

Detection and Assessment of Clarithromycin Inducible Resistant Strains Among Korean *Mycobacterium abscessus* Clinical Strains: PCR Methods

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Background: *Mycobacterium abscessus* group belongs to a group of rapidly growing mycobacteria (RGM) and, following *Mycobacterium avium* complex, is the second most common pathogen responsible for lung disease caused by nontuberculous mycobacteria (NTM). Clarithromycin is known to be the key drug in the treatment of *M. abscessus* group disease, but a high failure rate of treatment response is reported due to clarithromycin inducible resistance. **Methods:** Using the results from a clarithromycin susceptibility test we examined the proportion of clarithromycin inducible resistant *M. abscessus* (sensu stricto; hereafter referred to as *M. abscessus*) clinical strains. Also, we attempted to detect the clarithromycin resistant strains, using the amplification refractory mutation system-PCR (ARMS-PCR) and real-time PCR methods for rapid de-

tection of single-nucleotide polymorphisms (SNPs) at position 28 (T or C) of the *erm(41)* gene of *M. abscessus* leading to resistance to clarithromycin. **Results:** Of the 157 *M. abscessus* clinical strains, clarithromycin susceptible, resistant, and inducible resistant strains accounted for 10.83% ($n = 17$), 22.29% ($n = 35$), and 66.88% ($n = 105$), respectively. Clarithromycin resistant strains were able to separate from clarithromycin susceptible strains by ARMS-PCR and real-time PCR identical to DNA sequence analysis. **Conclusion:** Most *M. abscessus* clinical strains in Korea are resistant to clarithromycin, and ARMS-PCR and real-time PCR are useful tools for the rapid detection of single-nucleotide polymorphisms (SNPs) at position 28 of the *erm(41)* gene. J. Clin. Lab. Anal. 28:409–414, 2014. ©

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Key words: amplification refractory mutation system-PCR; real-time PCR; *erm(41)*; clarithromycin

INTRODUCTION

Mycobacterium abscessus group accounts for approximately 65–85% of lung disease caused by rapidly growing mycobacteria (RGM; 1–3). It is the second most common pathogen, following *Mycobacterium avium* complex, responsible for lung disease caused by nontuberculous mycobacteria (NTM) in South Korea (4, 5). Infections due to *M. abscessus* are difficult to treat because this mycobacterium is intrinsically resistant, not only to the classical antituberculous drugs, but also to most of the antibiotics that are currently available (6–9). Combination therapy of intravenous amikacin with cefoxitin or imipenem and oral macrolide (clarithromycin or azithromycin) have been rec-

ommended by the American Thoracic Society/Infectious Disease Society of America (ATS/IDSA; 10).

Clarithromycin is known to be the key drug in the treatment of *M. abscessus* group disease, but its application

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is undermined by clarithromycin inducible resistance of *M. abscessus*, associated with single-nucleotide polymorphisms (SNPs) in the *erm(41)* gene at position 28 (T or C) recently (11, 12). For these reasons, the Clinical and Laboratory Standards Institute (CLSI) recommendations were based on the presence of the *erm(41)* gene and inducible resistance whatever the polymorphism at nucleotide 28 is (13).

In the present study, we examined the proportion of clarithromycin inducible resistant *M. abscessus* clinical strains, by using the results from clarithromycin susceptibility test and the correlation between the mutation of the *erm(41)* at position 28 and the minimum inhibitory concentrations (MICs) of clarithromycin for *M. abscessus* strains. In addition, we applied to the rapid detection methods for clarithromycin resistance using amplification refractory mutation system and polymerase chain reaction (ARMS-PCR) and real-time PCR methods for detecting *erm(41)* gene mutation.

MATERIALS AND METHODS

Strains

A total of 157 *M. abscessus* clinical strains, submitted to the drug susceptibility test from July 2009 to December 2010, were obtained for this study. All clinical strains were identified using a polymerase chain reaction and restriction length polymorphism (PCR-RFLP) method based on the *rpoB* gene (4). Also, they were differentiated from *Mycobacterium massiliense* and *Mycobacterium bolletii* based on the PCR product size and -35 promoter sequence for *erm(41)* gene in the *M. abscessus* group (11).

Clarithromycin Susceptibility Test

A broth microdilution method was used to determine the MIC of clarithromycin for *M. abscessus* strains at pH 7.4, and were interpreted according to the CLSI document M24-A2 in 2011 (13). Strains were considered resistant if the MIC of clarithromycin was ≥ 8 mg/l for 3 days, and susceptible if the MIC of clarithromycin was ≤ 2 mg/l for 14 days. Also, strains were considered inducible resistant if the MIC of clarithromycin was ≤ 2 mg/l for 3 days and ≥ 8 mg/l for 14 days.

Sequence Analysis of the *erm(41)* Gene at Position 28

The mycobacterial DNA preparations for 157 *M. abscessus* strains were obtained by heating method (100°C, 15 min). PCR was performed to amplify the *erm(41)* gene according to the method described by Nash

et al. (14). The PCR products were sequenced using the BigDye Terminator v3.1 Cycle Sequencing kit and sequencing was performed on a 3730XL DNA analyzer (Applied Biosystems, Foster city, CA). Using BLAST, the *erm(41)* gene sequences from clinical strains were analyzed for homology with the *erm(41)* gene sequence from *M. abscessus* strain MAB30 (GenBank accession number EU590129), which is susceptible to clarithromycin (MIC ≤ 0.5 mg/l).

ARMS-PCR

The mycobacterial DNA preparations for 157 *M. abscessus* strains were obtained by heating method (100°C, 15 min). Primers were designed using Primer3 software based on the *erm(41)* sequence in *M. abscessus* MAB30 (GenBank accession number EU590129; Table 1). For the detection of clarithromycin-resistant strains, *ermf1* and *ermR* were used for amplification of 522 bp product. Also, *ermf3* and *ermR* were used for amplification of 300 bp for the *erm(41)* gene control. The reaction mixtures were prepared by adding the following to 1 μ l of DNA solution: 0.5 (*ermf1*), 0.25 (*ermf3*), 0.0625 (*ermR*) pmol of each primer; 1 U of EX Taq polymerase (5 U/ μ l [Takara Bio, Inc., Otsu, Japan]; 2 μ l of 2.5 mM dNTP mixture; 2 μ l of 10 \times PCR buffer; and using sterile purified water were made to a total volume of 20 μ l. The reactions were performed using a GeneAmp 9700 PCR system (Applied Biosystems). PCR conditions were as follows: 1 cycle of 95°C for 2 min; 35 cycles of 95°C for 30 sec, 69°C for 30 sec, 72°C for 30 sec; and 1 cycle of 72°C for 5 min. The PCR products were electrophoresed in a 2% agarose gel. When 522 bp and 300 bp were present, the strain was judged to be a clarithromycin-resistant (T28 type). But the strain was judged to be a clarithromycin susceptible when only 300 bp was present (C28 type).

TABLE 1. Primer Sequences and Probe Sequences Used ARMS-PCR and Real-Time PCR

	Sequence (5' \rightarrow 3')
Primers for ARMS-PCR	
<i>ermf1</i>	GGCCAATGGTCGTGCCGCCacT ^a
<i>ermf3</i>	GCGAGCCCCGCCCTACCAAGTCA
<i>ermR</i>	GACTTCCCCGCACCGATTCCAC
Primers for real-time PCR	
<i>erm-real-F</i>	GGAGCATGGGCATATTCA
<i>erm-real-R</i>	TGCGCCCAGATCCACAA
Probes	
<i>erm28T-sencor</i>	LC Red 640-TACCAGCCCCACTGGCG-Phosphate
<i>erm28T-anchor</i>	CCGCCAGTCATCAGTGAGCG-fluorescein

Small letter indicate mismatched nucleotides.

^aNucleotide at position 28.

Real-Time PCR of *erm(41)* and Melting Curve Analysis

The mycobacterial DNA preparations for 67 *M. abscessus* strains were obtained by heating method (100°C, 15 min). Real-time PCR and melting curve analysis were performed using a LighCycler® 480 instrument and Gene Scanning software version 1.5 (Roche Diagnostics, Mannheim, Germany). Primers and probes sequences were shown in Table 1. For real-time PCR, reaction volumes of 20 µl were applied containing 0.5 µM of each primer, 0.2 µM of each HybProbe probe, 4 µl Genotyping Master Mix (LightCycler® Genotyping Master version 7.0, Roche Diagnostics), 8 µl of PCR grade water, and 5 µl of target DNA template. Real-time PCR conditions were 95°C for 10 min and 45 cycles of 95°C for 10 sec, 54°C for 10 sec and 72°C for 10 sec; after amplification a melting curve was obtained by one cycle of 95°C for 1 min, 40 for 2 min, and 95°C for 0 sec (acquisition mode continuous, 0.1°C/s), followed by a cooling step (40°C for 30 sec).

All PCR products were confirmed by gel electrophoresis and sequenced using the BigDye Terminator v3.1 Cycle Sequencing kit and sequencing was performed on 3730XL DNA analyzer (Applied Biosystems).

RESULTS

Clarithromycin Susceptibility Test

Clarithromycin susceptibility was determined for 157 strains by the broth microdilution method. Of the 157 *M. abscessus* clinical strains, clarithromycin-susceptible, clarithromycin-resistant, and clarithromycin inducible resistant strains accounted for 10.83% (*n* = 17), 22.29% (*n* = 35), and 66.88% (*n* = 105), respectively (Table 2).

Sequence Analysis of the *erm(41)* Gene at Position 28

We carried out DNA sequence analysis to confirm the association of clarithromycin resistance with single-nucleotide polymorphism (SNP) in the *erm(41)* gene at position 28 (T or C). We confirmed that 17 susceptible strains had nucleotide C and 140 resistant strains had nucleotide T at position 28, and these results were identical to those of the susceptibility test.

ARMS-PCR

The 300 bp amplification products, which all strains yielded, were used as an *erm(41)* positive control, and clarithromycin-resistant strains (T28 type) yield 522 bp amplification products with 300 bp as a result. We observed 522 and 300 bp amplification products in 140 of the

TABLE 2. Clarithromycin Susceptibility Test Results by Microdilution Method of 157 *M. abscessus* Clinical Strains

Result Number (%)	MIC (µg/ml)		Number
	3 Days	14 Days	
Susceptible 17 (10.83)	≤0.5	≤0.5	13
	1	1	1
	2	2	3
Resistant 35 (22.29)	8		11
	16		3
	32		2
	64		7
	>64		12
Inducible resistant 105 (66.88)	≤0.5	8	5
		16	12
		32	9
		64	13
		>64	16
		1	8
	1	16	4
		32	4
		64	7
		>64	8
		2	64
	>64	11	

140 clarithromycin-resistant strains, while we observed only 300 bp amplification products in 17 clarithromycin-susceptible strains (C28 type; Fig. 1, Table 3).

Real-Time PCR of *erm(41)* and Melting Curve Analysis

In order to investigate the usefulness of real-time PCR for detecting SNPs at position 28 in *erm(41)* gene, 17 clarithromycin-susceptible and 50 clarithromycin-resistant strains, determined by the clarithromycin

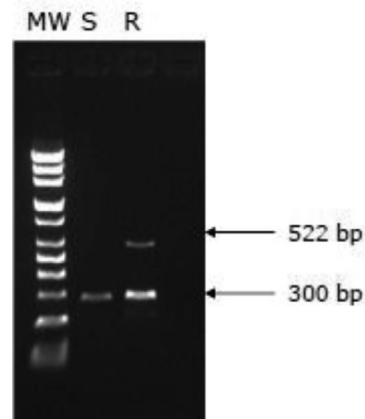


Fig. 1. Products of ARMS-PCR. S, clarithromycin-susceptible strain; R, clarithromycin-resistant strain; MW, molecular weight marker.

TABLE 3. Comparison of the Results of Sequence Analysis of *erm(41)* Gene, ARMS-PCR and Real-Time PCR With the Clarithromycin Susceptibility Test 157 *M. abscessus* Clinical Strains

Clarithromycin susceptibility	Total number	Sequence analysis		ARMS-PCR		Real-time PCR	
		C28	T28	C28	T28	C28	T28
Susceptible	17	17	0	17	0	17	0
Resistant	140	0	140	0	140	0	50

susceptibility test, sequence analysis, and ARMS-PCR, were applied to melting curve analysis using a LightCycler® 480 instrument and Gene Scanning software version 1.5.

Susceptible and resistant strains displayed different melting curves (Fig. 2, Table 3). Melting peaks of 47°C were displayed by all clarithromycin-susceptible strains and 69°C melting peaks were displayed by all clarithromycin-resistant strains. This result illustrated that 47°C is the melting peak of susceptible (C28 type) and 69°C is the melting peak of resistant (T28 type) strains.

DISCUSSION

ATS/IDSA has recommended the administration of multidrug therapy, including a macrolide and one or more parenteral agents (amikacin, cefoxitin, or imipenem) over several months, in order to help to control symptoms and progression of *M. abscessus* lung disease (10). Since Nash et al. published the mechanism of resistance to macrolides in the *M. abscessus* group as clarithromycin inducible re-

sistance conferred by the *erm(41)* gene, several reports showed the association with SNPs in the *erm(41)* gene at position 28 (T or C; 11, 14, 15). Some report revealed that the lack of efficacy of antibiotic therapy against *M. abscessus* is due to the clarithromycin inducible resistance, that is, although a strain may appear susceptible after 3 days of in vitro incubation, the strain becomes clarithromycin-resistant on increasing the incubation period to 14 days (12). For these reasons, it is recommended in the newest CLSI document that in order to ensure detection of inducible macrolide resistance in RGM, including *M. abscessus*, the final reading for clarithromycin is to be taken after 14 days, unless resistance (MIC ≥ 8 mg/l) is recognized earlier (13).

In this study, we aimed to examine the proportion of clarithromycin inducible resistant *M. abscessus* clinical strains in Korea using a comparison of the results from clarithromycin susceptibility tests, and confirm the correlation between the results of drug susceptibility tests and mutations in the *erm(41)* gene in *M. abscessus* strains. In addition, we evaluated the usefulness of two systems for

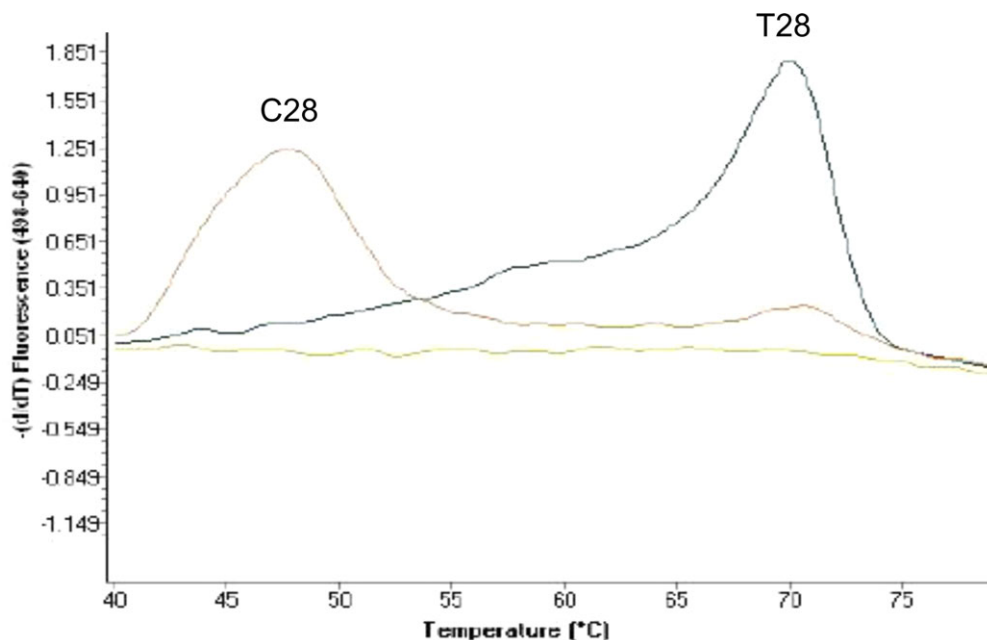


Fig. 2. Melting peaks for genotyping of SNP in *erm(41)* gene. Melting peak for clarithromycin-susceptible strain (C28 type) at 47°C, melting peak for clarithromycin-resistant strain (T28 type) at 69°C.

the rapid detection of clarithromycin resistance, using the ARMS-PCR and real-time PCR methods, reducing the reading time from that recommended by the newest CLSI document.

M. abscessus clinical strains were identified using a PCR-RFLP method based on the *rpoB* gene, and were differentiated from *M. massiliense* and *M. bolletii* based on their PCR product size and -35 promoter sequence for *erm(41)* gene in the *M. abscessus* group (11). *M. abscessus* strains were present in almost equal proportions with *M. massiliense* in the *M. abscessus* group in Korea (data not shown). We excluded *M. massiliense* clinical strains because they have nonfunctional methyltransferase based on the deleted *erm(41)* gene, and are able to interpret the results for drug susceptibility test for 3~5 days. In the *M. bolletii* strain, we identified one clinical strain (T28 type) and applied it to ARMS and real-time PCR methods. We found same results in *M. bolletii* as those of *M. abscessus*.

This study was the largest of its kind, performed with 157 clinical strains of *M. abscessus*, and also isolated the most number of clarithromycin-susceptible strains (17 strains), conferring and confirming those strains identified by Bastian et al. (10/43 strains), Nash et al. (2/11 strains), and Kim et al. (5/39 strains; 11,14,15). This study identified that clarithromycin inducible resistant strains accounted for 66.88% of Korean *M. abscessus* clinical strains. It was also identified that clarithromycin-resistant strains occupied almost 90% of Korean *M. abscessus* clinical strains, adding supportive data to the previous report that it is very difficult to treat *M. abscessus* lung disease (12).

By performing the drug susceptibility test and *erm(41)* gene sequencing analysis, it was confirmed that C28 type *erm(41)* gene is clarithromycin-susceptible and T28 type is clarithromycin-resistant. However, Bastian et al. reported that two *M. abscessus* strains acquired resistance to clarithromycin with mutation in *rrl* (23S rRNA gene) were C28 type and that no strains in the *M. abscessus* with T28 type had mutations in *rrl*. They mentioned that there might be less selection of resistant *rrl* mutants for those strains expressing inducible resistance, than for strains not expressing inducible resistance (11). In this study, we found all clarithromycin-resistant strains had the T28 type *erm(41)* gene, and two of them also had mutations in the *rrl* gene. These results are inconsistent with Bastian et al. (11).

For the rapid detection of *M. abscessus* clarithromycin resistance, we applied ARMS-PCR and real-time PCR methods for a point mutation at nucleotide position 28 within *erm(41)* gene. ARMS-PCR method was first described by Newton et al. as a general technique for the analysis of any point mutation (16). A typical ARMS test, which can detect a known SNP polymorphism, consists of two complementary reactions: one containing an ARMS

primer specific for the normal DNA sequence that cannot amplify mutant DNA at a given locus, and the other one containing a mutant-specific primer that also does not amplify normal DNA. We applied the latter only within this study.

Melting curve analysis is an assessment of the dissociation characteristics of double-stranded DNA during heating. The information gathered can be used to infer the presence and identity of SNPs. The probe-based technique is sensitive enough to detect SNP and can distinguish between homozygous wild type, heterozygous, and homozygous mutant alleles by virtue of the dissociation patterns produced. With higher resolution instruments and advanced dyes, amplicon melting analysis of base variants is now possible with several instruments commercially available.

In this study, both methods were shown to be able to differentiate clarithromycin-resistant strains (T28 type) from clarithromycin-susceptible strains (C28 type). The differences between the two methods lay in the required cost and time. While ARMS-PCR is a simple PCR application by changing of only primer sequence, real-time PCR needs an expensive real-time PCR instrument and probes. In terms of the required time, real-time PCR has advantage in detecting the result simultaneously without any additional method. The time required for real-time PCR takes 2 hr and for ARMS-PCR takes 3~4 hr. Our results showed both methods are simple, rapid, and effective methods for the detection of clarithromycin resistance, especially when compared to conventional drug susceptibility test.

In conclusion, both methods can likely be used for the detection of clarithromycin resistance and can contribute to the appropriate and rapid management of patients with *M. abscessus* infections. In light of the proportion of clarithromycin-resistant clinical strains, treatment response rates for clarithromycin-based antibiotic therapy are low in patients with *M. abscessus* lung disease. Clearly, further study is needed to investigate more effective antibiotics against *M. abscessus* infections.

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