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# A Molecular-Beacon-Based Multiplex Real-Time PCR Assay To Distinguish *Mycobacterium abscessus* Subspecies and Determine Macrolide Susceptibility

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**ABSTRACT** *Mycobacterium abscessus* is a rapidly growing nontuberculous mycobacterial species that comprises three subspecies: *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *massiliense*, and *M. abscessus* subsp. *bolletii*. These predominantly environmental microorganisms have emerged as life-threatening chronic pulmonary pathogens in both immunocompetent and immunocompromised patients, and their acquisition of macrolide resistance due to the *erm*(41) gene and mutations in the 23S *rrl* gene has dramatically impacted patient outcome. However, standard microbiology laboratories typically have limited diagnostic tools to distinguish *M. abscessus* subspecies, and the testing for macrolide resistance is often not done. Here, we describe the development of a real-time multiplex assay using molecular beacons to establish a robust, rapid, and highly accurate method to both distinguish *M. abscessus* subspecies and to determine which strains are susceptible to macrolides. We report a bioinformatic approach to identify robust subspecies sequence targets, the design and optimization of six molecular beacons to identify all genotypes, and the development and application of a 2-tube 3-color multiplex assay that can provide clinically significant treatment information in less than 3 h.

**KEYWORDS** *Mycobacterium abscessus*, macrolide resistance, molecular beacon

**M**ycobacterium abscessus is a rapidly growing nontuberculous mycobacterial species that comprises three subspecies: *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *massiliense*, and *M. abscessus* subsp. *bolletii* (1–3). These predominantly environmental microorganisms are opportunistic pathogens that have been associated with skin and soft tissue infections resulting from contaminated equipment and hospital water supplies; but, more recently, they have emerged as life-threatening chronic pulmonary pathogens in both immunocompetent and immunocompromised patients (1, 3, 4). Among persons with cystic fibrosis (CF), *M. abscessus* has become a significant cause of chronic lung infections, and treatment is confounded by their intrinsic resistance to antibiotics, including antituberculosis agents. The inability to successfully treat *M. abscessus* infections with macrolides, clarithromycin, or azithromycin, as a result of the *erm*(41) gene and mutations in the 23S *rrl* gene, has dramatically impacted patient outcomes, as cure rates drop to 25 to 40% against clarithromycin-resistant *M. abscessus* (5–8).

As a result of the increased number of high-risk immunocompromised patient populations, including bone marrow transplant patients, those receiving solid organs, and

those with underlying lung disease, such as cystic fibrosis patients, there is an increase in *M. abscessus* chronic infections and a dire clinical need to improve patient treatment. Macrolide antibiotics remain one of the most effective classes of antibiotics against susceptible *M. abscessus* strains; however, standard microbiology laboratories typically have limited diagnostic tools to distinguish subspecies of *M. abscessus*, and the testing for macrolide resistance is often not done (9).

There is significant literature that links subspecies of *M. abscessus* and genotypic resistance to macrolides, including the important observation that nearly all *M. abscessus* subsp. *massiliense* strains have two deletions (one of 2 bp and the other of 274 bp, bases 64 to 65 and 159 to 432, respectively) in the *erm*(41) gene that lead to susceptibility to clarithromycin (10). Similarly, there are clinical isolates of *M. abscessus* subsp. *abscessus* with a single base change from T to C in codon 28 in the *erm*(41) gene that correlates with clarithromycin susceptibility (11).

The diagnostic challenge of developing a rapid and simple assay to both distinguish *M. abscessus* subspecies and genotype macrolide resistance revolves around the requirement to sequence the various subspecies and drug susceptibility gene targets. A number of examples, including genetic changes in *rpoB*, *hsp65*, and *secA1*, have been used to distinguish *M. abscessus* subspecies with relatively high accuracy (12–14), but, in each case, the assay requires PCR amplification followed by DNA sequencing. The same issue applies to defining the resistance mutations in the 23S *rrl* gene and the *erm* (41) genetic alterations that are associated with macrolide susceptibility in *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *massiliense*. PCR approaches that require gel electrophoresis have been developed (15, 16); but, these research techniques are labor intensive, are subjective in interpreting the results, and are not amenable to the routine clinical microbiology laboratory.

Here, we describe the development of a real-time multiplex assay using molecular beacons to establish a robust, rapid, and highly accurate method to both distinguish *M. abscessus* subspecies and determine which strains are susceptible to macrolides. We report a bioinformatic approach to identify robust subspecies sequence targets, the design and optimization of six molecular beacons to identify all genotypes, and the development and application of a 2-tube 3-color multiplex assay that can provide clinically significant treatment information in less than 3 h.

## MATERIALS AND METHODS

**Clinical isolates and clarithromycin testing.** A collection of 115 *M. abscessus* clinical isolates, including 100 recovered from patients with cystic fibrosis and catalogued and archived at National Jewish Health, was utilized for this study. The strains included 69 *M. abscessus* subsp. *abscessus* strains, 38 *M. abscessus* subsp. *massiliense* strains, and 10 *M. abscessus* subsp. *bolletii* strains. All strains were analyzed by whole-genome sequencing (BioProject accession no. PRJNA319839 and PRJNA549322). A collection of 22 strains from 20 different nontuberculous *Mycobacterium* species, including *M. aurum*, *M. avium*, *M. gallinarum*, *M. gastri*, *M. gordonae*, *M. haemophilum*, *M. intracellulare*, *M. marinum*, *M. scrofulaceum*, *M. simiae*, *M. szulgai*, *M. terrae*, *M. ulcerans*, *M. chelonae*, *M. fortuitum*, *M. kansasii*, *M. phlei*, *M. simiae*, plus *M. tuberculosis* and *M. microti*, archived at the Center for Discovery and Innovation, was used to evaluate the specificity of the assay.

A panel of six test strains, which includes all three subspecies and genotypic diversity associated with clarithromycin resistance, was analyzed for response to clarithromycin. Susceptibility and resistance were assessed according to the CLSI recommendations (17, 18). Constitutive clarithromycin resistance was defined by a MIC of ≥8 μg/ml at day 5. Inducible resistance was defined by an increase in clarithromycin MIC from ≤2 μg/ml at day 5 to ≥8 μg/ml at day 14 (17, 18).

**Design of the molecular probes and PCR primers.** For the identification and development of subspecies-specific sequence targets that are amenable for PCR and molecular beacon probe detection, we interrogated 33 completely sequenced *M. abscessus* genomes from the NCBI (www.ncbi.nlm.nih.gov/assembly, as of January 2020), including 12 *M. abscessus* subsp. *abscessus*, 19 *M. abscessus* subsp. *massiliense*, and 2 *M. abscessus* subsp. *bolletii* genomes. The genomes were aligned by Parsnp from the Harvest suite, and subspecies-specific single nucleotide polymorphisms (SNPs) were extracted. A core genome analysis was conducted using Roary (19) to identify conserved genes across different *M. abscessus* subspecies. Core SNP numbers within a 20-nucleotide (nt) sliding window across *M. abscessus* core genes were calculated using Bedtools (20) to identify putative regions for molecular beacon probe design.

We next used blastn to examine the *in silico* sensitivity and specificity of putative molecular beacon probes and their neighboring sequences against 1,930 *Mycobacterium abscessus* complex (MABC) draft genomes in the NCBI assembly database (www.ncbi.nlm.nih.gov/assembly) and among our in-house

**TABLE 1** Molecular beacon probe and primer sequences

| Oligonucleotide[a] | Sequence (5′–3′)[b] |
| --- | --- |
| *MAB2248* for | GTGACCGGTCTCGATCAGTT |
| *MAB2248* rev | CATTGTCTGGCGTCAATGGC |
| *MAB2248* bol MB | CFR610-<u>CCGTGC</u> CTCGGGAACCGGATGACT <u>GCACGG</u>-BHQ-2 |
| *MAB2830* for | CCTCATCGAGGACGGTCAGA |
| *MAB2830* rev | CACGAATCCGGGCAGCAATA |
| *MAB2830* abs MB | FAM-<u>CCGTCG</u> CGAGGCCGGCATCGGCGCA <u>CGACGG</u>-BHQ-1 |
| *MAB2830* mas MB | Q670-<u>CCGTCG</u> GAAGCTGGTATCGGTCCAGG <u>CGACGG</u>-BHQ-2 |
| 23S *rrl* for | CGGCGAAATTGCACTACGAG |
| 23S *rrl* rev | CCGAACCAAACGCCAATACC |
| 23S *rrl* WT MB | FAM-<u>CGCTGC</u> CAGGACGAAAAGACCCC <u>GCAGCG</u>-BHQ-1 |
| Δ*erm*(41) for | TTCAGGGGAGTTCGTTGTGA |
| Δ*erm*(41) rev | AAAGTGCTTCGCGGCAATG |
| Δ*erm*(41) MB | CFR610-<u>CACGG</u> TGGACGCTCCGGGC <u>CCGTG</u>-BHQ-2 |
| *erm*(41) T28C for | GCATGCCCCGATATCTTTGG |
| *erm*(41) T28C rev | ATCCACAACGAACTCCCCTG |
| *erm*(41) T28C MB | Q670-<u>CGCTG</u> ACGCCAGCGGGGCTG <u>CAGCG</u>-BHQ-2 |

[a]Forward (for) and reverse (rev) primers and molecular beacon probe (MB) sequences for each of the target genes. abs, *M. abscessus* subsp. *abscessus*; bol, *M. abscessus* subsp. *bolletii*; mas, *M. abscessus* subsp. *massiliense*; WT, wild-type.

[b]BHQ-1, Blackhole Quencher 1; BHQ-2, Blackhole Quencher 2; CFR610, Cal Fluor Red 610; FAM, fluorescein; Q670, Quasar 670; underlined nucleotides represent arm sequences of the molecular beacon probe.

sequenced genomes (21). In addition, the specificity of the molecular beacons and primers was also examined against 476 representative or completely sequenced genomes of nontuberculous mycobacterial species in the NCBI Refseq database (www.ncbi.nlm.nih.gov/refseq/), including genomes from 119 unique mycobacterial species or subspecies.

Acquired macrolide resistance in *M. abscessus* is largely associated with the point mutations A2058 and A2059 in the *rrl* gene encoding the peptidyltransferase domain of the 23S rRNA (22, 23), and inducible macrolide resistance is linked to the ribosomal methylase gene *erm*(41) (10). The *erm*(41) gene with a T-to-C polymorphism at nucleotide 28 is associated with macrolide susceptibility. Currently, most clinical isolates of *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *bolletii* express inducible macrolide resistance; but, in contrast, *M. abscessus* subsp. *massiliense* is nearly always associated with clarithromycin susceptibility due to two deletions (bases 64 to 65 and 159 to 432) within *erm*(41). This association correlates with a favorable clinical outcome with macrolide treatment (24). In this study, we designed and validated three sets of primer pairs and molecular beacon probes to detect the *rrl* macrolide susceptible (wild-type) gene, the *erm*(41) deletion, and the *erm*(41) T28C mutation in order to specifically and rapidly genotype macrolide resistance in different *M. abscessus* subspecies. A region in gene *MAB2248* and two regions in *MAB2830* were used to design three primer pairs and molecular beacon probes to specifically identify *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *bolletii*, and *M. abscessus* subsp. *massiliense*, respectively (see below).

PCR primers were designed utilizing Primer-BLAST (25). Molecular beacon probes were designed to identify genes or species-specific single nucleotide polymorphisms using guidelines described by Vet and Marras (26). PCR primers and molecular beacon probes were evaluated with Integrated DNA Technologies (IDT)'s OligoAnlyzer tool software (https://www.idtdna.com/calc/analyzer) for compatibility in multiplex PCR assays. PCR primers were obtained from Integrated DNA Technologies (Coraville, IA), and molecular beacon probes were obtained from LGC Biosearch Technologies (Novato, CA). The sequences of the PCR primers and molecular beacon probes are listed in Table 1.

**Denaturation profile analysis.** Molecular beacon probes were characterized in a denaturation profile analysis to determine their specificity. For each molecular beacon probe, dependent on the number of DNA target sequences they have to discriminate (wild-type gene sequences versus mutant gene sequences or multiple species to distinguish), three or four 20-$\mu$l reaction volumes were prepared containing 1× Platinum Hot Start PCR master mix (Thermo Fisher Scientific, Waltham, MA) and 400 nM molecular beacon probe. To one tube, 1,000 nM final concentration of the matched reverse complement target oligonucleotide was added, and 1,000 nM final concentration of mismatched reverse complement targets was added to other tubes (corresponding to the DNA sequences to be discriminated against). As a control, the target oligonucleotide was left out of one tube. The denaturation profile analysis was performed in 200-$\mu$l white polypropylene PCR tubes (USA Scientific, Ocala, FL) in a CFX96 Touch real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA). The hybridization mixtures were incubated for 2 min at 95°C to denature the oligonucleotides and molecular beacon probes, followed by a decrease in temperature from 90°C to 30°C in −1°C steps and holding each step for 10 s while monitoring the fluorescence intensity of the molecular beacon probes.

**Assay design.** A two-tube real-time PCR assay was developed to be able to both distinguish the three *M. abscessus* subspecies and identify genetic changes in the *erm*(41) and 23S *rrl* genes that predict macrolide resistance. In PCR Assay I, molecular beacons were targeted to the wild-type 23S *rrl* sequence, the subspecies *M. bolletii*-specific sequence, and the *erm*(41) T28C mutation and labeled with

fluorophores: (FAM) fluorescein, (CFR610) Cal Fluor Red 610, and (Q670) Quasar 670, respectively. In Assay II, molecular beacons were designed for *M. abscessus* subspecies *abscessus*, the *erm*(41) deletion specific for the *M. massiliense* subspecies, and the sequence-specific target that distinguishes the *M. massiliense* subspecies. The three molecular beacons were labeled with fluorophores: (FAM) fluorescein, (CFR610) Cal Fluor Red 610, and (Q670) Quasar 670, respectively. The molecular beacon probes and the PCR primers for the three subspecies are listed in Table 1 as well as the primers and molecular beacon probes to identify the T28C mutation in *erm*(41), the unique deletion of *erm*(41) found in *M. abscessus* subsp. *massiliense*, and the wild-type target sequence in the 23S *rrl* gene.

**Real-time polymerase chain reactions (RT-PCR).** For each DNA sample, two RT-PCR assays were performed. One assay (Assay I) contained PCR primers and molecular beacon probes to determine the presence of *M. abscessus* subsp. *bolletii*, the wild-type gene for 23S *rrl* 2058-2059, and the *erm*(41) T28C mutation. The second assay (Assay II) contained PCR primers and molecular beacon probes to determine the presence of either *M. abscessus* subsp. *abscessus* or *M. abscessus* subsp. *massiliense* and the *erm*(41) deletion. Each RT-PCR assay was carried out in 20-$\mu$l volumes that contained 1× Platinum Hot Start PCR master mix (Thermo Fisher Scientific, Waltham, MA). For Assay I, 250 nM *MAB2248* forward (for), 250 nM *MAB2248* reverse (rev), 500 nM *MAB2248 M. abscessus* subsp. *bolletii* (bol) molecular beacon probe (MB), 250 nM 23S *rrl* for, 250 nM 23S *rrl* rev, 500 nM 23S *rrl* MB, 100 nM *erm*(41) T28C for, 1,000 nM *erm*(41) T28C rev, and 500 nM *erm*(41) T28C MB were added. For Assay II, 250 nM *MAB2830* for, 250 nM *MAB2830* rev, 500 nM *MAB2830 M. abscessus* subsp. *abscessus* (abs) MB, 250 nM *MAB2830 M. abscessus* subsp. *massiliense* (mas) MB, 250 nM Δ*erm*(41) for, 250 nM Δ*erm*(41) rev, and 500 nM Δ*erm*(41) MB was added. Each assay was initiated with a 5-$\mu$l DNA template. The PCR assays were performed in 200-$\mu$l white polypropylene PCR tubes (USA Scientific, Ocala, FL) in a CFX96 Touch real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA). The reaction mixtures were incubated for 2 min at 95°C to activate the DNA polymerase, followed by 40 thermal cycles that consisted of denaturation at 95°C for 15 s and annealing and chain elongation at 60°C for 60 s. Molecular beacon fluorescence intensity was monitored during the 60°C annealing and chain elongation stage of each thermal cycle.

## RESULTS

**Target selection and *in silico* sensitivity and specificity.** Genomic analysis of 33 whole-genome sequenced (WGS) *M. abscessus* genomes identified candidate genetic regions that were amenable to distinguish *M. abscessus* subspecies isolates using molecular beacon probes. Two targets, *MAB2248* and *MAB2830*, were selected based on their conservation and specificity to differentiate the three subspecies. *MAB2248* encodes a putative peptide synthetase MbtE protein, and *MAB2830* is the dihydroorotase PyrC gene. An 18-bp sequence within the *MAB2248* gene, specific to *M. abscessus* subsp. *bolletii*, was targeted for the molecular beacon probe to distinguish this subspecies. The *MAB2248* beacon differs from the sequences of *M. abscessus* subsp. *abscessus* or *M. abscessus* subsp. *massiliense* by three SNPs (3/18, 16.7% variation), providing the specificity to detect *M. abscessus* subsp. *bolletii* in an allele-specific RT-PCR assay (Table 1). *MAB2830* homologous sequences in *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *massiliense* were used to design the two subspecies-specific molecular beacons, and the target regions can be amplified by the same PCR primer pairs in the two subspecies. An 18-bp *MAB2830* sequence specific for *M. abscessus* subsp. *abscessus* was used as a molecular beacon probe, and this sequence is distinguishable from *M. abscessus* subsp. *massiliense* and *M. abscessus* subsp. *bolletii* by 4 to 5 SNPs (22.2 to 27.8% variations), respectively. Similarly, a 19-bp *MAB2830* homolog sequence specific to *M. abscessus* subsp. *massiliense* was targeted for the design of the molecular beacon probe and differed from the *MAB2830* sequences in *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *bolletii* by 5 and 3 SNPs (16.7 to 27.8% variations), respectively (Table 1).

We then examined the *in silico* sensitivity and specificity of the three designed molecular beacon probes and primers against 1,930 *M. abscessus* genomes from NCBI and from our own collection. The 1,930 genomes were assigned to three subspecies, and they include 1,192 *M. abscessus* subsp. *abscessus*, 609 *M. abscessus* subsp. *massiliense*, and 129 *M. abscessus* subsp. *bolletii* genomes. *MAB2248* and *MAB2830* sequences were found in all 1,930 MABC genomes. The *in silico* sensitivities were 100%, 99.41%, and 99.67% for *M. abscessus* subsp. *bolletii*-, *M. abscessus* subsp. *massiliense*-, and *M. abscessus* subsp. *abscessus*-specific molecular beacons, and the specificities were 99.72%, 100%, and 99.92% for the three subspecies, respectively (Table 2).

With the intent of developing this assay to ultimately diagnose primary patient specimens, we checked the *in silico* specificity of the three subspecies molecular

**TABLE 2** *In silico* sensitivity and specificity of PCR probes used to identify subspecies[a]

| MAB2248 bol MB | WGS | | MAB2830 abs MB | WGS | | MAB2830 mas MB | WGS | |
|---|---|---|---|---|---|---|---|---|
| | + | - | | + | - | | + | - |
| PCR + | 129 | 5 | PCR + | 1185 | 0 | PCR + | 607 | 1 |
| PCR - | 0 | 1796 | PCR - | 7 | 738 | PCR - | 2 | 1320 |
| Sensitivity | 100 | | Sensitivity | 99.41 | | Sensitivity | 99.67 | |
| Specificity | 99.72 | | Specificity | 100 | | Specificity | 99.92 | |
| PPV | 96.27 | | PPV | 100 | | PPV | 99.84 | |
| NPV | 100 | | NPV | 99.06 | | NPV | 99.85 | |

[a]abs, *M. abscessus* subsp. *abscessus*; bol, *M. abscessus* subsp. *bolletii*; mas, *M. abscessus* subsp. *massiliense*; MB, molecular beacon probe; NPV, negative predictive value; PPV, positive predictive value; WGS, whole-genome sequencing.

beacon probes and two primer pairs against 476 representative or completely sequenced *Mycobacterium* genomes deposited in the NCBI Refseq database. The results showed that the primers and probes are highly specific to *M. abscessus* strains, showing no or low sequence identities to other *Mycobacterium* species. Among the most clinically relevant nontuberculous *Mycobacterium* species listed in the Materials and Methods, the *MAB2248* primer and probe sequences were absent in all but the five species that belong to the *M. chelonae* complex (*M. chelonae*, *M. saopaulense*, *M. immunogenum*, *M. salmoniphilum*, and *M. franklinii*), and, when present, the molecular beacon target region only showed 61 to 82% (4 to 7 mismatches) similarities. Similarly, the *MAB2830* target was absent in most species and revealed 70 to 80% (4 to 6 mismatches) nucleotide identity against only genomes from *M. chelonae*, *M. saopaulense*, *M. immunogenum*, *M. salmoniphilum*, and *M. franklinii*. In addition, the two targets were absent in other CF- and lung-associated pathogens, including *Staphylococcus aureus*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, *Burkholderia cepacia* complex, and *Stenotrophomonas maltophilia*. The *in silico* specificity examination of the primers and probes for the *erm*(41) T28C mutation and the deletion were specific for the *M. abscessus* genomes, and there are no sequence matches against other queried genomes. The 23S *rrl* primers and molecular beacon were designed to target the wild-type alleles (A2058 and A2059), and this region showed 100% identity to other species of *M. chelonae*, *M. saopaulense*, *M. immunogenum*, *M. salmoniphilum*, and *M. franklinii*.

**Denaturation profile analysis.** Each molecular beacon design used in this assay was characterized in a denaturation profile analysis to determine that they only elicit a fluorescent signal if their intended perfect-matched target is present at the annealing temperature of the PCR and remain nonfluorescent if a nonintended mismatched target is present at the annealing temperature of the PCR. For each of the three molecular beacon probes that distinguish the *M. abscessus* subspecies present in a sample, three oligonucleotide targets were designed (Table 3): one oligonucleotide is identical to the target region of the probe to *M. abscessus* subsp. *abscessus*, one to the target region of the *M. abscessus* subsp. *massiliense* probe, and one to the target region of the probe for *M. abscessus* subsp. *bolletii*. Each analysis also included a control in which no oligonucleotide target was added, and this control shows at which temperature the molecular beacon probe stem opens, resulting in an increase in the background fluorescence of the probe.

The top row in Fig. 1 shows the denaturation profiles of the three subspecies molecular beacon probes. The plots show that at the annealing temperature of the PCR assay (60°C, represented by a dashed line), a fluorescent signal only arises from the subspecies-specific molecular beacon probe and its intended perfect-matched target oligonucleotide, and that, at this temperature, the probes do not elicit a fluorescent signal from the other two mismatched targets. Furthermore, the stem of the molecular beacon probes is closed at this temperature, and there is no increased background

**TABLE 3** Denaturation profile analysis target oligonucleotides

| Oligonucleotide[a] | Sequence (5'–3') |
|---|---|
| *MAB2248* abs | CCGATCGGAGTCAACCGGTTCGCGGGCATCGAAC |
| *MAB2248* bol | CCGATCGGAGTCATCCGGTTCCCGAGCATCGAAC |
| *MAB2248* mas | CCGATCGGAGTCAACCGGTTCGCGGGCATCGAAC |
| *MAB2830* abs | TCGATGCCTGCGCCGATGCCGGCCTCGATGCGGG |
| *MAB2830* bol | TCGATGCCTGGGCCGATACCGGCCTCGATGCGGG |
| *MAB2830* mas | TCGATGCCTGGACCGATACCGGCTTCGATGCGGG |
| 23S *rrl* 2058-2059 wt | AAGGTCCCGGGGTCTTTTCGTCCTGCCGCGCGT |
| 23S *rrl* 2058-2059 AG mut | AAGGTCCCGGGGTCTCTTCGTCCTGCCGCGCGT |
| 23S *rrl* 2058-2059 GA mut | AAGGTCCCGGGGTCTTCTCGTCCTGCCGCGCGT |
| *erm*(41) T28C wt | GCGGATACCAGCCCCACTGGCGTCGCGACCG |
| *erm*(41) T28C mt | GCGGATACCAGCCCCGCTGGCGTCGCGACCG |
| Δ*erm*(41) wt | TGATTCCGGCCCGTAGCGTCCAATGG |
| Δ*erm*(41) mut | TGATTCCGGCCCGGAGCGTCCAGCGG |

[a]abs, *M. abscessus* subsp. *abscessus*; bol, *M. abscessus* subsp. *bolletii*; mas, *M. abscessus* subsp. *massiliense*; mut, mutant; wt, wild-type.

fluorescence from the probes. Similar denaturation profile analyses were carried out for the three molecular beacon probes designed to identify the wild-type *rrl* gene, the *erm*(41) T28C mutation, and the *erm*(41) deletion. For these probes, oligonucleotide targets were designed as a perfect match for the 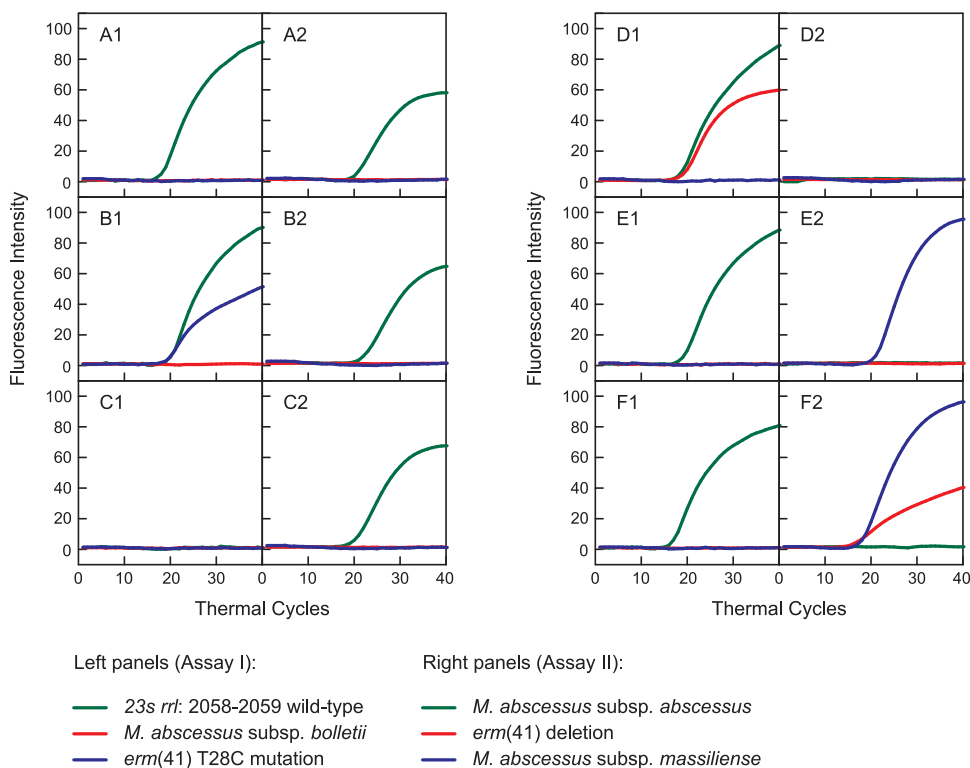intended target and with mismatches compared to nonintended targets. The bottom row of Fig. 1 shows the denaturation profiles of these three molecular beacon probes. For example, the left plot shows the denaturation profile for the wild-type *rrl* gene molecular beacon probe in the presence of its intended wild-type target (green curve), in the presence of the two possible mismatched targets, a GA mutant (red curve) or AG mutant (blue curve), and in the absence of any target (black curve) as a control to determine the stability of the stem hybrid of the probe. The plot shows that at the 60°C annealing temperature, a fluores-
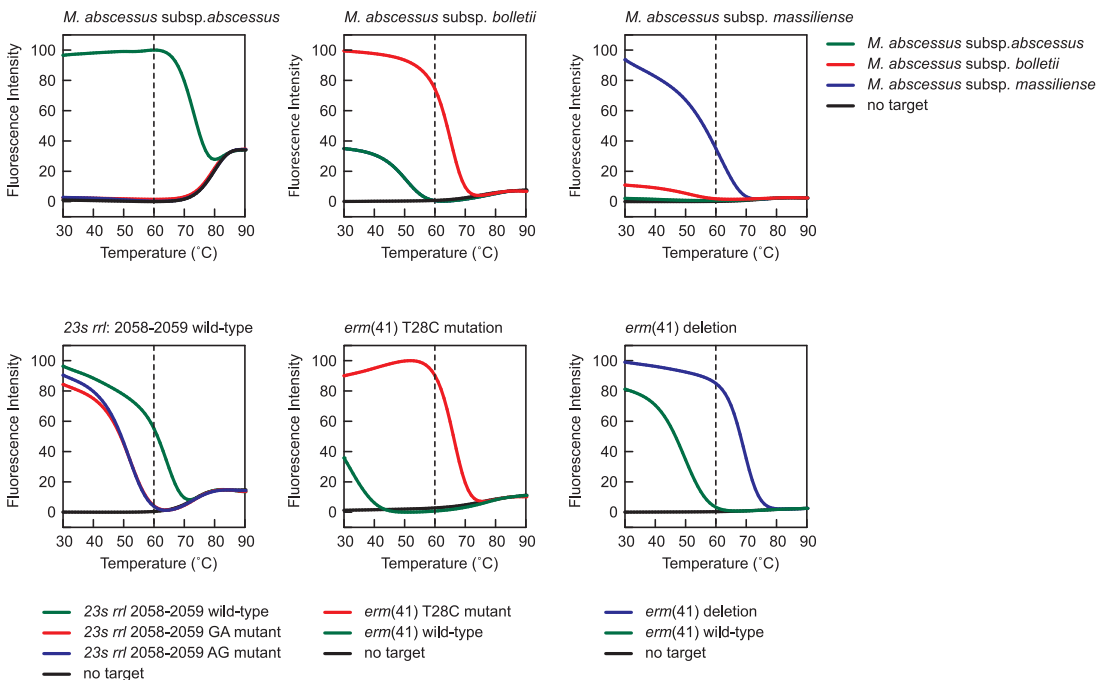


**FIG 1** Denaturation profile analysis of the six molecular beacon probes utilized in this assay. For each molecular beacon, a denaturation profile analysis was carried out in the presence of its intended perfectly matched target sequence, its mismatched target sequence, and a no target control. The dashed vertical line in each plot shows that at the 60°C annealing temperature step in the PCR assay, where the fluorescence intensity of the molecular beacon probes is recorded, a fluorescent signal is only generated from the molecular beacon probe hybridized to its intended target, and no fluorescent signal is generated in the presence of a mismatched target or when no target is present.

**Left panels (Assay I):**

— *23s rrl*: 2058–2059 wild-type
— *M. abscessus* subsp. *bolletii*
— *erm*(41) T28C mutation

**Right panels (Assay II):**

— *M. abscessus* subsp. *abscessus*
— *erm*(41) deletion
— *M. abscessus* subsp. *massiliense*

**FIG 2** Two-tube real-time PCR assay. Assay I, left plots (A1 to F1), determines if the sample contains the wild-type sequences for the 23S *rrl* gene at position 2058-2059 (green curves), if the sample contains *M. abscessus* subsp. *bolletii* (red curves), and if the sample contains the *erm*(41) T28C mutation (blue curves). Assay II, right plots (A2 to F2), determines if the sample contains *M. abscessus* subsp. *abscessus* (green curves) or *M. abscessus* subsp. *massiliense* (blue curves) and if the sample contains the *erm*(41) deletion (red curves). The following six different samples are shown: (A) *M. abscessus* subsp. *abscessus*, with a wild-type 23S *rrl* gene at position 2058-2059, no *erm*(41) T28C mutation, and no *erm*(41) deletion; (B) *M. abscessus* subsp. *abscessus*, with a wild-type 23S *rrl* gene at position 2058-2059, but with an *erm*(41) T28C mutation and no *erm*(41) deletion; (C) *M. abscessus* subsp. *abscessus*, with a mutation in the 23S *rrl* gene at position 2058-2059, no *erm*(41) T28C mutation, and no *erm*(41) deletion; (D) *M. abscessus* subsp. *bolletii* with a wild-type 23S *rrl* gene at position 2058-2059, no *erm*(41) T28C mutation, and no *erm*(41) deletion; (E) *M. abscessus* subsp. *massiliense* with a wild-type 23S *rrl* gene at position 2058-2059, no *erm*(41) T28C mutation, and no *erm*(41) deletion; (F) *M. abscessus* subsp. *massiliense* with a wild-type 23S *rrl* gene at position 2058-2059, no *erm*(41) T28C mutation, but with a deletion in the *erm*(41) gene.

cent signal from the probe only arises when its intended perfect-matched, wild-type target is present. At this temperature, the molecular beacon probe does not show any fluorescence in the presence of the two nonintended mutant targets, indicating the perfect discrimination of this molecular beacon probe between the wild-type and mutant targets. This is also the case for the other molecular beacon probes in this assay.

**Assay analysis.** Analysis of the real-time PCR results combines the information for both multiplex assays to predict the *M. abscessus* subspecies and to determine whether the strain is macrolide resistant or susceptible. The assay is designed with the understanding that all isolates have the *erm*(41) gene, and its wild-type structure correlates with inducible macrolide resistance. Therefore, those strains that do not have the T28C mutation or the 274-bp deletion are predictively resistant. Similarly, the wild-type 23S *rrl* gene is associated with macrolide susceptibility, and rare strains have a mutation that correlate with constitutive macrolide resistance. In the current assay, the absence of the *rrl*-positive molecular beacon predicts resistance.

The multiplex real-time PCR results of Assays I and II are shown in Fig. 2, and the genotypes of 6 representative clinical strains are described in Table 4. For each strain, the real-time PCR profiles of Assay I are shown in the left panel and the profiles of Assay II are shown in the right panel. The results for strain A reveal the presence of the wild-

**TABLE 4** Interpretation of real-time PCR results for six MABC isolates

| Strains | Real-time PCR Assay I[a] | | | Real-time PCR Assay II[a] | | | Results interpretation | |
|---|---|---|---|---|---|---|---|---|
| | *MAB2248* bol (MB) | 23S *rrl* WT (MB) | *erm*(41) T28C (MB) | *MAB2830* abs (MB) | *MAB2830* mas (MB) | Δ*erm*(41) (MB) | Subspecies | Macrolide phenotype |
| A | − | + | − | + | − | − | *abscessus* | Inducible resistance |
| B | − | + | + | + | − | − | *abscessus* | Susceptible |
| C | − | − | − | + | − | − | *abscessus* | Constitutive resistance |
| D | + | + | − | − | − | − | *bolletii* | Inducible resistance |
| E | − | + | − | − | + | − | *massiliense* | Inducible resistance |
| F | + | + | + | + | + | + | *massiliense* | Susceptible |

[a]+, positive amplification for the molecular beacon; −, negative amplification for the molecular beacon; abs, *M. abscessus* subsp. *abscessus*; bol, *M. abscessus* subsp. *bolletii*; mas, *M. abscessus* subsp. *massiliense*; MB, molecular beacon probe; WT, wild-type.

type *rrl* gene and the identification of *M. abscessus* subsp. *abscessus*. The interpretation is that this strain has the wild-type *erm*(41) gene, wild-type *rrl* gene, and, consequently, it has inducible macrolide resistance. The results for strain B show the wild-type *rrl* gene but also the presence of the *erm*(41) T28C mutation, indicating that this strain is susceptible to macrolides, and the positive FAM molecular beacon profile in the right panel indicates it is an *M. abscessus* subsp. *abscessus* strain. The absence of a positive molecular beacon profile for strain C in the left panel indicates that it has the *rrl* gene mutation, predictive of constitutive macrolide resistance, and the positive FAM molecular beacon profile in the right panel indicates it is an *M. abscessus* subsp. *abscessus* strain. The results for strain D reveal the wild-type *rrl* gene and a positive CFR610 molecular beacon fluorescence in the left panel, predictive of *M. abscessus* subsp. *bolletii*. The absence of any molecular beacon profile in the right panel confirms the subspecies identification, and the overall interpretation is that this is an *M. abscessus* subsp. *bolletii* strain with inducible resistance to macrolides as a result of having the wild-type *erm* (41) gene. The results for strains E and F reveal the wild-type *rrl* gene and a positive Q670 fluorescence in the right panel, predictive of *M. abscessus* subsp. *massiliense*. For strain E, the *erm*(41) gene shows a wild-type genotype, indicating that this strain has inducible resistance to macrolides, whereas strain F has the definitive deletion that is predictive of a macrolide-susceptible strain.

**Technical sensitivity and specificity.** The sensitivity of the two-tube multiplex assay was determined by initiating PCR assays with different quantities of DNA obtained from strains B, D, and F. These strains were chosen because together they contain target regions for all six molecular beacon probes utilized in this assay. The amount of DNA added as a template to each of the reactions was calculated to be equivalent to 1,000, 100, 10, and 1 copies of genomic DNA. The results show that Assays I and II, for each strain, were able to detect and identify 1 or 10 copies of genomic DNA (data not shown).

We tested the specificity of the assay using DNA from 19 different *Mycobacterium* species. No positive amplifications were observed for *MAB2248* and *MAB2830* molecular beacon probes, which is consistent with our above *in silico* data-mining results. The bioinformatic and experimental approaches confirm that the assay is highly specific for the detection of *M. abscessus*. In addition, none of the 19-sample panel were positive for the *erm*(41) T28C mutation or *erm*(41) 274-bp deletion. For the 23S *rrl* molecular beacon, only DNA from *M. chelonae* gave a positive amplification with a threshold cycle ($C_T$) value of <30 cycles, while other species only showed very late amplification signals ($C_T > 33$). It is significant to note that the 23S *rrl* primer and molecular beacon regions are 100% identical between *M. abscessus* and *M. chelonae*, and, in agreement with the *in silico* data, the *rrl* RT-PCR result was positive.

**Clinical isolate testing.** The two-tube multiplex assay was used to evaluate a large collection of genetically characterized clinical *M. abscessus* isolates from National Jewish Health, representing nontuberculous mycobacterial (NTM) isolates from CF centers across the United States, and from selected strains archived at the Center for Discovery and Innovation. Blinded DNA samples were assayed and compared to the subspecies and the macrolide genotype of each strain as determined by whole-

**TABLE 5** Performance of the real-time PCR in 115 clinical MABC isolates in comparison to the whole-genome sequencing results

| | | Whole-genome sequencing analysis results[a] | | | | | | | | | | | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | | Abs | | Mas | | Bol | | 23s *rrl*: 2058-2059 mutants[b] | | *erm*(41) truncation | | *erm*(41) T28C mutation | |
| | | Yes | No | Yes | No | Yes | No | Yes | No | Yes | No | Yes | No |
| MB-real-time PCR results | *MAB2830* abs MB pos | 69 | 0 | | | | | | | | | | |
| | *MAB2830* abs MB neg | 0 | 46 | | | | | | | | | | |
| | *MAB2830* mas MB pos | | | 36 | 0 | | | | | | | | |
| | *MAB2830* mas MB neg | | | 0 | 79 | | | | | | | | |
| | *MAB2248* bol MB pos | | | | | 10 | 0 | | | | | | |
| | *MAB2248* bol MB neg | | | | | 0 | 105 | | | | | | |
| | 23S *rrl* MB pos | | | | | | | 4 | 0 | | | | |
| | 23S *rrl* MB neg | | | | | | | 0 | 111 | | | | |
| | Δ*erm*(41) MB pos | | | | | | | | | 35 | 0 | | |
| | Δ*erm*(41) MB neg | | | | | | | | | 0 | 80 | | |
| | *erm*(41) T28C MB pos | | | | | | | | | | | 18 | 0 |
| | *erm*(41) T28C MB neg | | | | | | | | | | | 0 | 97 |

[a]abs, *M. abscessus* subsp. *abscessus*; bol, *M. abscessus* subsp. *bolletii*; mas, *M. abscessus* subsp. *massiliense*; MB, molecular beacon probe; neg, negative; pos, positive.
[b]The 23S *rrl* MB was designed to detect wild-type 2058-2059 alleles, and mutations in 2058 or 2059 will fail to show an amplification curve. The negative amplification curve for 23S *rrl* MB is regarded as a 23S *rrl* 2058-2059 mutation result.

genome sequencing. A summary of each of the molecular beacon results is shown in Table 5 for a total of 115 strains that were assayed. The results showed 100% specificity and sensitivity in determining the subspecies and the macrolide genotype, and the individual results are shown in Table S1 in the supplemental material.

## DISCUSSION

In addressing the need for a simple, yet comprehensive, assay to improve the treatment of *M. abscessus*, we have developed a robust and rapid molecular beacon diagnostic that can determine both the identification of the *M. abscessus* subspecies and the genotypic profile associated with macrolide resistance. Several sequencing-based methods using regular PCR followed by Sanger sequencing to characterize sequence variations among some housekeeping genes, including *hsp65*, *rpoB*, *secA*, and 16S, either individually or in combination, were developed previously to identify the three *M. abscessus* subspecies (12, 27, 28). Additionally, multiplex PCR-based targeted next-generation sequencing (29) and Cas12a/single guide RNA (sgRNA)-based nucleic acid detection (30) platforms were recently developed for distinguishing *M. abscessus* subspecies. Commercial nucleic acid amplification tests, such as the line probe GenoType NTM-DR assay, which distinguishes the *M. abscessus* subspecies and macrolide resistance, are also available (31, 32); however, all of these approaches involve post-PCR procedures, which require longer turnaround times. Due to the high level of genetic relatedness, it is a challenge to differentiate strains from *M. abscessus* based on a single gene sequence (33).

To facilitate our ability to select alternative targets for *M. abscessus* detection and identification of subspecies, we analyzed 1,930 *M. abscessus* genomes and selected two targets for SNP-based real-time PCR design. The subspecies identification PCR targets are highly specific for *M. abscessus* based on both *in silico* analysis and comparison of the public nontuberculous mycobacterial genomes and our experimental testing of representative isolates from clinically frequently encountered mycobacterial species, supporting their future use in testing primary specimens where other confounding species could be present.

The molecular epidemiology of the three subspecies varies significantly, and infections caused by *M. abscessus* subsp. *bolletii* are rarer than the other two subspecies (3, 34, 35). However, clinical disease and outbreaks caused by *M. abscessus* subsp. *bolletii* have been documented (34). In contrast to *M. abscessus* subsp. *bolletii*, *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *massiliense* postsurgical wound

infections and respiratory disease are frequently reported globally (36–40), and differences in clinical outcomes commonly correlate to the strain's susceptibility to macrolides (41). Infections due to *M. abscessus* subsp. *massiliense* respond more favorably to macrolide therapy. However, as shown in the current study and in other publications, not all *M. abscessus* subsp. *massiliense* harbor the truncated *erm*(41), and *M. abscessus* subsp. *massiliense* isolates with a wild-type *erm*(41) have been described (15). The *erm*(41) C28 sequevar *M. abscessus* subsp. *abscessus* is associated with significantly higher culture conversion rates with treatment, since these isolates do not exhibit inducible resistance to macrolides (5). Finally, *M. abscessus* subsp. *massiliense* with the truncated *erm*(41) gene has been identified with mutations in the *rrl* 2058 and 2059 sites, making them fully resistant to macrolides (42). Collectively, identification of subspecies and genotyping macrolide susceptibility are necessary to guide "precise treatment" for clinical *M. abscessus* infections (41, 43). It is noted that our assay was designed to detect the most common mutations associated with macrolide resistance in MABC with the understanding that rare resistance mechanisms (44) could be targeted in the future and added to the assay if they become more prevalent among clinical isolates.

Besides the identification of subspecies, our assay has strong predictive ability to determine whether to use a macrolide in the patient's treatment regimen. Compared to the 3 to 14 days needed for clarithromycin phenotypic susceptibility testing, our real-time PCR method can be completed in 2 to 3 h and provides timely and definitive laboratory results. Results from current and other studies (5, 45) also suggested that the *erm*(41) gene truncation is not a reliable marker to distinguish *M. abscessus* subsp. *massiliense*. Importantly, our assay showed 100% specificity and sensitivity among 115 tested *M. abscessus* strains from multiple health care facilities in the United States, further supporting its clinical application. The ability for health care facilities to extend their diagnostic capability in the treatment of *M. abscessus* infections would dramatically improve patient care by providing appropriate antibiotic treatment and establishing epidemiological markers to monitor and track *M. abscessus* infections.

## SUPPLEMENTAL MATERIAL

Supplemental material is available online only.
**SUPPLEMENTAL FILE 1**, XLSX file, 0.01 MB.

## ACKNOWLEDGMENTS

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