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New Real-Time PCR Assays for Detection of Inducible and Acquired Clarithromycin Resistance in the *Mycobacterium abscessus* Group

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Members of the *Mycobacterium abscessus* group (MAG) cause lung, soft tissue, and disseminated infections. The oral macrolides clarithromycin and azithromycin are commonly used for treatment. MAG can display clarithromycin resistance through the inducible *erm*(41) gene or via acquired mutations in the *rrl* (23S rRNA) gene. Strains harboring a truncation or a T28C substitution in *erm*(41) lose the inducible resistance trait. Phenotypic detection of clarithromycin resistance requires extended incubation (14 days), highlighting the need for faster methods to detect resistance. Two real-time PCR-based assays were developed to assess inducible and acquired clarithromycin resistance and tested on a total of 90 clinical and reference strains. A SYBR green assay was designed to distinguish between a full-length and truncated *erm*(41) gene by temperature shift in melting curve analysis. Single nucleotide polymorphism (SNP) allele discrimination assays were developed to distinguish T or C at position 28 of *erm*(41) and 23S rRNA *rrl* gene mutations at position 2058 and/or 2059. Truncated and full-size *erm*(41) genes were detected in 21/90 and 69/90 strains, respectively, with 64/69 displaying T at nucleotide position 28 and 5/69 containing C at that position. Fifteen isolates showed *rrl* mutations conferring clarithromycin resistance, including A2058G (11 isolates), A2058C (3 isolates), and A2059G (1 isolate). Targeted sequencing and phenotypic assessment of resistance concurred with molecular assay results. Interestingly, we also noted cooccurring strains harboring an active *erm*(41), inactive *erm*(41), and/or acquired mutational resistance, as well as slowly growing MAG strains and also strains displaying an inducible resistance phenotype within 5 days, long before the recommended 14-day extended incubation.

Rapidly growing mycobacteria (RGM) are important emerging human pathogens. Three closely related taxa of RGM with a controversial species/subspecies status—i.e., *Mycobacterium abscessus* subsp. *abscessus* (here *M. abscessus*), *M. abscessus* subsp. *massiliense* (*M. massiliense*), and *M. abscessus* subsp. *bolletii* (*M. bolletii*)—comprise the so-called "*M. abscessus* group" (MAG) (1–3). MAG is commonly associated with chronic lung infections in susceptible hosts, such as cystic fibrosis (CF) (4), as well as wound infection and postsurgical site infections (5, 6). Macrolides such as clarithromycin and azithromycin are frequently the only oral antibiotics that are active against MAG and are commonly used to treat pulmonary infections (7, 8). Members of the MAG differ in their susceptibility to clarithromycin. *M. abscessus* and *M. bolletii*, commonly display inducible macrolide resistance conferred by the ribosomal methyl transferase *erm*(41) gene (9). In contrast, most *M. massiliense* strains do not show inducible resistance due to a large, 274-bp deletion in the ribosomal methyl transferase *erm*(41) gene that renders it nonfunctional (9, 10), and clinical studies have shown a better response to clarithromycin in *M. massiliense* compared to *M. abscessus* (8, 10). Besides this major truncation, *erm*(41) can lose functionality by a T-to-C substitution at position 28 (T28C) yielding a Trp-to-Arg amino acid change (9, 11). The second mechanism of clarithromycin resistance is acquired through mutations in the drug-binding pocket of the 23S rRNA *rrl* gene at nucleotide positions 2058 and 2059 (12–15, 27). Phenotypic detection of clarithromycin resistance requires incubation of MAG with clarithromycin for up to 14 days. While MAG strains displaying acquired resistance show high MICs to clarithromycin after 3 to 5 days, those with an inducible active *erm*(41) gene typically show low MICs at 3 to 5 days and require longer incubation times (up to 14 days) for induction of resistance (16).

Targeted sequencing is routinely used in the clinical laboratory to determine the intraspecies genetic diversity in mycobacterial isolates and discriminate among closely related taxa. Multiple genes, including *rpoB*, *hsp65*, *secA*, and others (17–22), as well as PCR-based assays (10, 23) have been evaluated as tools to discriminate between the closely related subspecies of the MAG. Since a truncated *erm*(41) gene was described as a hallmark of *M. massiliense*, size differences in PCR-amplified *erm*(41) PCR products were proposed as a simple method to differentiate *M. massiliense* from *M. abscessus* and *M. bolletii* (10). However, this method can misclassify some strains, such as two recently described *M. massiliense* strains harboring a full-length and functional *erm*(41) gene (23).

Since standard susceptibility testing of clarithromycin requires up to 14 days, there is a need for faster methods to detect clari-

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TABLE 1 Primers used in the full and truncated *erm*(41) gene discrimination assay, single nucleotide polymorphism, and sequencing of the *erm*(41) and 23S rRNA *rrl* genes of the *M. abscessus* group

Primer	Sequence	Purpose	Gene ID no.	Product size (bp)
erm225 Forward	5′-GGCACATCTGGTTGCCGC-3′	SYBR green *erm*(41) real-time assay	MAB_2297 *M. abscessus*	321 or 47
erm546Rev_RC Reverse	5′-CGGCAATGTGATTCCGGCC-3′			
MycoSNP_Fex Forward	5′-GAGCATGGGCATATTCATGATGG-3′	*erm*(41) position 28 SNP real-time assay	MAB_2297 *M. abscessus*	125
MycoSNP_Rex Reverse	5′-TGAGCGAACACCGGATTCG-3′			
23S_SNPF Forward	5′-GCGAAATTGCACTACGAGTAAAG-3′	23S rRNA position 2058/2059 SNP real-time assay	MAB_5052 *M. abscessus*	110
23S_SNPR Reverse	5′-CCTATCCTACACAAACCGAACC-3′			
ermF Forward	5′-GACCGGGGCCTTCTTCGTGAT-3′	*erm*(41) sequencing	MAB_2297 *M. abscessus*	672 or 396
ermR1 Reverse	5′-GACTTCCCCGCACCGATTCC-3′			
23S_18 Forward	5′-AGTCGGGACCTAAGGCGAG-3′	23S rRNA sequencing	MAB_5052 *M. abscessus*	1,525
23S_21 Reverse	5′-TTCCCGCTTAGATGCTTTCAG-3′			
23SrRNAF_207 Forward	5′-AGCGAAATTCCTTGTCGGGT-3′	23S rRNA sequencing	MAB_5052 *M. abscessus*	207
23SrRNAR_207 Reverse	5′-CTGCTTCACAGTCTCCCACC-3′			

thromycin resistance. The present study describes the development and evaluation of two novel rapid real-time PCR assays for assessment of clarithromycin resistance on 87 clinical and 3 reference MAG strains. The first assay discriminates between full-length and truncated *erm*(41) based on differences in the melting temperature (T_m) of amplified products. The second assay identifies mutations associated with clarithromycin susceptibility and resistance using fluorescent locked nucleic acid (LNA) probes, including single nucleotide polymorphisms (SNPs) in the 23S rRNA *rrl* A2058G/C and A2058G positions (*Escherichia coli rrl* numbering) and T/C at the position 28 of the *erm*(41) gene. Our assays decreased the turnaround time of detection of resistance from 14 days to ~3 h, and results were concordant with targeted sequencing and phenotypic testing. This assay is feasible to use in laboratories set up to perform molecular tests and can help overcome technical challenges of determining MICs in slowly growing strains.

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MATERIALS AND METHODS

DNA isolation and identification of clinical isolates. Eighty-seven clinical isolates belonging to the *M. abscessus* group were obtained between 2005 and 2015 from sputum, bronchoalveolar lavage fluid, blood cultures, skin, or lymph node from patients with bronchiectasis, cystic fibrosis, and interleukin-21 receptor gene primary immunodeficiency. In addition, 3 reference strains were used, for a total of 90 isolates: *M. abscessus* ATCC 19977^T, *M. massiliense* CCUG 48898^T, and *M. bolletii* CCUG 50184^T. The bacterial strains were stored at −80°C in Tween albumin broth (Remel, Thermo Scientific, Lenexa, KS). Prior to use, the organisms were subcultured onto Middlebrook 7H11 agar (Remel, Lenexa, KS). DNA was extracted from a 10-μl loopful of each mycobacterial colony with the Ultra Clean microbial DNA isolation kit (MoBio Laboratories, Solana Beach, CA), according to the manufacturer's instructions. Most clinical isolates before 2011 were previously identified by multilocus genomic sequencing with *secA1*, *rpoB*, and *hsp65* (22). More recent isolates were identified as MAG by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) followed by subspecies-level identification either by multilocus genomic sequencing (22) or a recently developed PCR method (23). All primers (Table 1) and probes (Table 2) in this study were purchased from IDT (Integrated DNA Technologies, Coralville, IA).

Real-time SYBR green assay to discriminate between full-length and truncated *erm*(41) genes. Primers erm225 and erm546Rev_RC (Table 1) were designed using the *M. abscessus* ATCC 19977^T genome *erm*(41) gene (MAB_2297), spanning the 274-bp region, which is truncated (nonfunctional) in most *M. massiliense* strains. The SYBR green assay was carried out in a 20-μl reaction mixture, which consisted of 1 μl of the forward and reverse primers (10 μM stock) at a final concentration of 500 nm each and 2 to 3 μl of genomic DNA (30 to 50 ng), nuclease-free water (5 to 6 μl), and 10 μl of the 2× Fast SYBR green master mix (Thermo Fisher Scientific, Life Technologies, Grand Island, NY). The cycling conditions were as follows: a pre-PCR step of 95°C for 20 s followed by 40 cycles of 95°C for 10 s, 60°C for 30 s and 72°C for 30 s. This was followed by a melt curve step of 2 cycles of 95°C for 15 s and 60°C for 1 min in a 96-well PCR plate and processing on a 7500 Fast thermocycler (Thermo Fisher Scientific, Applied Biosystems, Grand Island, NY). The *M. abscessus* and *M. massiliense* type strains were used as positive controls for the full-length and truncated *erm*(41) genes, respectively. A negative control containing nuclease-free water instead of DNA template was included in all experiments. The reaction was set up in triplicate in a 96-well PCR plate and processed on a 7500 Fast thermocycler (Thermo Fisher Scientific, Applied Biosystems, Grand Island, NY). *In silico* PCR was done using NCBI BLASTn (http://blast.ncbi.nlm.nih.gov/), and the publicly available *M. abscessus* ATCC

TABLE 2 SNP detection probes of the 23S rRNA *rrl* gene 2058A-to-G or -C and 2059A-to-G mutation and the *erm*(41) gene 28T-to-C mutation

Probe	Gene	Nucleotide position(s)	Base change	LNA probe sequence[a]	T_m (°C)
WT_MAB	23S rRNA	2058–2059	AA to AA	5′-HEX-ACG+A+A+A+AGA+C+CC-IABkFQ-3′	65.1
AA_2058G	23S rRNA	2058	AA to GA	5′-Cy5-ACG+A+G+A+AGA+CCC-IABkFQ-3′	68.2
AA_2058C	23S rRNA	2058	AA to CA	5′-6FAM-ACG+A+C+A+AGA+CCC-IABkFQ-3′	66.8
AA_2059G	23S rRNA	2059	AA to AG	5′-6FAM-ACG+A+A+G+AGA+CCC-IABkFQ-3′	68.2
C Allele	*erm*(41)	28	T to C	5′-6FAM-CCA+G+C+GGGGC/IABkFQ-3′	67.4
T Allele	*erm*(41)	28	T to T	5′-HEX-CCA+G+T+GGGGC-IABkFQ-3′	69.3

[a] HEX, hexachlorofluorescein; 6FAM, 6-carboxyfluorescein; IABkFQ, Iowa Black fluorescent quencher.

19977^T and *M. massilense* CCUG 48898^T genomes yielded the expected PCR product sizes for each subspecies.

Real-time 28C/T SNP detection in the inducible clarithromycin resistance *erm*(41) gene. Primers (MycoSNP_Fex and MycoSNP_Rex (Table 1) were designed using the *M. abscessus* ATCC 19977^T genome spanning a 124-bp region of the *erm*(41) gene containing the position 28 C/T SNP. Probes were reconstituted in Tris-EDTA (TE) buffer at a stock concentration of 100 μM and stored at −70°C before use. The probes were then diluted to a working concentration of 10 μM, with a final concentration in the reaction mixture of 250 nM for each probe. Allele-specific fluorescent-labeled LNA probes with a C or T nucleotide at position 28 of the *erm*(41) gene with LNA bases located on the SNP base and the flanking nucleotides were designed (probes T Allele and C Allele in Table 2). The SNP assay was carried out in a 20-μl reaction mixture, which consisted of 1 μl of the forward and reverse primers at a concentration of 20 μM (1,000 nM final concentration), 0.5 μl of each of the Allele T and Allele C LNA probes at a concentration of 10 μm (250 nM final concentration), 2 to 3 μl of genomic DNA (30 to 50 ng), nuclease-free water (5 to 6 μl), and 10 μl of the TaqMan GTXexpress master mix (Thermo Fisher Scientific, Life Technologies, Grand Island, NY). The reaction was set up in a 96-well PCR plate and processed on a 7500 Fast thermocycler (Applied Biosystems, Life Technologies, Grand Island, NY). The cycling conditions for the assays were a pre-PCR step at 95°C for 3 min, followed by 40 cycles of 95°C for 10 s, 60°C for 30 s, and 72°C for 30 s, followed by a post-PCR step at 72°C for 2 min. The extent of binding and the resulting fluorescence signal during the PCR at each time point are measured as Rn values for each probe (where Rn is the normalized reporter value). The fluorescence intensity for each probe is shown in allelic discrimination plots; the probe showing a high-intensity signal represents the allele on the DNA template. Allelic discrimination plots were generated using the Applied Biosystems 7500 Fast real-time PCR system software (Thermo Fisher Scientific, Applied Biosystems, Grand Island, NY).

Real-time single nucleotide polymorphism detection for acquired clarithromycin resistance on the *rrl* (23S rRNA) gene. Primers 23S_SNPF and 23S_SNPR (Table 1) were designed using the *M. abscessus* ATCC genome 23S rRNA *rrl* gene (MAB_r5052). Allele-specific fluorescent-labeled LNA probes with the wild-type allele AA at nucleotides 2058 to 2059 and G or C at the nucleotide 2058 position or G at 2059 were designed (Table 2) with LNA bases located on the SNP base and the flanking bases. The SNP assay was carried out with the pairs WT_MAB and AA_2058G to detect the A2058G SNP, WT_MAB and AA_2058C to detect the A2058C SNP, and WT_MAB and AA_2059G to detect the A2059G SNP. The PCR, cycling conditions, and analysis of results were as described for the real-time 28C/T SNP detection assay.

Sequencing of the *erm*(41) and *rrl* genes to confirm deletions and SNPs. PCR was performed for the *erm*(41) and *rrl* genes on 87 clinical isolates and three reference strains using Qiagen *Taq* polymerase (Qiagen, Carlsbad, CA) in a 25-μl total reaction volume consisting of 12.5 μl master mix comprised of buffer, deoxynucleoside triphosphates (dNTPs), *Taq* polymerase, and 2 to 3 μl (~30 to 50 ng) of extracted DNA. Primers ermF and ermR1 (Table 1) (10) were used for amplification and sequencing of the *erm*(41) gene. Primers 23S_18 and 23S_21 were used for partial amplification of a region of the *rrl* gene spanning nucleotides 2058 and 2059, and sequencing was performed using the PCR primers and the internal primers 23SrRNAF_207 and 23SrRNAR_207. PCR products from the *erm*(41) and *rrl* genes were purified for sequencing using Amicon Ultra-0.5 ml 100K centrifugal filters (Millipore, Ltd., Carrigtwohill, Ireland). Sequencing of PCR amplicons was performed on the ABI Prism 3100 genetic analyzer (Applied Biosystems, Carlsbad, CA), and data were assembled using Lasergene SeqMan Pro technology (DNAStar, Inc., Madison, WI). Sequences were compared against the GenBank database using NCBI BLASTn (24; http://blast.ncbi.nlm.nih.gov/).

Clarithromycin susceptibility testing. Clarithromycin MIC values were determined in Mueller-Hinton medium by the broth microdilution method, using Sensititre RAPMYCO plates for strains isolated after July 2010 (Trek Diagnostic Systems, Thermo Fisher Scientific, Oakwood Village, OH). Earlier isolates were tested with frozen panels from PML Microbiologicals (Wilsonville, OR). Plates were evaluated at 3 to 5 days and were further incubated for 14 days at 30°C for a final reading to ensure detection of inducible resistance, according to the CLSI M24-A2 document (25) requiring extended incubation. The interpretative breakpoints used were those recommended by the CLSI (25): ≤2 μg/ml, susceptible; 4 μg/ml, intermediate; and ≥8 μg/ml, resistant (see Table S1 in the supplemental material).

RESULTS

A total of 90 clinical and reference isolates of *M. abscessus* subspecies were used to develop and validate two real-time PCR-based assays aimed at the detection of clarithromycin resistance, which include differentiation between full-length and truncated inducible clarithromycin *erm*(41) and SNP detection in *erm*(41) and 23S rRNA *rrl* genes.

Discrimination between full-length and truncated *erm*(41) genes by real-time SYBR green assay. This assay takes advantage of the size difference between a full-length *erm*(41) gene (characteristic of *M. abscessus* and *M. bolletii*) versus a truncated *erm*(41) gene (seen in most *M. massiliense* strains) (9, 11). In this assay, primers were designed spanning the 274-bp deletion region of the *erm*(41) gene, generating a 321-bp product for the full-length *erm*(41) gene or a 47-bp product with a truncated product as observed by *in silico* PCR on *M. abscessus* ATCC 19977^T and *M. massiliense* CCUG 48898^T genomes. This size difference results in an ~7°C change in T_m in real-time PCR melting curve analysis (Fig. 1). Most of our isolates (69/90 [77%]), including *M. abscessus* and *M. bolletii* type strains, showed a T_m of 85.8°C (±0.9°C), corresponding to a full-length *erm*(41) gene, while (21/90 [23%]) of the isolates (including the *M. massiliense* type strain) showed a T_m of 78.4°C (±0.4°C), consistent with a truncated *erm*(41) gene. The water negative control had a melt curve temperature of 73.9°C, about 5°C lower than the T_m of the truncated *erm*(41) gene melt curve. The standard deviations of T_m values of replicates were 0.09 to 0.43. Sequencing of the *erm*(41) gene performed on all isolates confirmed the results of the real-time PCR assay (see Table S1 in the supplemental material). Most of the 69 isolates with the full-length *erm*(41) gene were *M. abscessus*. Four isolates (CI5-9, CI7-8 CI20-27, and CI20-38) were identified as *M. bolletii*, and three isolates (CI20-39, CI20-40, and CI80-82) with full-length *erm*(41) were identified as *M. massiliense*.

Real-time assay for T/C SNP at position 28 of the *erm*(41) gene. Another mechanism by which the inducible clarithromycin *erm*(41) gene can lose functionality (besides a major truncation) is through a T-to-C substitution at position 28 (T28C) (9, 11). The *erm*(41) SNP allele discrimination assay was designed to differentiate between both nucleotides at position 28. Out of a total of 90 isolates in this study, the majority had a T at position 28 (85/90 [95%]), and 5/90 (5%) had a C (see Table S1 in the supplemental material). Sixty-four of 69 isolates with full-length *erm*(41) contained a T at position 28 characteristic of active *erm*(41) genes, while 5/69 contained a C (strains CI6-2, CI6-6, CI7-9, CI12-1, and CI20-43—all *M. abscessus*). An allelic discrimination plot of T and C alleles at position 28 is shown in Fig. 2. Isolates with T are seen in the far right quadrant along the *x* axis (CI6-3, CI20-19, CI20-25, CI20-30, CI20-31, CI20-35, CI20-36, CI20-37, CI20-38, CI20-42, and CI20-44), and an isolate with C is located in the upper left quadrant on the *y* axis. The negative control with water instead of

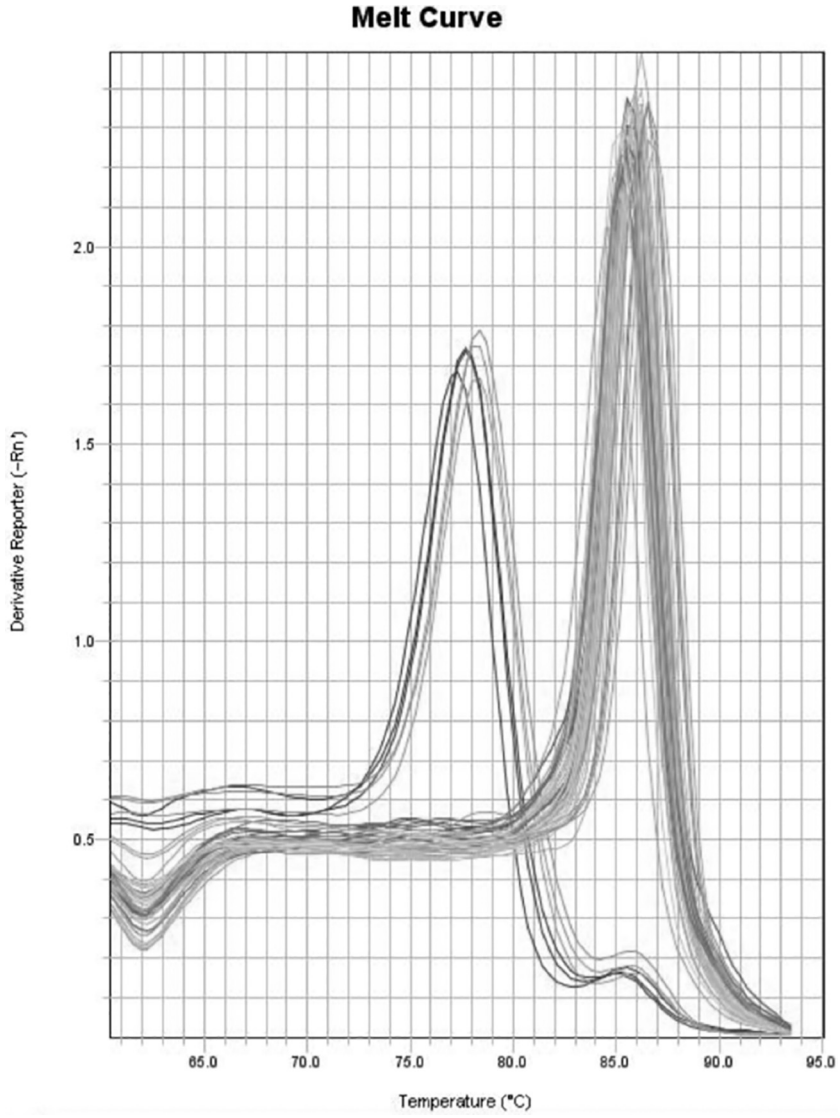

FIG 1 Melting curve peaks for the full-length *erm*(41) gene at 85.8°C (median) for 69/90 (77%) of isolates and truncated *erm*(41) gene at 78.4°C (median) for 21/90 (23%) isolates. The assay was run in triplicates. Isolates with a median T_m of 85.8°C include CI12-1, CI12-8, CI12-9, CI12-13, CI12-14, CI12-15, CI12-16, CI12-19, CI12-20, CI4-1, CI6-7, CI6-5, CI6-4, and CI6-3, *M. abscessus* ATCC 19977^T, and *M. bolletii* 501898^T. The isolates with a median T_m of 78.4°C are CI12-10, CI12-2, and *M. massiliense* 48898^T.

genomic DNA is located in the left lower quadrant of the graph close to zero.

SNP assignment by this assay was confirmed by partial amplification and sequencing of *erm*(41). Susceptibility testing of most of the isolates with a T at position 28 of *erm*(41) yielded initial MIC values between 0.5 and 2.0 μg/ml (susceptible) at 3 to 5 days and MIC values of ≥8 μg/ml (resistant) at 14 days (see Table S1 in the supplemental material). Similar results were obtained with *M. bolletii* isolates (CI7-8, CI20-38, and CCUG 50184^T) and *M. massiliense* full-length *erm*(41) isolates CI20-40 and CI81-82). In contrast, all 5 isolates with C at position 28 of *erm*(41) (CI6-2, CI6-6, CI7-9, CI12-1, and CI20-43) remained susceptible to clarithromycin even at 14 days incubation, with MIC values of 0.25 to 2.0 μg/ml.

All 24 *M. massiliense* isolates in the study had a T at position 28, of which 21 harbored a truncated *erm*(41). Of the 21 strains with truncated *erm*(41), 13/21 (62%) had no acquired resistance and displayed clarithromycin MIC values of 0.12 to 0.5 μg/ml at 3 to 5 days after the initial reading and remained susceptible to clarithromycin at 14 days.

Eight MAG isolates harboring a full *erm*(41) gene with T at position 28 and no SNPs associated with acquired resistance showed clarithromycin MICs above the susceptible range at the initial reading. These isolates were categorized into two groups: (i) isolates with a MIC of 4 (intermediate) to clarithromycin at the initial reading, including 3 *M. abscessus* strains CI20-2, CI20-15, and CI20-24, and (ii) isolates with a MIC of ≥8 (resistant) to clarithromycin at 3 to 5 days. This group includes 5 isolates: *M. abscessus* CI20-32, CI20-33, and CI20-44, *M. bolletii* isolate CI20-27, and *M. massiliense* isolate CI20-39.

Another noteworthy finding is the presence of very-slow-growing MAG strains with a rough-colony morphology requiring

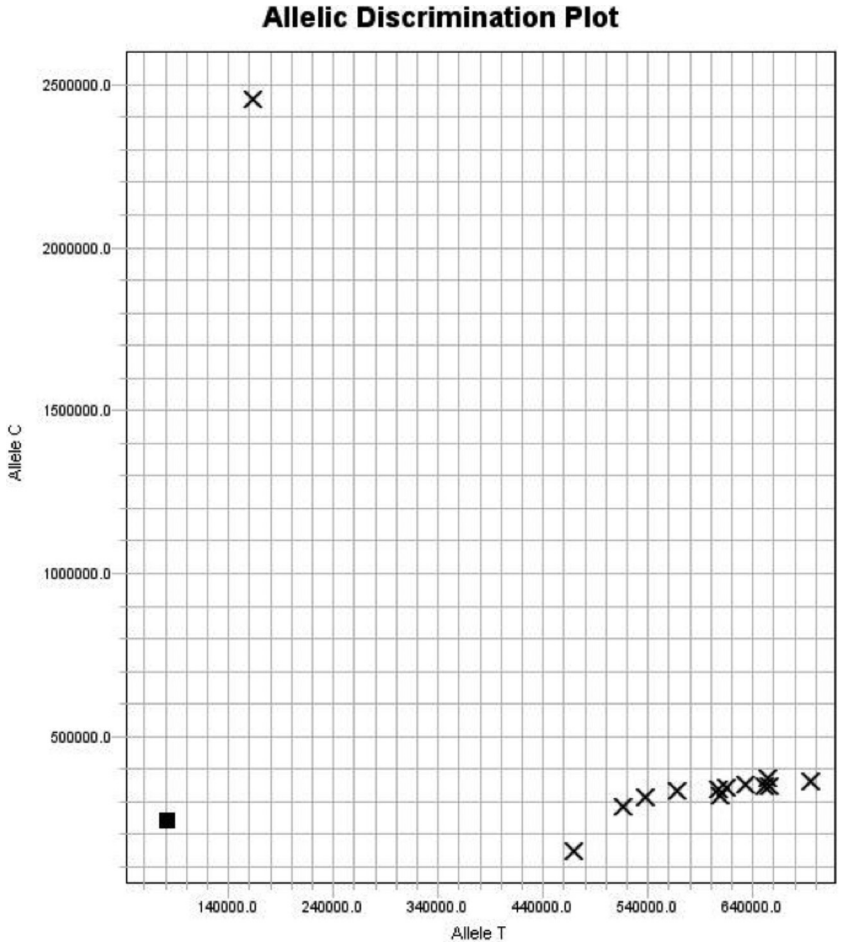

FIG 2 SNP allelic discrimination plot for SNP at position 28 in the *erm*(41) gene. This is a representative figure of allelic discrimination between probes Allele C and Allele T with isolate CI20-43 with C28 shown in the upper quadrant of the *y* axis. The isolates with T28 are located in the far right quadrant along the *x* axis: CI6-3, CI20-19, CI20-25, CI20-30, CI20-31, CI20-35, CI20-36, CI20-37, CI20-38, CI20-42, CI20-44, and *M. abscessus* ATCC 19977^T . The negative control with water instead of genomic DNA is located in the left lower quadrant of the graph close to zero.

6 to 8 days of incubation for the first clarithromycin MIC reading, due to insufficient growth at 3 to 5 days. The slow-growing isolates in this group are *M. abscessus* CI20-2, CI20-6, CI20-8, CI20-10, CI20-11, and CI20-12. Interestingly, isolates CI20-2, CI20-6, CI20-8, and CI20-10 belong to 4 patients from which earlier isolates (7 to 11 years prior) with a standard growth pattern were available (CI20-1, CI20-5, CI20-7, and CI20-9, respectively) and were included in this study. Isolates CI20-11 and CI20-12 are an early strain and later strain from a 5th patient (5 years apart), both showing slow growth. All of these isolates showed inducible resistance to clarithromycin by day 14. Repeat antibiotic susceptibility tests on some of the slow-growing isolates showed faint growth in the positive-control well but no growth in any of the clarithromycin wells within the 5-day initial reading period. This highlights the technical difficulty in antibiotic susceptibility testing of slowly growing isolates.

Real-time assays for 23S rRNA SNPs associated with acquired clarithromycin resistance. Allele determination for common mutations conferring acquired clarithromycin resistance in the 23S rRNA *rrl* gene was performed using probes targeting an A-to-G or -C change at position 2058 and an A-to-G change at position 2059. This assay was done in a pairwise manner in 3 reactions comprising WT_MAB with mutant probe AA_2058G, WT_MAB with mutant probe AA_2058C, and WT_MAB with mutant probe AA_2059G, respectively. We identified 15/90 (17%) isolates harboring resistance mutations in the 23S rRNA gene, including 11/90 (12%) isolates with the A2058G mutation comprising 8 *M. massiliense* isolates with a truncated *erm*(41) gene (CI6-14, CI6-15, CI7-3, CI1-1, CI20-42, CI15-1, CI20-26, and CI20-34) and 3 *M. abscessus* isolates with a full-length *erm*(41) gene (CI5-7, CI20-16, and CI20-18). Figure 3A is a representative allelic discrimination plot of an A or G SNP at position 2058, with isolate CI20-42 containing the 2058G mutation in the upper quadrant of the *y* axis, isolates with A at position 2058 (wild-type allele) in the far right quadrant of the *x* axis (CI20-19, CI20-30, CI20-31, CI20-35, CI20-38, CI20-43, and CI20-44, *M. abscessus* ATCC 19977^T), and the negative control (water) or isolates with a different mutation (2058C or 2059G, strains CI6-3, CI20-25, CI20-36, and CI20-37) on the *x*-*y* coordinate close to zero. Three isolates in this study (CI6-3, CI20-25, and CI20-37) harbored the A2058C mutation in the 23S rRNA gene (Fig. 3B), and one isolate (CI20-36) contained the A2059G mutation (Fig. 3C). Again, strains harboring an SNP different from the wild type or the one

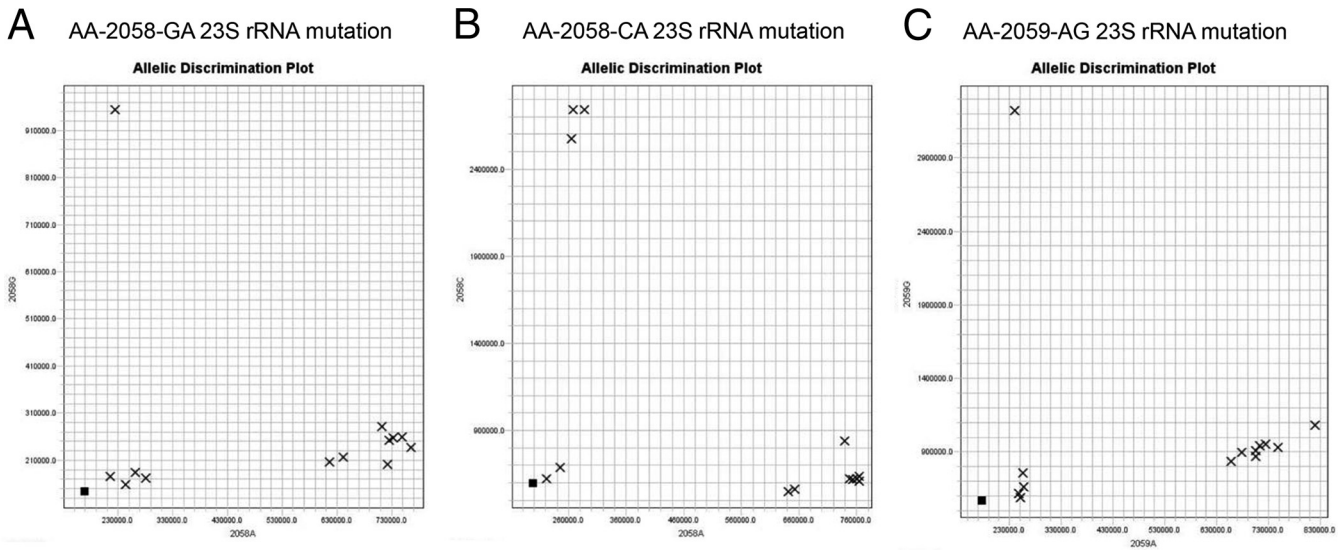

FIG 3 (A) SNP allelic discrimination plot for SNP A2058G at position 2058 in the *rrl* (23S rRNA) gene. This is a representative figure of allelic discrimination between probes 2059A (WT) and 2058G (mutation). The upper quadrant of the *y* axis includes isolate CI20-42 with the A2058G mutation. Strains CI20-19, CI20-30, CI20-31, CI20-35, CI20-38, CI20-43, CI20-44, and *M. abscessus* ATCC 19977^T are A2058A and cluster in the right lower quadrant. Isolates CI6-3, CI20-25, CI20-36, and CI20-37, which have A2058C or A2059G, are in the lower quadrant with the negative-control water. (B) SNP allelic discrimination plot for SNP A2058C at position 2058 in the *rrl* (23S rRNA) gene. This is a representative figure of allelic discrimination between probes 2058A (WT) and 2058C (mutation). Strains CI6-3, 20-25, and 20-37 with the A2058C mutation are clustered in the upper quadrant of the *y* axis. Similar to panel A, isolates with the A2058A mutation are clustered in the right lower quadrant. CI20-42 and CI20-36 have A2058G or A2059G and appear in the left lower quadrant with the negative-control water. (C) SNP allelic discrimination plot for SNP A2059G at position 2059 in the *rrl* (23S rRNA) gene. This is a representative figure of allelic discrimination between probes 2059A (WT) and 2059G (mutation). The upper quadrant of the *y* axis includes isolate CI20-36 with the A2059G mutation. Similar to Fig. 3A, A2059A isolates cluster in the right lower quadrant. Isolates CI6-3, CI20-25, CI20-37, and CI20-42, which have A2058G or A2058C, are in the left lower quadrant with the negative-control water.

assayed, A2058C (Fig. 3B) or A2059G (Fig. 3C), respectively, clustered next to the negative control, close to zero. SNP assignments by these assays were confirmed by partial amplification and sequencing of the 23S rRNA gene.

All 15 isolates, *M. abscessus* or *M. massiliense* with *rrl* mutations conferring clarithromycin resistance, showed phenotypic resistance to clarithromycin at 3 to 5 days of incubation, with MIC values of ≥8 μg/ml (see Table S1 in the supplemental material). There was 100% concordance between SNP assay results, sequencing of the 23S rRNA gene, and phenotypic detection of resistance by the broth microdilution method.

DISCUSSION

Macrolides such as clarithromycin and azithromycin are key antimicrobials in the treatment of pulmonary infections by members of the *M. abscessus* group (MAG) (7, 8, 26). Clarithromycin resistance can be acquired via point mutations in the *rrl* gene encoding the peptidyl transferase domain of the 23S rRNA (27) via A2058G/C and/or A2059G transition or can be induced by the drug via a functional full-length inducible clarithromycin resistance *erm*(41) gene with a T at position 28 (9). In this study, we developed a highly reproducible real-time PCR-based assay to differentiate between full-length and truncated *erm*(41) genes and SNP detection assays for mutations associated with clarithromycin resistance on the 23S rRNA and *erm*(41) genes using 87 clinical isolates of MAG obtained between 2005 and 2015 and 3 reference strains. All isolates were identified to the subspecies level by genomic sequencing and/or a PCR-based assay (23).

Our SYBR green real-time PCR-based assay distinguished between the full-length (77%) and truncated (23%) *erm*(41) genes with a sensitivity of 100% compared to sequencing of the *erm*(41) gene. The assay is highly reproducible and does not require setting reactions with replicates based on the large, 7°C, difference in T_m between the two products and the low standard deviation observed when tested in triplicate. Similarly, assignment of the T or C SNP at position 28 of *erm*(41) by our real-time SNP assay showed 100% concordance to *erm*(41) gene sequencing. In this study, the majority (93% [64/69]) of the isolates with full-length *erm*(41) had a T at position 28, and 7% (5/69) had a C. Thus, the rate of inactive C28 *erm*(41) in our study (7%) is lower than the recently reported rate of 18% of *M. abscessus* with an inactive C28 *erm*(41) sequevar (28).

Current CLSI guideline M24-A2 (25) recommends reading the clarithromycin MIC at 3 days (or 4 or 5 days if there is insufficient growth) and a final reading at 14 days. All isolates in this study harboring full-length *erm*(41) with the T28 nucleotide (and no acquired resistance) showed inducible resistance to clarithromycin within 14 days. While most isolates showed MIC values of ≤2 μg/ml (sensitive) in the first reading at 3 to 5 days, some strains showed a MIC of >2 (i.e., CI20-32, CI20-33, CI20-44, CI20-27, and CI20-39). One hypothesis is the possibility of an earlier induction of the *erm*(41) gene upon incubation with the drug for some strains. Another explanation lies in the technical challenges of this test. In a recent article, Brown-Elliott et al. (28) reported that some C28 isolates with a MIC of 8 upon extended incubation showed a MIC of ≤2 μg/ml upon repeat testing. In the same study, the authors proposed to change clarithromycin susceptibility breakpoints from ≤2 μg/ml to ≤4 μg/ml. Using those newly proposed breakpoints, our 3 isolates with a MIC of 4 at 3 to 5 days would be

considered susceptible at initial reading. In any case, more important than determination of the exact days of incubation at which inducible resistance appears is to make sure inducible resistance is phenotypically detected by day 14, when the final reading of the clarithromycin MIC occurs.

A more challenging issue is the slow-growing MAG strains often seen in multiply treated chronically infected patients for which incubation for 3 to 5 days in Mueller-Hinton broth yields insufficient growth for recording MICs and for which, according to CLSI M24-A2 (25), testing should be repeated. Examples of these isolates are CI20-2, CI20-6, CI20-8, and CI20-10. In the absence of guidelines for phenotypic testing of slowly growing isolates of MAG, sequencing of the *erm*(41) gene as suggested by Brown-Elliott et al. (28) could be particularly useful for predicting clarithromycin susceptibility patterns for these MAG strains.

In the work-up of cultures for identification and susceptibility testing, it is important to note any evidence of mixed populations on the culture plate reflecting a heterogeneous population of MAG in the patient sample. This is best illustrated by *M. abscessus* strains CI20-43 and CI20-44 and *M. abscessus* strains CI20-15 and CI20-16, which were coisolated on the same cultures from sputum samples. CI20-43 is a smooth-colony-morphology strain that harbors a C28-inactive *erm*(41) gene and remains sensitive to clarithromycin upon extended incubation, while the rough morphotype CI20-44 contains a T28-active *erm*(41) gene conferring inducible resistance to the clarithromycin MIC. Isolate CI20-15 is a rough-colony-morphology isolate with an active *erm*(41) gene and no mutational resistance, while CI20-16 is a smooth-colony-morphology isolate with acquired mutational resistance to clarithromycin (A2058G mutation in the 23S rRNA gene).

In this study, we have identified 15 (out of 90 MAG) isolates with mutations on the 23S rRNA gene comprising 7/61 (11%) *M. abscessus* and 8/24 (33%) *M. massiliense*. The most common mutation was A2058G, seen in all *M. massiliense* isolates and 3 *M. abscessus* isolates with acquired resistance, followed by A2058C (three *M. abscessus* isolates) and A2059G (one *M. abscessus* isolate). The high frequency of mutational resistance among *M. massiliense* isolates should not be surprising, as this is the only known mechanism of resistance available for organisms harboring a truncated *erm*(41) gene. This study included early (CI15-4 and CI20-41) and late (CI15-1 and CI20-42) *M. massiliense* isolates (4 to 7 years apart) from the 2 cystic fibrosis (CF) patients. Early isolates were susceptible to clarithromycin, with MIC values of 0.25 μg/ml after 14 days of incubation, as expected for strains harboring truncated *erm*(41) genes; however, late isolates from each patient showed MIC values of >16 μg/ml due to the acquisition of the A2058G mutation on the 23S rRNA gene. Acquisition of mutational resistance to clarithromycin on patients treated with macrolides has been documented in serial isolates of *M. abscessus* (29) and *M. massiliense* (2). Our study confirms this finding with these 2 CF patients who received prolonged treatment with azithromycin and/or clarithromycin.

There are few studies describing molecular assays for clarithromycin resistance that are amenable to implementation in a clinical laboratory. In a recent study, Lee et al. (30) described an amplification refractory mutation system and PCR (ARMS-PCR) and a real-time PCR melting peak analysis method for detection of *erm*(41) T28 and C28 mutations in *M. abscessus* clinical isolates. The ARMS-PCR method (cheaper but more time-consuming than real-time PCR) detected all 17 C28 isolates and all 140 T28 isolates, in agreement with sequence analysis, which was used as a reference standard. Their real-time PCR melting peak method also detected all 17 C28 isolates; however, the authors only reported the results from 50 of the 140 T28 isolates. Curry et al. developed a real-time assay consisting of two duplex PCRs for identification of both *erm*(41) and *rrl* clarithromycin mutations using SYBR green chemistry and melt curve analysis (C. Curry, R. Luo, and N. Banaei, poster 118, presented at the 114th General Meeting of the American Society for Microbiology, Boston, MA, 17 to 20 May 2014). Phenotypic testing (either Etest or broth MIC) was used as the reference standard to define clarithromycin resistance. They reported a sensitivity of 88% (23/26 isolates) for detection of resistance due to a functional *erm*(41) gene and 100% sensitivity for detection of isolates with a nonfunctional or truncated *erm*(41) gene in 37/37 isolates. The assay was 100% sensitive in detecting *rrl* mutations in 3/3 isolates.

In our study, the SYBR green and position 28 T/C SNP real-time PCR-based assays were 100% sensitive in differentiating between functional and nonfunctional *erm*(41) compared to *erm*(41) gene sequencing and correctly predicted the presence and absence of inducible clarithromycin resistance by phenotypic testing. These assays were particularly helpful for predicting resistance in slowly growing strains of MAG. Our real-time SNP assay for acquired mutational clarithromycin resistance in the 23S rRNA gene was 100% sensitive compared to 23S rRNA gene sequencing and also predicted the phenotypic resistance observed in all 15 strains. Of note, both the RAPMYCO and PML Microbiologicals susceptibility testing systems test a limited range of clarithromycin MIC values (up to 8 or 16 μg/ml) far below the MICs (>128 μg/ml) reported in MAG with 23S rRNA mutations (10, 11, 27).

Based on this study, our proposed testing algorithm includes an initial assessment of the full or truncated *erm*(41) gene using the SYBR green assay followed by SNP detection in isolates that have an intact *erm*(41) gene for the presence of a C or T base at position 28. This is followed by detection of SNPs of acquired clarithromycin resistance in the 23S rRNA gene. The most common mutation in this cohort was the A2058G substitution on the 23S rRNA gene and should be tested first. Moreover, the A2058G SNP assay by itself will also distinguish non-wild-type isolates with other mutations at position 2058 and/or 2059, as they will be clustering with the negative control in SNP allelic discrimination plots. In conclusion, our real-time melting curve analysis and SNP detection assays for inducible and acquired clarithromycin resistance are easy to implement in the laboratory and provide clinically useful results within ~3 h, a significant improvement in turnaround time compared to the 14-day incubation required by phenotypic detection of resistance by broth microdilution.

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