcomes, we evaluated the distribution and drug resistance characteristics of the strains that had acquired inducible resistance.

There is still an obvious lack of studies investigating the *M. abscessus* group genes involved in resistance to antibiotics other than clarithromycin. Therefore, this study aimed to contribute to the diagnosis and treatment of *M. abscessus* group infections by analyzing drug resistance against other antibiotics.

Table 1. The primers used in this study

Genes	Primer sequence
erm(41)	ermF: 5′ GAG CGC CGT CAC AAG ATG CAC A 3′
	ermR: 5′ GAC TTC CCC GCA CCG ATT CCA C 3′
rrl	23SF: 5′ AAT GGC GTA ACG ACT TCT CAA CTG T 3′
	23SR: 5′ GCA CTA GAG GTT CGT CCG TCC C 3′
rrs	rrsF: 5′ CAG TAC AGA GGG CTG CGA ACG 3′
	rrsR: 5′ AAG GAG GTG ATC CAG CCG CA 3′
gyrA	gyrAF: 5′ GGG CAT CTA AAG CCG CTG AGA 3′
	gyrAR: 5′ GAC GAT GGC GCG CTG ACG T 3′
gyrB	gyrBF: 5′ GCA GAT GCT AAA ACG GTT GTG A 3′
	gyrBR: 5′ CTC GTA AGT ACG ACG GCA CAA 3′

METHODS

1. Strain selection and culture

This study was conducted using 413 *M. abscessus* group clinical strains that were submitted for nontuberculous mycobacteria susceptibility testing from July 2009 to December 2010 at the Korean Institute of Tuberculosis. The selected strains were cultured in Lowenstein-Jensen medium. Of the 413 strains, 3 mixed strains and 6 contaminated strains were excluded from the study, and a total of 404 *M. abscessus* group clinical strains were analyzed.

2. Differentiation and distribution of *M. abscessus* and *M. massiliense* on the basis of the *erm(41)* gene

M. abscessus and *M. massiliense* were differentiated by comparing the PCR product size of the *erm(41)* gene, and the distribution of these strains in *M. abscessus* group was examined. Tables 1 and 2 show the primer sets and PCR conditions [11].

3. Clarithromycin susceptibility testing and differentiation of *M. abscessus* clinical strains with inducible resistance to clarithromycin

Drug susceptibility was tested in 404 *M. abscessus* group clinical strains (202 *M. abscessus* strains, 199 *M. massiliense* strains, and 3 *M. bolletii* strains) using the micro-dilution method [14].

Approximately 100 μL/well of cation-adjusted Muller Hinton II (Becton Dickinson, San Jose, CA, USA) broth containing 50 mg/L 2,3-diphenyl-5-(2-thienyl)-tetrazolium chloride (STC: Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan) and 0.25 mg/L of clarithromycin, obtained by serial 0.5-fold dilutions from an initial concentration of 512 mg/L, was loaded onto microplate wells. The strains were then inoculated at a density of 10^4-10^5 cell/

Table 2. PCR conditions used in this study

Genes	Denaturation	Amplification				Extension
		Denaturation	Annealing	Extension	Cycles	
erm(41)	95°C, 2 min	95°C, 30 sec	60°C, 30 sec	72°C, 30 sec	35	72°C, 5 min
rrl	94°C, 5 min	94°C, 1.5 min	60°C, 2 min	72°C, 2 min	35	72°C, 10 min
rrs	95°C, 2 min	95°C, 30 sec	60°C, 30 sec	72°C, 30 sec	35	72°C, 5 min
gyrA	94°C, 5 min	94°C, 1 min	63°C, 1.5 min	72°C, 2 min	35	72°C, 10 min
gyrB	94°C, 5 min	94°C, 1 min	61°C, 1 min	72°C, 2 min	35	72°C, 10 min

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well, incubated at 30°C, and the minimal inhibitory concentration (MIC) was examined.

The strains were cultured for 3, 7, or 14 days and then the strains with MIC≤2 mg/L, MIC=4 mg/L, and MIC≥8 mg/L were considered susceptible, intermediate, and resistant, respectively. The strains showing clarithromycin susceptibility on day 3 and resistance after 7 days were determined to have inducible resistance.

4. Sequence analysis of clarithromycin resistance-related genes

Acquired resistance to clarithromycin is associated with point mutations (at positions A2,058 and A2,059) in a region of the *rrl* gene encoding the peptidyltransferase domain of the 23S rRNA [15]. It has also been reported that the *erm* gene is associated with inducible resistance to clarithromycin. Therefore, characteristics of the susceptible and resistant clinical strains were compared and analyzed by sequencing the *rrl* and *erm(41)* genes. The primer sets and PCR conditions for amplification of two genes are outlined in Tables 1 and 2 [11, 16].

5. Distribution of amikacin-resistant strains and sequence analysis of the resistance-related genes

Drug susceptibility for amikacin was tested to investigate the distribution of the resistant strains. The strains were cultured for 3 days and strains with MIC≤16 mg/L, MIC=32 mg/L, and MIC≥64 mg/L were considered susceptible, intermediate, and resistant, respectively. The resistant strains were subjected to PCR and sequence analysis to confirm the base substitution (A→G) at position 1,408 (*E. coli* numbering) of the 16S ribosomal RNA (rRNA) gene *rrs*, which is an amikacin resistance-related gene (Tables 1 and 2) [17].

6. Distribution of quinolone derivative-resistant strains and sequence analysis of the resistance-related genes

Drug susceptibility for ciprofloxacin and moxifloxacin was tested to examine the distribution of resistant strains. The strains were cultured for 3 days, and then strains with MIC≤1 mg/L, MIC=2 mg/L, and MIC≥4 mg/L were considered susceptible, intermediate, and resistant, respectively. We selected approximately one-third of the resistant strains with the highest MICs. PCR and sequence analysis were performed to investigate the mutations in the quinolone resistant-dependent region (QRDR) of the *gyraseA* (*gyrA*) and *gyraseB* (*gyrB*) genes (Tables 1 and 2) [18].

Table 3. Strain distribution among the *M. abscessus* group clinical strains

Species	PCR amplicon size	N of strains (%)
M. abscessus	892 bp	202 (50.00)
M. massiliense	616 bp	199 (49.26)
M. bolletii	892 bp	3 (0.74)
Total		404 (100.00)

RESULTS

1. The distinction and distribution of *M. abscessus* and *M. massiliense* on the basis of *erm(41)* gene

Amplification of the *erm(41)* gene resulted in an 892-bp PCR product for *M. abscessus* and a 616-bp product for *M. massiliense*, which is the *erm(41)*-deletion mutant. It was therefore possible to differentiate the 2 species according to their PCR product sizes. Additionally, *M. bolletii* was isolated from the *M. abscessus* strain and was separated by a -35 sequence difference in *erm(41)* gene promoter. Table 3 shows the distribution of the *M. abscessus, M. massiliense*, and *M. bolletii* clinical strains.

2. Clarithromycin susceptibility testing and the differentiation of *M. abscessus* clinical strains with inducible resistance to clarithromycin

Clarithromycin susceptibility testing of 202 *M. abscessus* strains identified 31 susceptible stains, 48 resistant strains, 120 inducible resistance-bearing strains, and 3 intermediate strains. Indeed, most of the clinical strains (168 strains, 83%) were resistant to clarithromycin, which was most commonly utilized for treatment (Table 4). Among the 199 *M. massiliense* strains, 184 were susceptible and 15 were resistant, while among the 3 *M. bolletii* strains, 1 was resistant and 2 were inducible-resistant.

3. Sequence analysis of clarithromycin resistance-related genes

Of the 404 *Mycobacterium abscessus* group clinical strains, the sequencing results (for the *rrl* and *erm(41)* genes) were inconclusive for 51 strains, which were excluded from further analysis. Therefore, resistance-related gene analysis was performed on 157 *M. abscessus* strains, 194 *M. massiliense* strains, and 2 *M. bolletii* strains. Among the clarithromycin-resistant strains (140 *M. abscessus*, 12 *M. massiliense*, and 2 *M. bolletii*), 2 *M. abscessus* strains and 4 *M. massiliense* strains harbored point mutations in the peptidyltransferase domain of the 23S rRNA gene. These 6 strains showed resistance, with high MICs (>64

mg/L) (Table 5).

Thymine (T)/cytosine (C) point mutations were detected at position 28 of the *M. abscessus erm(41)* gene. Of the 157 *M. abscessus* strains, 140 strains showed thymine 28 (T28 *M. abscessus* sequevar, Trp10 codon) mutations, while 17 had cytosine 28 (C28 *M. abscessus* sequevar, Arg10 codon) mutations. All the C28 strains were susceptible, whereas the T28 strains showed either resistance or inducible resistance. Of the 140 T28 *M. abscessus* strains, 78 strains presented an amino acid substitution at codon 80 (Val→Ile), and 35 strains presented an amino acid substitution at codon 140 (Pro→Leu; Table 6).

4. Distribution of amikacin-resistant strains and sequence analysis of the resistance-related genes

The amikacin resistant strains accounted for 69% (138 *M. abscessus*, 139 *M. massiliense*) of the study population (Table 4). Furthermore, *rrs* mutations were present in 2 *M. abscessus* and 2 *M. massiliense* strains. These 4 strains showed MIC values higher than 2,048 mg/L, thereby suggesting that the mutations occurred in highly resistant strains.

5. Distribution of the strains resistant to quinolone derivatives and sequence analysis of the resistance-related genes

The strains resistant to ciprofloxacin or moxifloxacin accounted for over 70% (≥149 strains) of the study population (Table 4). In the mutation analysis of the QRDR in the *gyrA* and *gyrB* genes, 5 mutants were identified among the *M. abscessus* and *M. massiliense* resistant strains (Table 7). In terms of the *gyrA*

Table 5. Distribution of *rrl* gene mutants among the clarithromycin-resistant strains

Strain	N of DST resistant strains	N of *rrl* gene mutants
M. abscessus	140	2
M. massiliense	12	4

Abbreviation: DST, drug susceptibility test.

Table 4. Drug susceptibility for the *M. abscessus* group clinical strains

Strain (N)	Susceptibility	Clarithromycin N (%)	Amikacin N (%)	Ciprofloxacin N (%)	Moxifloxacin N (%)
M. abscessus (202)	Susceptible	31 (15.35)	138 (68.32)	4 (1.98)	14 (6.93)
	Intermediate	3 (1.49)	39 (19.30)	14 (6.93)	21 (10.39)
	Resistant	48 (23.76)	25 (12.38)	184 (91.09)	167 (82.68)
	Inducible resistant	120 (59.40)	-	-	-
M. massiliense (199)	Susceptible	184 (92.46)	139 (69.85)	9 (4.52)	17 (8.54)
	Intermediate	0 (0.00)	48 (24.12)	16 (8.04)	33 (16.58)
	Resistant	15 (7.54)	12 (6.03)	174 (87.44)	149 (74.88)
M. bolletii (3)	Susceptible	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
	Resistant	1 (33.33)	3 (100.00)	3 (100.00)	3 (100.00)
	Inducible resistant	2 (66.67)	-	-	-

Table 6. Mutation patterns and distribution of the clarithromycin-resistant *M. abscessus erm(41)* gene

Nucleotide amino acid	C28T Arg→Trp	A120G* Ala→Ala	C159T* Gly→Gly	G238A Val→Ile	G255A* Leu→Leu	G279T* Arg→Arg	C330A* Ile→Ile	T336C* Ser→Ser	C419T Pro→Leu	N (%)
Pattern 1	T	-	T	A	-	-	A	-	-	78 (55.71)
Pattern 2	T	-	-	-	A	T	-	C	T	35 (25.00)
Pattern 3	T	G	-	-	A	T	-	C	-	9 (6.43)
Others	T	-	-	-	-	-	-	-	-	18 (12.86)

*Silent mutation.

Table 7. Mutation analysis of quinolone-resistant dependent region (QRDR) in *gyrA* and *gyrB* genes

Species	Quinolone susceptibility	N of sequences (*gyrA*-QRDR)	N of mutants	N of sequences (*gyrB*-QRDR)	N of mutants
M. massiliense	Susceptible	3	0	3	0
	Resistant	61	2	61	1
M. abscessus	Susceptible	1	0	1	0
	Resistant	53	2	54	0

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gene, alanine at amino acid position 92 (*M. abscessus* numbering) was converted to valine in 1 strain, and aspartic acid at position 96 was mutated to asparagine in 3 strains. Arginine at amino acid position 492 of the *gyrB* gene was also converted to cysteine in 1 strain. Additionally, these 5 strains showed MIC values greater than 16 mg/L, thereby indicating that mutations are more likely to occur in the highly resistant strains.

DISCUSSION

Despite the continuous change in the taxonomic status of *M. chelonae* and *M. abscessus*, *M. abscessus* is considered as a separate species rather than a subspecies of *M. chelonae*. However, interspecific relationships have been identified within the *M. abscessus* group through genotype analysis, such as PCR restriction analysis (PRA) and sequencing of *hsp65* and *rpoB*. Moreover, it has been recently reported that *M. massiliense* and *M. bolletii* are very closely related to *M. abscessus* [10-12, 19, 20]. In the present study *M. abscessus* and *M. massiliense* strains were differentiated on the basis of the *erm(41)* gene, and *M. abscessus* and *M. bolletii* were differentiated utilizing the -35 sequence difference in *erm(41)* gene promoter. As a result, out of the 202 strains of *M. abscessus* (50.00%), 199 strains of *M. massiliense* (49.26%) and 3 strains of *M. bolletii* (0.74%) were isolated, which is in good agreement with the distribution rate reported by Kim et al. [10].

Several mechanisms of antibiotic resistance have been proposed, including (1) changes in the target and receptors, (2) changes in membrane permeability, and (3) the active drug efflux pump [21-24].

In mycobacteria, clinically acquired macrolide resistance is caused by a point mutation at position 2,058 or 2,059 (*E. coli* numbering) in the 23S rRNA [25]. However, such mutations were rarely observed in the treatment of *M. abscessus* or *M. chelonae* infection [15]. According to a recent report, the *erm(41)* gene is involved in the acquisition of inducible-clarithromycin resistance by *M. abscessus*, thereby resulting in susceptibility at day 3 of incubation, and resistance after a maximum incubation period of 14 days. It was also confirmed that position 28 in the *erm(41)* gene in the resistant strains was mutated from C to T [11, 12, 26]. The results of the susceptibility testing of the 202 *M. abscessus* clinical strains demonstrated that the resistant strains accounted for 23.76% of the strains at day 3 of incubation, whereas their frequency increased to 59.40% after a maximum incubation period of 14 days. In addition, it was observed that among the 199 strains of *M. massiliense*, 184 strains were susceptible (92.5%) and 15 were resistant (7.5%); whereas among the *M. bolletii*, 1 was resistant, and 2 others showed inducible resistance. These results confirmed that the majority of the clinical strains of *M. abscessus* were resistant to clarithromycin (83.16%), which is a known therapeutic agent, and most of the clinical strains of *M. massiliense* were susceptible. These results support the findings of Koh et al. [13] that the treatment regimen containing clarithromycin was more effective in patients with *M. massiliense* pulmonary disease than in those with *M. abscessus* pulmonary disease, and the inducible resistance to clarithromycin shown in *M. abscessus* clinical strains played a role in the lack of efficacy of clarithromycin containing antibiotic therapy. Based on these results, the 2011 CLSI guidelines recommended that the incubation period of the strains be extended up to 14 days in the cases where the day 3 test indicates susceptibility [14].

In the analysis of the clarithromycin resistance-related 23S rRNA gene, 2 *M. abscessus* and 4 *M. massiliense* had *rrl* mutations, and both showed resistance at high MICs (MIC≥64 mg/L). Although it was not confirmed whether these strains had acquired drug resistance, the frequency of 23S rRNA mutants was low. In terms of the *erm(41)* sequence analysis, all resistant strains showed a T at nucleotide position 28, whereas all susceptible strains had a C (Table 6). Such results are in agreement with previous studies reporting that all *erm(41)* gene T28 type strains are resistant to clarithromycin [11, 12, 26]. Although the number of strains examined in this study was higher than that in previous studies, we could not identify any C28-type 23S rRNA mutants as reported by Bastian et al. [26]. It is noteworthy that 2 amino acid changes (Ile80 and Leu140 codon) were found in the *erm(41)* gene T28-type strains, except the Trp10 codon. Most of the resistant strains (80.71%) showed 2 distinct *erm(41)* gene sequence patterns that included silent mutations. Further studies need to be investigated whether these 2 amino acid changes facilitate resistance.

To the best of our knowledge, only a small number of studies have investigated the resistance characteristics of the drugs utilized for therapy, with the exception of clarithromycin. In this study, drug susceptibility and the characteristics of the drug resistance-related genes were examined to investigate the resistance to other drugs used for treatment.

In the amikacin susceptibility test, 6.03% of *M. massiliense* strains were resistant, whereas 12.38% of the strains in the *M. abscessus* showed resistance. Of the resistant strains, 4 mutations were found in both species when investigating the base mutation (A→G) at position 1,408 (*E. coli* numbering) of the 16S

ribosomal RNA gene associated with amikacin resistance. In addition, the amikacin MIC values of all the mutant strains were remarkably high (>2,048 mg/L). Most of the strains showed susceptibility, and the distribution of the mutant strains was low. In previous reports, Prammananan et al. [17] identified that 16 out of the 17 examined *M. abscessus* strains were mutant with high MIC values because most strains were from the patients who had received aminoglycoside therapy. Another study also identified a new mutation that was not at the 1,408 position [27].

Fluoroquinolone antibiotics have been applied as effective therapeutic agents for infections induced by rapidly growing mycobacteria. Resistance to these antibiotics is mainly mediated by *gyrA* and *gyrB* gene mutations. Monego et al. [28] found that 31 out of 35 ciprofloxacin-resistant *M. massiliense* isolates showed mutations at amino acid position 90 (*M. tuberculosis* numbering, 92 *M. abscessus* numbering) but no mutation at position 94 (96 *M. abscessus* numbering) of *gyrA*. They stated that amino acid 90 of *gyrA* gene plays an important role in antibiotic resistance to fluoroquinolone. In this study, both *M. massiliense* and *M. abscessus* strains showed over 74% resistance to ciprofloxacin and moxifloxacin. Unlike previous studies, when investigating *gyrA* and *gyrB* mutations in one-third of the resistant strains, mutations at position 92 (alanine→valine, *M. abscessus* numbering), 96 of *gyrA* (aspartic acid→asparagine), and 492 of *gyrB* (arginine→cysteine) were observed in 1 strain (1 *M. abscessus*), 3 strains (1 *M. abscessus*, 2 *M. massiliense*), and 1 strain (1 *M. massiliense*), respectively. Amino acids at positions 90 and 94 in the A subunit (*M. tuberculosis* numbering system), and at positions 495, 516, and 533 in the B subunit (*M. tuberculosis* numbering) are frequently substituted in strains with acquired resistance to quinolones [18]. The mutation rate in this study was lower than that reported by Monego et al. [28], even though all the samples used in that study were collected for microbial culture before initial antibiotic treatment of patients and none of the patients has received quinolones for at least 4 weeks before the surgical procedures. They presumed that the incidence of ciprofloxacin-resistant *M. massiliense* may be due to selective pressure caused by drug abuse before the occurrence of the present cases. Further studies are required to fully establish the *M. abscessus* group susceptibility to fluoroquinolone antibiotics. Taken together, the results suggested that the mutations in the sequence encoding amino acid 96 of the *gyrA* gene and amino acid 492 of the *gyrB* gene are also involved in the resistance mechanisms, along with that encoding amino acid 92 of the *gyrA* gene (*M. abscessus* numbering).

In conclusion, we confirmed the characteristics of resistance related-genes in the *M. abscessus* group through drug susceptibility testing and analyses of resistance related-genes. The findings that most *M. massiliense* strains are susceptible to clarithromycin and amikacin, and most *M. abscessus* strains are susceptible to amikacin will aid the prescription of antibiotics for patients with infectious diseases.

Authors' Disclosures of Potential Conflicts of Interest

No potential conflicts of interest relevant to this article were reported.

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