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GenoType NTM-DR Performance Evaluation for Identification of *Mycobacterium avium* Complex and *Mycobacterium abscessus* and Determination of Clarithromycin and Amikacin Resistance

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ABSTRACT We evaluated the GenoType NTM-DR (NTM-DR) line probe assay for identifying *Mycobacterium avium* complex (MAC) species and *Mycobacterium abscessus* subspecies and for determining clarithromycin and amikacin resistance. Thirty-eight reference strains and 145 clinical isolates (58 MAC and 87 *M. abscessus* isolates), including 54 clarithromycin- and/or amikacin-resistant strains, were involved. The performance of the NTM-DR assay in rapid identification was evaluated by comparison with results of multigene sequence-based typing, whereas performance in rapid detection of clarithromycin and amikacin resistance was evaluated by comparison with sequencing of the *erm*(41), *rrl*, and *rrs* genes and drug susceptibility testing (DST). The accuracies of MAC and *M. abscessus* (sub)species identification were 92.1% (35/38) and 100% (145/145) for the 38 reference strains and 145 clinical isolates, respectively. Three MAC strains other than *M. intracellulare* were found to cross-react with the *M. intracellulare* probe in the assay. Regarding clarithromycin resistance, NTM-DR detected *rrl* mutations in 52 isolates and yielded 99.3% (144/145) and 98.6% (143/145) concordant results with sequencing and DST, respectively. NTM-DR sensitivity and specificity in the detection of clarithromycin resistance were 96.3% (52/54) and 100% (91/91), respectively. The NTM-DR yielded accurate *erm*(41) genotype results for all 87 *M. abscessus* isolates. Regarding amikacin resistance, NTM-DR detected *rrs* mutations in five isolates and yielded 99.3% (144/145) and 97.9% (142/145) concordant results with sequencing and DST, respectively. Our results indicate that the NTM-DR assay is a straightforward and accurate approach for discriminating MAC and *M. abscessus* (sub)species and for detecting clarithromycin and amikacin resistance mutations and that it is a useful tool in the clinical setting.

KEYWORDS GenoType NTM-DR, nontuberculous mycobacteria, amikacin, antibacterial susceptibility, clarithromycin, identification, mutation

The incidence of infection with nontuberculous mycobacteria (NTM) in humans is steadily increasing although the clinical significance and treatment outcomes of NTM infections differ according to etiologic organism and resistance profile (1–3). Thus,

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differential and accurate identification of NTM species, together with drug susceptibility testing (DST), is of great interest in the clinical setting. Specifically, precise *Mycobacterium avium* complex (MAC) species identification and *Mycobacterium abscessus* subspecies identification in clinical isolates are becoming increasingly important due to different treatment outcomes and epidemiological implications (4–7). However, identification of these organisms is currently limited to specialized laboratories (8). Furthermore, the gold standard method for NTM DST is broth microdilution (BMD), which is a time-consuming method, especially with slow-growing mycobacteria (9).

GenoType NTM-DR (NTM-DR; Hain Lifescience, Nehren, Germany) is a line probe assay (LPA) that enables species- or subspecies-level identification of the major clinically encountered NTM, including MAC species (*M. avium*, *M. intracellulare*, and *M. chimera*), *M. chelonae*, and subspecies belonging to *M. abscessus*, i.e., *M. abscessus* subspecies *abscessus*, *M. abscessus* subspecies *massiliense*, and *M. abscessus* subspecies *bolletii* (10–12). The NTM-DR assay also allows for detection of antibiotic resistance to macrolides and aminoglycosides. Specifically, macrolide resistance is identified by polymorphisms (T28 or C28) at position 28 in the *erm*(41) gene and mutations at positions 2058/2059 in the *rrl* gene (11). Likewise, aminoglycoside resistance is detected by examining positions 1406 to 1408 of the *rrs* gene (10, 11). The mutation probes for the NTM-DR assay were designed to hybridize to alleles containing specific mutations: four mutations in the *rrl* gene, including A2058C (MUT1 probe), A2058G (MUT2), A2059C (MUT3), and A2059G (MUT4), and one mutation in the *rrs* gene (A1408G).

So far, studies on the performance of NTM-DR (10–12), especially ones examining a large number of isolates and antibiotic-resistant clinical strains, have been limited. The aim of this study was to evaluate the analytical and clinical performance of the NTM-DR assay for identifying (sub)species of MAC and *M. abscessus* and determining their resistance to macrolides and aminoglycosides. We evaluated the performance of the assay by comparison of the results to those with multigene sequence-based identification, BMD for clarithromycin (CLR) and amikacin (AMK), and sequencing of the *erm*(41), *rrl,* and *rrs* genes.

MATERIALS AND METHODS

NTM isolates. This study was carried out at a tertiary care hospital in Seoul, South Korea, and was approved by the Institutional Review Board of Samsung Medical Center. The study involved 145 clinical NTM isolates and a total of 38 reference strains, including 33 mycobacterial strains and 5 strains from the *Gordonia*, *Nocardia*, *Rhodococcus*, and *Tsukamurella* genera. The clinical NTM isolates consisted of 58 MAC and 87 *M. abscessus* isolates that were used in our previous studies and subcultured from frozen pure cultures stored at −70°C (13–16). All clinical isolates were obtained from patients who fulfilled the diagnostic criteria of NTM pulmonary disease (1).

Multigene sequencing for identification and molecular detection of antibiotic resistance. Subcultures grown in 1 ml of liquid mycobacteria growth indicator tube culture medium (Becton, Dickinson, Sparks, MD, USA) were centrifuged at 20,000 × *g* for 15 min. The precipitates were resuspended in 100 to 300 μl of distilled water and heated to 95°C for 20 min to extract the DNA. After centrifugation, the supernatants were used for PCR and sequencing. NTM species were identified by multigene sequencing analysis of *rrs*, *rpoB*, and *hsp65*, as described previously (17–19). The amplified sequences were analyzed using the GenBank database with the Basic Local Alignment Search Tool (BLAST), available from the National Center for Biotechnology Information (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The regions of genes involved in CLR and AMK resistance were evaluated by PCR and sequencing as described previously (20–22). Each *M. abscessus* isolate was genotyped for the presence of a T-to-C mutation at position 28 of the *erm*(41) gene, a mutation associated with loss of inducible macrolide resistance (20). The presence of point mutations at position 2058 or 2059 (*Escherichia coli* numbering) in the *rrl* gene was also investigated (21). Last, the *rrs* gene was sequenced from position 1200 to 1501 (22).

GenoType NTM-DR assay. After DNA extraction as described above, amplification and hybridization were performed using the NTM-DR assay according to the manufacturer's recommended protocol. Briefly, a master mix containing amplification mixes A and B was prepared, and 5 μl of extracted DNA was added for PCR. PCR amplification was carried out under the following conditions: 1 cycle at 95°C for 15 min, followed by a first round of amplification consisting of 10 cycles of 30 s at 95°C and 120 s at 65°C. The second round of amplification consisted of 20 cycles of 25 s at 95°C, 40 s at 50°C, and 40 s at 70°C, with a final extension at 70°C for 8 min. Reverse hybridization and detection were carried out in a shaking water bath (TwinCubator; Hain) according to the manufacturer's instructions. The developed strips were attached to an evaluation sheet and interpreted with the help of the chart provided by the manufacturer. Three separate observers independently interpreted the results in a blinded fashion.

TABLE 1 Analytical specificity of the GenoType NTM-DR assay

Microbial species	Reference strain[a]	GenoType NTM-DR assay	
		Band patterns	Interpretation[c]
Mycobacterium spp.			
M. abscessus subsp. *abscessus*	ATCC 19977	CC, UC, SP 4, 5, 6, 9, 10[b]	*M. abscessus* subsp. *abscessus*
M. alvei	ATCC 51304	CC, UC	Other species
M. avium	KMRC 00136-41011	CC, UC, SP 1	*M. avium*
M. abscessus subsp. *bolletii*	CCUG 50184	CC, UC, SP 4, 5, 6, 7, 9, 10	*M. abscessus* subsp. *bolletii*
M. celatum	ATCC 51131	CC, UC, SP 4	Other species
M. chelonae	ATCC 35752	CC, UC, SP 4, 5	*M. chelonae*
M. conceptionense	CCUG 50187	CC, UC, SP 8, 9	Other species
M. cosmeticum	ATCC BAA-878	CC, UC, SP 4	Other species
M. flavescens	ATCC 14474	CC, UC	Other species
M. fortuitum	KMRC 00136-60004	CC, UC	Other species
M. gordonae	ATCC 14470	CC, UC, SP 4	Other species
M. interjectum	ATCC 51457	CC, UC, SP 2, 4	Other species
M. intracellulare	ATCC 13950	CC, UC, SP 2	*M. intracellulare*
M. kansasii	ATCC 12478	CC, UC, SP 4	Other species
M. marinum	KMRC 00136-21108	CC, UC, SP 4	Other species
M. marseillense	KMRC 00136-83001	CC, UC, SP 2	***M. intracellulare***
M. abscessus subsp. *massiliense*	CCUG 48898	CC, UC, SP 4, 5, 8, 9	*M. abscessus* subsp. *massiliense*
M. mucogenicum	ATCC 49650	CC, UC, SP 4	Other species
M. neoaurum	ATCC 25795	CC, UC, SP 4	Other species
M. nonchromogenicum	KMRC 00136-46002	CC, UC, SP 4	Other species
M. obuense	ATCC 27023	CC, UC	Other species
M. paraintracellulare	KCTC 29084	CC, UC, SP 2	***M. intracellulare***
M. parascrofulaceum	ATCC BAA-614	CC, UC, SP 2, 3, 4	Other species
M. paraterrae	KCTC 19556	CC, UC, SP 4	Other species
M. peregrinum	KMRC 00136-75003	CC, UC	Other species
M. phlei	ATCC 11758	CC, UC	Other species
M. senuense	KCTC 19147	CC, UC, SP 4	Other species
M. smegmatis	ATCC 19420	CC, UC	Other species
M. szulgai	ATCC 35799	CC, UC, SP 4	Other species
M. terrae	ATCC 15755	CC, UC	Other species
M. tuberculosis	ATCC 27294	CC, UC, SP 4	Other species
M. vaccae	ATCC 15483	CC, UC, SP 4	Other species
M. yongonense	KCTC 19555	CC, UC, SP 2	***M. intracellulare***
Nonmycobacterial *Corynebacterineae* spp.			
Gordonia polyisoprenivorans	ATCC BAA-14	CC, UC	Other species
Gordonia terrae	ATCC 25594	CC, UC	Other species
Nocardia brasiliensis	ATCC 19296	CC, UC	Other species
Rhodococcus ruber	ATCC 27863	CC, UC	Other species
Tsukamurella pulmonis	ATCC 700081	CC, UC	Other species

[a]ATCC, American Type Culture Collection; CCUG, Culture Collection University of Göteborg; KCTC, Korean Collection for Type Cultures; KMRC, Korea Mycobacterium Resource Center.
[b]Numbers refer to species-specific probes (SP). CC, conjugate control; UC, universal control.
[c]Bold type indicates species misidentification.

Drug susceptibility testing. DST for CLR and AMK was performed at the Korean Institute of Tuberculosis, a World Health Organization-designated supranational reference laboratory, using BMD as described by the Clinical and Laboratory Standards Institute (CLSI) (23). The tested concentration ranges were 0.5 to 64 μg/ml and 1 to 256 μg/ml for CLR and AMK, respectively. The MICs were interpreted according to the CLSI M64 protocol (24). The breakpoints for CLR were as follows: (i) for MAC, $\leq$8 μg/ml, 16 μg/ml, and $\geq$32 μg/ml indicated susceptible, intermediate, and resistant isolates, respectively; (ii) for *M. abscessus*, $\leq$2 μg/ml, 4 μg/ml, and $\geq$8 μg/ml indicated susceptible, intermediate, and resistant isolates, respectively. For both MAC and *M. abscessus*, isolates with AMK MICs of $\leq$16 μg/ml, 32 μg/ml, and $\geq$64 μg/ml were considered susceptible, intermediate, and resistant, respectively.

RESULTS

NTM identification. The analytical specificity test results for the NTM-DR assay using reference strains are summarized in Table 1. The accuracy for the 38 reference strains was 92.1% (35/38; 95% confidence interval [CI], 77.5 to 97.9%); three mycobacterial strains, namely, *M. marseillense*, *M. paraintracellulare*, and *M. yongonense*, were all misidentified as *M. intracellulare*.

TABLE 2 Comparison of GenoType NTM-DR assay results with *rrl* gene sequencing and DST for detecting clarithromycin resistance

GenoType NTM-DR identification	*rrl* gene sequencing result (no. of isolates)[a]		Clarithromycin drug susceptibility testing (no. of isolates)[a]		
	Wild type	Mutant	Susceptible	Inducibly resistant	Resistant
MAC					
M. avium					
Wild type	14	0	14		0
Mutant	0	9	0		9
M. intracellulare					
Wild type	19	0	19		0
Mutant	0	16	0		16
M. abscessus					
M. abscessus subsp. *abscessus*					
Wild type	37 (13, 24)	1 (0, 1)	12 (12, 0)	25 (1, 24)	1 (0, 1)
Mutant	0	9 (1, 8)	0	0	9 (1, 8)
M. abscessus subsp. *massiliense*					
Wild type	20 (0, 20)	0	20 (0, 20)	0	0
Mutant	0	18 (0, 18)	0	0	18 (0, 18)
M. abscessus subsp. *bolletii*					
Wild type	2 (0, 2)	0	0	1 (0, 1)	1 (0, 1)
Mutant	0	0	0	0	0
Total					
Wild type	92	1	65	26	2
Mutant	0	52	0	0	52

[a]Values in parentheses represent the number of *erm*(41) C28 sequevars and the number of *erm*(41) T28 sequevars, respectively.

A total of 145 clinical isolates were studied, of which 100% (145/145; 95% CI, 96.8 to 100%) of the species or subspecies identification results obtained from the NTM-DR assay were concordant with the results obtained by multigene sequence-based identification. Among the 58 MAC isolates tested, 23 and 35 isolates were identified as *M. avium* and *M. intracellulare*, respectively. A total of 87 *M. abscessus* isolates were identified at the subspecies levels, consisting of 47 *M. abscessus* subsp. *abscessus*, 38 *M. abscessus* subsp. *massiliense*, and two *M. abscessus* subsp. *bolletii*.

Detection of CLR resistance. The results of the NTM-DR assay, sequencing, and DST for detection of CLR resistance are presented in Table 2. Among 58 MAC isolates, 25 (9 *M. avium* and 16 *M. intracellulare*) showed mutations in the *rrl* gene by sequencing. The NTM-DR assay identified a corresponding genotypic pattern for all 58 MAC isolates (Table 2 and Table 3). Two mutant (MUT) bands (MUT1 and MUT3) were observed for one *M. avium* isolate harboring three mutations: A2058C, A2058T, and A2059C (Table 3). One *M. intracellulare* isolate harboring the A2059T mutation revealed neither wild-type (WT) nor MUT bands by the NTM-DR assay, which was attributed to the lack of a specific MUT probe for this mutation. The *rrl* genotype results obtained from the NTM-DR assay were 100% concordant (58/58; 95% CI, 92.2 to 100%) with the CLR DST results. NTM-DR sensitivity and specificity in the detection of CLR resistance of MAC were 100% (25/25; 95% CI, 83.4 to 100%) and 100% (33/33; 95% CI, 87.0 to 100%), respectively.

Among the 87 *M. abscessus* isolates, 28 (10 *M. abscessus* subsp. *abscessus* and 18 *M. abscessus* subsp. *massiliense*) showed mutations in the *rrl* gene by sequencing. Of 47 *M. abscessus* subsp. *abscessus* isolates, 14 had the *erm*(41) C28 sequevar, including 13 isolates with the WT *rrl* gene and a single isolate with an *rrl* mutation, and the remaining 33 were of the *erm*(41) T28 sequevar, including 24 isolates with the WT *rrl* gene, a single isolate with an *rrl* mutation identified by gene sequencing, and an additional eight isolates with *rrl* mutations detected by both the NTM-DR assay and sequencing (Table 2). The NTM-DR assay matched 100% (87/87; 95% CI, 94.7 to 100%) of the *erm*(41) sequencing results and 98.9% (86/87; 95% CI, 92.9 to 99.9%) of the *rrl* sequencing results (Table 2). The *M. abscessus* subsp. *abscessus* isolate with a discordant

TABLE 3 Isolates with an *rrl* mutation detected by the GenoType NTM-DR assay and/or sequencing

Species	GenoType NTM-DR assay result[b]	*erm*(41) sequevar	No. of isolates	Sequencing result	Clarithromycin MIC (μg/ml)[a]
M. avium	MUT1 (A2058C)		3	A2058C	≥64
	MUT2 (A2058G)		2	A2058G	>64
			1	A2058G, A2058T	>64
	MUT3 (A2059C)		2	A2059C	≥64
	MUT1 (A2058C), MUT3 (A2059C)		1	A2058C, A2058T, A2059C	>64
M. intracellulare	MUT1 (A2058C)		2	A2058C	>64
	MUT2 (A2058G)		4	A2058G	≥64
	MUT3 (A2059C)		1	A2059C	>64
	MUT4 (A2059G)		8	A2059G	≥64
	Other		1	A2059T	>64
M. abscessus subsp. *abscessus*	MUT1 (A2058C)	T28	4	A2058C	>64
	MUT2 (A2058G)	T28	3	A2058G	≥64
		C28	1	A2058G	≥64
	MUT4 (A2059G)	T28	1	A2059G	64
	WT	T28	1	A2058T, WT	>64
M. abscessus subsp. *massiliense*	MUT1 (A2058C)	T28	2	A2058C	>64
	MUT2 (A2058G)	T28	5	A2058G	≥64
	MUT4 (A2059G)	T28	8	A2059G	≥64
		T28	1	A2059G	8
	Other	T28	1	A2058T	16
		T28	1	A2057G, A2058G	32

[a]All isolates were resistant.
[b]Other, wild-type band missing.

rrl result harbored an A2058T mutation in a heterogeneous pattern with the WT allele, from which only the *rrl* WT band was detected by the NTM-DR assay (Table 3). One *M. abscessus* subsp. *massiliense* isolate harboring the A2057G and A2058G mutations revealed an absence of the WT band by the NTM-DR assay.

The *rrl* genotype of *M. abscessus* isolates obtained with the NTM-DR assay was 97.7% (85/87; 95% CI, 91.2 to 99.6%) concordant with the CLR DST results (Table 2). NTM-DR sensitivity and specificity in the detection of CLR resistance of *M. abscessus* were 93.1% (27/29; 95% CI, 75.8 to 98.8%) and 100% (58/58; 95% CI, 92.3 to 100%), respectively. Two isolates, one each of *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *bolletii*, were WT by the NTM-DR assay but resistant to CLR (MIC, >64 μg/ml). Although the *M. abscessus* subsp. *abscessus* isolate harbored an A2058T mutation, no *rrl* mutations were detected by sequencing in the *M. abscessus* subsp. *bolletii* isolate. Phenotypic inducible CLR resistance was detected in 26 *M. abscessus* isolates (25 *M. abscessus* subsp. *abscessus* and 1 *M. abscessus* subsp. *bolletii*), which all had the WT *rrl* gene accompanied by the *erm*(41) T28 genotype, except for one isolate with the C28 genotype.

Overall, the concordance between the NTM-DR assay results and those of sequencing was 99.3% (144/145; 95% CI, 95.6 to 99.9%), and the concordance with DST results was 98.6% (143/145; 95% CI, 94.6 to 99.8%) for detection of CLR resistance. NTM-DR sensitivity and specificity in the detection of CLR resistance were 96.3% (52/54; 95% CI, 86.2 to 99.4%) and 100% (91/91; 95% CI, 95.0 to 100%), respectively.

Detection of AMK resistance. The results of the NTM-DR assay and corresponding sequencing and DST results for detection of AMK resistance are presented in Table 4. Among 58 MAC isolates, 3 (2 *M. avium* and 1 *M. intracellulare*) showed mutations in the *rrs* gene by sequencing. The NTM-DR assay showed a concordant genotypic pattern for two MAC isolates with an A1408G mutation. One *M. avium* isolate produced a discordant *rrs* result and comprised a heterogeneous population carrying the T1406A mutation and WT alleles, from which only the WT band was observed (Table 5). The *rrs* genotype obtained with the NTM-DR assay was 96.6% (56/58; 95% CI, 87.0 to 99.4%) concordant with the results of AMK DST (Table 4). NTM-DR sensitivity and specificity in

TABLE 4 Comparison of GenoType NTM-DR assay results with *rrs* gene sequencing and DST for detecting amikacin resistance

GenoType NTM-DR *rrs* gene result	*rrs* gene sequencing result (no. of isolates)		Amikacin drug susceptibility testing (no. of isolates)		
	Wild type	Mutant	Susceptible	Intermediate	Resistant
MAC					
M. avium					
Wild type	21	1	12	8	2
Mutant	0	1	0		1
M. intracellulare					
Wild type	34	0	19	15	0
Mutant	0	1	0		1
M. abscessus					
M. abscessus subsp. *abscessus*					
Wild type	46	0	34	12	0
Mutant	0	1	0	0	1
M. abscessus subsp. *massiliense*					
Wild type	36	0	25	10	1
Mutant	0	2	0	0	2
M. abscessus subsp. *bolletii*					
Wild type	2	0	2	0	0
Mutant	0	0	0	0	0
Total					
Wild type	139	1	92	45	3
Mutant	0	5	0	0	5

the detection of AMK resistance of MAC were 50% (2/4; 95% CI, 9.2 to 90.8%) and 100% (54/54; 95% CI, 91.7 to 100%), respectively. The MICs for the two discordant *M. avium* isolates were both 64 μg/ml, while those for isolates with the A1408G mutation (detected as a positive MUT1 probe) were $>$256 μg/ml.

Among the 87 *M. abscessus* isolates, 3 (1 *M. abscessus* subsp. *abscessus* and 2 *M. abscessus* subsp. *massiliense*) showed an A1408G mutation in the *rrs* gene by sequencing. The NTM-DR assay revealed a corresponding genotypic pattern for all *M. abscessus* isolates (Table 4 and Table 5). The *rrs* genotype determined by the NTM-DR assay was concordant with AMK DST results with the exception of one *M. abscessus* subsp. *massiliense* isolate with a WT *rrs* gene and an MIC of 64 μg/ml. NTM-DR sensitivity and specificity in the detection of AMK resistance of *M. abscessus* were 75% (3/4; 95% CI, 21.9 to 98.7%) and 100% (83/83; 95% CI, 94.5 to 100%), respectively.

Overall, the concordance of results of the NTM-DR assay with those of sequencing was 99.3% (144/145), and the concordance with DST results was 97.9% (142/145; 95% CI, 93.6 to 99.5%) for detection of AMK resistance. NTM-DR sensitivity and specificity in the detection of AMK resistance were 62.5% (5/8; 95% CI, 25.9 to 89.8%) and 100% (137/137; 95% CI, 96.6 to 100%), respectively. The MIC for isolates harboring the A1408G mutation was $>$256 μg/ml.

DISCUSSION

In this study, the NTM-DR assay exhibited excellent performance for identifying *M. abscessus* (sub)species in clinical isolates and good performance with most MAC

TABLE 5 Isolates with an *rrs* mutation detected by the GenoType NTM-DR assay and/or sequencing

Species	GenoType NTM-DR assay result	No. of isolates	Sequencing result	Amikacin MIC (μg/ml)
M. avium	MUT1 (A1408G)	1	A1408G	$>$256
	WT	1	T1406A, WT	32
M. intracellulare	MUT1 (A1408G)	1	A1408G	$>$256
M. abscessus subsp. *abscessus*	MUT1 (A1408G)	1	A1408G	$>$256
M. abscessus subsp. *massiliense*	MUT1 (A1408G)	2	A1408G	$>$256

species. However, we demonstrated that the assay has limitations in identifying MAC isolates at the species level. The MAC includes a total of 12 validly published species: *M. avium*, *M. intracellulare*, *M. chimera*, *M. colombiense*, *M. arosiense*, *M. vulneris*, *M. marseillense*, *M. timonense*, *M. bouchedurhonense*, *M. yongonense*, *M. paraintracellulare*, and *M. lepraemurium* (25). Among these, three MAC reference strains (*M. marseillense*, *M. paraintracellulare*, and *M. yongonense*) other than *M. avium* and *M. intracellulare* were included in our study, and all three strains were misidentified as *M. intracellulare*. Probes specific to these species that cross-reacted with the *M. intracellulare* probe are not available on the test strip. Similar misidentification of MAC species as *M. intracellulare* by LPAs has been reported previously (12, 25–27). Specifically, Mok et al. showed that *M. arosiense*, *M. timonense*, *M. bouchedurhonense*, and *M. marseillense* all cross-reacted with the *M. intracellulare* probe using the NTM-DR assay (12). These MAC species are infrequently encountered NTM species and are rarely associated with human infection, and thus the clinical spectra for these MAC species are not fully understood (28–30). However, the possibility of such misidentifications should be taken into account.

Acquired CLR resistance is almost always associated with mutations of the *rrl* gene at positions 2058/2059 (31). Indeed, these point mutations are present at high frequency in CLR-resistant MAC and *M. abscessus* isolates (32–35). In addition, inducible resistance to CLR has been reported in *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *bolletii* due to induced synthesis of an RNA methylase encoded by the *erm*(41) gene (36). *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *bolletii* exhibit a T/C polymorphism at position 28 of the *erm*(41) gene, with T28 strains showing inducible resistance and C28 strains lacking inducible resistance (36, 37). Furthermore, inducible resistance does not occur in *M. abscessus* subsp. *massiliense* because it has a partially deleted, nonfunctional *erm*(41) gene (35–37). Therefore, detection of acquired and intrinsic resistance is necessary prior to starting treatment or at recurrence of NTM disease. However, the time needed to perform NTM DST for detecting CLR resistance can be as long as 6 weeks for MAC and 14 days for *M. abscessus* (24). The results of our study indicate that the NTM-DR assay is a rapid and accurate tool for detecting resistance to both CLR and AMK. So far, two other studies have reported the results of the NTM-DR assay for molecular detection of antibiotic resistance in MAC and *M. abscessus* (10, 11). However, based on these previous studies, it was difficult to reliably assess the sensitivity of the assay for detecting antibiotic resistance among NTM isolates overall and with regard to MAC and *M. abscessus* isolates specifically due to their small number of CLR-resistant isolates. The study of Mougari et al. included nine and seven CLR-resistant MAC and *M. abscessus isolates*, respectively (11), and the study of Kehrmann et al. included only *M. abscessus* isolates, of which 11 were CLR resistant (10). In the present study, we included a sufficient number of CLR-resistant MAC and *M. abscessus* isolates (total of 54; 25 MAC and 29 *M. abscessus isolates*) to demonstrate that the sensitivity and specificity of the NTM-DR assay were excellent for detection of CLR resistance in both MAC and *M. abscessus*. However, we did observe two false WT results for two of the isolates. They were found to comprise a heterogeneous population with a WT allele and a resistance allele, as determined by sequencing. In order to check for reproducibility, the NTM-DR assay and sequencing were repeated, and the results were concordant with the original results. Consistent with this observation, Mougari et al. observed that a heterogeneous population containing a resistance mutation that is not screened for by a specific probe can be missed if a WT population is also present (11). Therefore, it is recommended that the NTM-DR assay should be used in tandem with phenotypic DST since this assay has the inherent limitations of LPAs: (i) the inability to exclude the possibility that a strain is resistant when the strain has a WT pattern and (ii) the inability to detect other mechanisms of resistance to CLR and AMK.

Mutations in the *rrs* gene responsible for AMK resistance occur mainly at position 1408 in both MAC and *M. abscessus* (35, 38). In our study, among eight isolates that were resistant to AMK, five harbored the A1408G mutation and had corresponding MICs of >256 μg/ml. On the other hand, the *rrs* A1408G mutation was not detected in isolates with an MIC of 64 μg/ml, in accordance with previous reports (10, 39, 40). Until

recently, no AMK breakpoints for MAC had been established (23). Brown-Elliott et al. suggested a resistance breakpoint of ≥64 μg/ml, which was derived from a mutation study on the *rrs* gene (39). However, Griffith et al. reported that treatment success correlates with the following MIC breakpoints: susceptible, ≤64 μg/ml; resistant, >64 μg/ml (41, 42). In guidelines published in November 2018, new AMK breakpoints for MAC were proposed by the CLSI, which need to be clinically validated (24). The CLSI guidelines included two separate sets of breakpoints for AMK, as follows: (i) for intravenous AMK, breakpoints of ≤16 μg/ml for susceptible, 32 μg/ml for intermediate, and ≥64 μg/ml for resistant; (ii) for liposomal inhaled AMK, breakpoints of ≤64 μg/ml for susceptible and ≥128 μg/ml for resistant. Although two separate sets of breakpoints for determining resistance were suggested, MAC isolates with MICs of >64 μg/ml almost always had mutations in the *rrs* gene regardless of the formulation of AMK, as shown by previous studies (39, 42) and the present study. Thus, the efficacy of therapy for isolates with AMK MICs of 64 μg/ml would require further clinical validation.

Apart from the A1408G mutation in the *rrs* gene, Nessar et al. demonstrated that mutations at positions 1406, 1409, and 1491 of *rrs* (T1406A, C1409T, and G1491T) are associated with AMK resistance in *M. abscessus* (22). In the present study, we describe for the first time a T1406A mutation in an *M. avium* isolate, previously only described in *M. abscessus*. The isolate harboring the T1406A mutation presented an AMK-susceptible phenotype (MIC, 32 μg/ml). However, it is not clear whether this substitution contributes to AMK resistance since the substitution was found in a heterogeneous pattern with a WT allele (Table 5).

A limitation of the present study was that we did not evaluate *M. chimera* and tested only two *M. abscessus* subsp. *bolletii* isolates since they are rarely isolated in South Korea (43–46). Thus, we cannot be sure about the performance of the NTM-DR assay for these two (sub)species in this study.

In conclusion, NTM-DR is a straightforward and accurate assay for discriminating MAC and *M. abscessus* (sub)species and for detecting CLR and AMK resistance mutations. We expect that the NTM-DR assay will be a useful and routine tool in the clinical setting.

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