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Genetic mutations in linezolid-resistant *Mycobacterium avium* complex and *Mycobacterium abscessus* clinical isolates

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ABSTRACT

There are no studies evaluating the mechanisms driving linezolid resistance in nontuberculous mycobacteria. The novel mutations G2599A and A2137T in the 23S rRNA gene and mutations A439G and G443A in the *rplD* gene associated with linezolid resistance were identified in linezolid-resistant *M. avium* complex isolates.

The incidence and prevalence of nontuberculous mycobacterial pulmonary disease (NTM-PD) are increasing worldwide (Prevots and Marras, 2015). Among NTM species, *Mycobacterium avium* complex (MAC), mainly composed of *M. avium* and *M. intracellulare*, is the most common pathogen; *M. abscessus* (MAB), predominantly composed of *M. abscessus* subspecies *abscessus* (hereafter referred to as *M. abscessus*) and *M. abscessus* subspecies *massiliense* (hereafter referred to as *M. massiliense*), is the second most common pathogen in many countries (Prevots and Marras, 2015).

Linezolid is the first antibiotic in the oxazolidinone class to be approved for treatment of tuberculosis. The Clinical and Laboratory Standards Institute (CLSI) guidelines suggested linezolid breakpoints for MAC and MAB (CLSI, 2011), and recent clinical guidelines have also recommended linezolid to manage NTM-PD (Floto et al., 2016; Haworth et al., 2017). However, studies reporting *in vitro* susceptibility of NTM clinical isolates to linezolid showed markedly different resistance rates according to species; the majority of MAB clinical isolates showed susceptibility to linezolid (Cho et al., 2018; Hatakeyama et al., 2017; Zhang et al., 2018), while less than 20% of MAC clinical isolates were susceptible to linezolid (Brown-Elliott et al., 2003; Schön and Chryssanthou, 2017).

Studies of linezolid resistance in *M. tuberculosis* (MTB) using *in vitro*–selected mutants and clinical isolates identified resistance-associated mutations in 23S rRNA (*rrl*) and in *rplC* and *rplD*, which encode the L3 and L4 ribosomal proteins, respectively (McNeil et al., 2017; Zong et al., 2018). However, there are no studies evaluating the mechanisms driving linezolid resistance in NTM clinical isolates (Huh et al., 2018). Therefore, we evaluated mutations of the potential target genes *rrl*, *rplC*, and *rplD* in clinical isolates of MAC and MAB.

Nonduplicate NTM isolates were obtained from 113 patients who fulfilled the diagnostic criteria of NTM-PD (Griffith et al., 2007) and who were enrolled in an institutional review board–approved observational cohort study investigating NTM-PD at Samsung Medical Center (IRB No. 2008-09-016). Drug-susceptibility testing for linezolid was performed at the Korea Institute of Tuberculosis using the broth microdilution method (CLSI, 2011). MAC and MAB isolates were considered susceptible (MIC ≤8 mg/L), intermediate (MIC = 16 mg/L), or resistant (MIC ≥32 mg/L) to linezolid (CLSI, 2011). Mycobacterial DNA extraction and NTM species identification were performed using a commercial diagnostic kit based on PCR-reverse-blot hybridization assay of the *rpoB* gene followed by multilocus sequence analysis of the 16S rRNA, *hsp*65, 16S-23S rRNA internal transcribed spacer, and *rpoB* as described previously (Kim et al., 2016). For amplification and sequencing of target genes, we used previously described primers for *rrl* (Richter et al., 2007) and designed new primers for whole *rplC* and

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Table 1
Primer sets used for target genes in this study.

Target gene	Etiological agent				Primer	PCR product size (bp)
	M. avium	*M. intracellulare*	*M. abscessus*	*M. massiliense*		
rrl	3111 bp	3113 bp	3112 bp	3114 bp	23SF2454: CCCAAACCAACACAGGTGGTCA 23SR3588: AGATGCTTTCAGCGGTTATCCT	1154[a] or 1152[b,c]
rplC	654 bp	654 bp	654 bp	654 bp	rplC F: GYACSCACAAGCGGCTSATC rplC R: ARCGCGATRTTGGCBGGG	869,[a] 887,[b] or 878[c]
rplD	654 bp	672 bp	642 bp	642 bp	rplD F: GSGTSACSRYYCARAACCTG rplD R: GTCTTGTTCGARTCSGGGTG	905,[a] 923,[b] or 907[c]

[a] Amplified gene fragment length in *M. avium*.
[b] Amplified gene fragment length in *M. intracellulare*.
[c] Amplified gene fragment length in *M. abscessus* and *M. massiliense*.

rplD sequencing (Table 1). The sequences of *rrl*, *rplC*, and *rplD* in clinical isolates were analyzed by alignment against corresponding sequences of *M. avium* subsp. *hominissuis* 104, *M. avium* subsp. *hominissuis* TH135, *M. intracellulare* ATCC 13950, *M. abscessus* ATCC 19977, and *M. massiliense* JCM 15300.

The 113 clinical isolates including 36 *M. avium*, 32 *M. intracellulare*, 22 *M. abscessus*, and 23 *M. massiliense* were examined. About half of the MAC isolates (51%, 35/68) were resistant to linezolid, whereas only 8 MAB isolates (18%) exhibited resistance (Table 2).

As shown in Table 2 and Fig. S1, 4 linezolid-resistant *M. avium* isolates had *rrl* mutations (A2358G, G2588A, A2358G/G2588A, and G2599A). The double mutation A2358G/G2588A was also found in 1 linezolid-susceptible isolate. Three linezolid-resistant *M. intracellulare* had a single mutation (A2137T or T2213C), but the T2213C mutation was also found in isolates susceptible to linezolid and with intermediate resistance. A T2131C mutation was only found in 1 *M. abscessus* isolate with intermediate resistance. Therefore, *rrl* mutations G2599A and A2137T found only in linezolid-resistant isolates are potentially involved in linezolid resistance.

M. avium and *M. massiliense* isolates had several *rplC* mutations, all of which were synonymous, whereas *M. intracellulare* and *M. abscessus* isolates had no mutations. For *rplD*, 3 linezolid-resistant *M. avium* isolates had a triple mutation (T138C/C519T/C618G) also found in 2 isolates with intermediate resistance. Interestingly, 2 linezolid-resistant *M. avium* isolates had a quadruple mutation (A439G/A489G/C570G/C618G), with the A439G mutation causing a Thr147Ala substitution. Six linezolid-resistant *M. intracellulare* isolates had *rplD* mutations, 5 of which were synonymous, with another (G443A) causing the amino

Table 2
Susceptibility to linezolid and mutations in the *rrl*, *rplC*, and *rplD* genes in 113 NTM clinical isolates.[a]

Etiological agent	Susceptibility to linezolid[b]	No. of isolates	No. of isolates with mutation	*rrl*[c]	*rplC*	*rplD*[e]	L4 protein
M. avium (*n* = 36)	S	9	1	A2358G/G2588A	-	-	
	I	8	2	-	C459T	T138C/C519T/C618G	
	R	19	1	A2358G	-	-	
			1	A2358G/G2588A	-	-	
			1	G2588A	-	-	
			1	**G2599A**	-	-	
			2	-	C459T	T138C/C519T/C618G	
			2	-	G237C/C459T/C492G	***A439G***/A489G/C570G/C618G	**T147A**
			1	-	-	T138C/C519T/C618G	
M. intracellulare (*n* = 32)	S	8	1	T2213C	-	-	
			1	-	-	*A337G*	I113V
			1	-	-	C366G	
			1	-	-	C644T	
	I	8	3	T2213C	-	-	
	R	16	1	**A2137T**[d]			
			2	T2213C			
			4	-	-	C366G	
			1	-	-	***G443A***/T534G	**R148K**
			1	-	-	G519A	
M. abscessus (*n* = 22)	S	10	2	-	-	C69T	
	I	8	1	T2131C	-	-	
			1	-	-	C69T	
	R	4	0	-	-	-	
M. massiliense (*n* = 23)	S	9	2	-	G258T	C225T/C576T	
			1	-	-	C219T/C576T	
			3	-	-	*G529C*	A177P
			3	-	-	C576T	
	I	10	2	-	G258T	C225T/C576T	
			4	-	-	*G529C*	A177P
			4	-	-	C576T	
	R	4	1	-	-	*G529C*	A177P
			2	-	-	C576T	

[a] Mutations found only in linezolid-resistant isolates are indicated in bold; -, no mutation.
[b] S, susceptible; I, intermediate; R, resistant.
[c] *E. coli* numbering.
[d] *M. intracellulare* nucleotide in *E. coli* numbering; T2137 in *E. coli*.
[e] Nonsynonymous mutations in *rplD* are denoted by italics. The corresponding amino acid changes are indicated in the L4 protein column. There is no a separate column for L3 protein because all the *rplC* mutants were synonymous at the amino acid level.

acid substitution Arg148Lys. Although some *rplD* mutations were found in MAB isolates, most were synonymous, and the G529C mutation, which causes an Ala177Pro substitution, was found in linezolid-resistant and -susceptible isolates and those with intermediate resistance. Therefore, of the mutations identified, only those resulting in the *rplD* substitutions Thr147Ala in *M. avium* and Arg148Lys in *M. intracellulare* may contribute to linezolid resistance.

The first linezolid-resistant MTB clinical isolates were reported in 2007, but no mutations were detected in potential target genes (Richter et al., 2007). In 2008, *rrl* mutations G2576T and G2061T were identified in linezolid-resistant MTB laboratory mutants (Hillemann et al., 2008). In 2012, the *rplC* mutation T460C was identified in 4 MTB laboratory mutants and 2 clinical isolates (Beckert et al., 2012). Additional *rrl* and *rplC* mutations were found in linezolid-resistant MTB clinical isolate and laboratory mutants (McNeil et al., 2017; Zhang et al., 2014, 2016; Zimenkov et al., 2017). In this study, we report 2 new *rrl* mutations, G2599A and A2137T, neither of which has been previously reported in linezolid-resistant MTB laboratory mutants or clinical isolates. Position 2599 is near the peptidyl transferase center in domain V of 23S rRNA and is therefore likely to be associated with linezolid resistance.

Although mutations in the L4 protein associated with linezolid resistance were commonly reported in Gram-positive bacteria (Mendes et al., 2014), there have been few studies on *rplD* mutations in linezolid-resistant mycobacteria. The first report of an *rplD* mutation G377A in a linezolid-resistant MTB clinical isolate was published in 2018 (Zong et al., 2018). Here, we identified the novel *rplD* mutations A439G and G443A in linezolid-resistant MAC clinical isolates, mutations not previously described either in MTB or in any other Gram-positive bacteria.

In conclusion, the novel *rrl* mutations G2599A and A2137T and *rplD* mutations A439G and G443A identified in this study are potentially involved in MAC resistance to linezolid. Structural studies to assess the impact of the mutated nucleotides on linezolid binding will help elucidate the molecular mechanisms of linezolid resistance. While the exact mechanisms of linezolid resistance remain unknown, these initial findings lay a foundation that future studies can build on to elucidate the mechanisms of resistance.

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Declaration of interest

Dr. Charles L. Daley has received grants from Insmed, Inc., and served on Advisory Boards for Insmed, Inc., Johnson and Johnson, Spero and Horizon, not associated with the submitted work. Dr. Won-Jung Koh has received a consultation fee from Insmed, Inc., for the Insmed Advisory Board Meeting, not associated with the submitted work. Otherwise, we have no conflicts of interest to declare.

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