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Utility of Sequencing the *erm*(41) Gene in Isolates of *Mycobacterium abscessus* subsp. *abscessus* with Low and Intermediate Clarithromycin MICs

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The *erm*(41) gene confers inducible macrolide resistance in *Mycobacterium abscessus* subsp. *abscessus*, calling into question the usefulness of macrolides for treating *M. abscessus* subsp. *abscessus* infections. With an extended incubation (14 days), isolates with MICs of ≥8 μg/ml are considered macrolide resistant by current CLSI guidelines. Our goals were to determine the incidence of macrolide susceptibility in U.S. isolates, the validity of currently accepted MIC breakpoints, and the *erm*(41) sequences associated with susceptibility. Of 349 isolates (excluding those with 23S rRNA gene mutations), 85 (24%) had clarithromycin MICs of ≤8 μg/ml. Sequencing of the *erm*(41) genes from these isolates, as well as from isolates with MICs of ≥16 μg/ml, including ATCC 19977^T, revealed 10 sequevars. The sequence in ATCC 19977^T was designated sequevar (type) 1; most macrolide-resistant isolates were of this type. Seven sequevars contained isolates with MICs of >16 μg/ml. The T28C substitution in *erm*(41), previously associated with macrolide susceptibility, was identified in 62 isolates (18%) comprising three sequevars, with MICs of ≤2 (80%), 4 (10%), and 8 (10%) μg/ml. No other nucleotide substitution was associated with macrolide susceptibility. We recommend that clarithromycin susceptibility breakpoints for *M. abscessus* subsp. *abscessus* be changed from ≤2 to ≤4 μg/ml and that isolates with an MIC of 8 μg/ml have repeat MIC testing or *erm* sequencing performed. Our studies suggest that macrolides are useful for treating approximately 20% of U.S. isolates of *M. abscessus* subsp. *abscessus*. Sequencing of the *erm* gene of *M. abscessus* subsp. *abscessus* will predict inducible macrolide susceptibility.

Three closely related taxa of rapidly growing mycobacteria (RGM) with a controversial species/subspecies status, i.e., *Mycobacterium abscessus* subsp. *abscessus*, "*M. abscessus* subsp. *massiliense*," and *M. abscessus* subsp. *bolletii*, comprise the heterogeneous *M. abscessus* group (1, 2). A 2013 phylogenetic analysis based on genomic sequencing corroborated previous recommendations that the three taxa should be separated into three subspecies (3). Of these three subspecies, *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *bolletii*, a relatively rare subspecies in the United States, demonstrate the presence of inducible macrolide resistance conferred by a novel *erm* gene, *erm*(41) (4).

In contrast, the *erm*(41) gene is generally present in isolates of *M. abscessus* subsp. *massiliense* but contains a large, 397-bp deletion (that includes position 28T) that results in a nonfunctional *erm*(41) gene, and thus isolates do not show inducible macrolide resistance (2).

In 2009, Nash and colleagues described two strains (MAB30 and MC1028) of *M. abscessus* subsp. *abscessus* which contained a nonfunctional *erm* gene (4); the strains were not inducibly resistant to clarithromycin, and even after extended incubation, they demonstrated susceptible clarithromycin MICs. The loss of function of the *erm* genes was associated with a T-to-C substitution at position 28 of the gene (T28C), leading to an amino acid change from Trp to Arg at codon 10 (4). Such strains are categorized as the MAB30 subgroup.

The presence of erythromycin ribosomal methylase (*erm*) genes has called into question the usefulness of macrolides for treatment of infections caused by some RGM, including *M. abscessus* subsp. *abscessus* (4). The prevalence of the MAB30 subgroup among U.S. isolates and their 14-day clarithromycin MICs have not been well studied. A relatively high prevalence would indicate that macrolides still have a role in therapy for *M. abscessus* subsp. *abscessus* disease. An initial evaluation of the MAB30 subgroup showed some isolates to have 14-day clarithromycin MICs of 4 μg/ml (considered intermediate in the current CLSI guidelines) and 8 μg/ml (considered resistant). Therefore, we initiated a study of these isolates of *M. abscessus* subsp. *abscessus* with clarithromycin MICs of 8 μg/ml or less following extended incubation.

(A portion of this study was presented at the 114th General Meeting of the American Society for Microbiology [5].)

MATERIALS AND METHODS

Isolates. All isolates of *M. abscessus* submitted for identification and antimicrobial susceptibility testing to the Mycobacteria/Nocardia Laboratory at the University of Texas Health Science Center at Tyler from Feb-

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ruary 2009 through September 2012 were identified. All isolates were submitted from laboratories in the United States, and some patients treated in our institution were known to have received prior macrolide therapy. Only one isolate per patient was counted within each *erm* gene sequevar. One isolate with a unique *erm* gene sequevar, received in 2014 from a patient treated in our institution, was included. This study was approved by the Institutional Review Board at the University of Texas Health Science Center at Tyler.

Identification of isolates. Isolates were identified as *M. abscessus* subsp. *abscessus* by PCR restriction fragment analysis (PRA) of a 441-bp *hsp65* gene sequence and/or sequencing of the *rpoB* gene, using an ABI genetic analyzer (Life Technologies, Grand Island, NY) (6–8). Isolates of *M. abscessus* subsp. *massiliense* and *M. abscessus* subsp. *bolletii* were excluded.

***erm*(41) gene sequence.** For *erm* gene sequencing, nested PCR was done on *M. abscessus* subsp. *abscessus* by using primers *erm*F (5′-GACCG GGGCCTTCTTCGTGAT-3′), *erm*R1 (5′-GACTTCCCCGCACCGATT CC-3′), and *erm*(41)-4 (5′-CCGGCCCGTAGCGTCCAATG-3′) to produce a 670-bp fragment. Primers *erm*F and *erm*R1 were used for amplification of the *erm*(41) gene of *M. abscessus* subsp. *abscessus* isolates, and cycle sequencing was performed using the *erm*F and *erm*(41)-4 primers (2, 4). All isolates of *M. abscessus* subsp. *abscessus* with 14-day clarithromycin MICs of ≤8 μg/ml, along with a select number of control isolates with clarithromycin MICs of ≥16 μg/ml, including *M. abscessus* subsp. *abscessus* ATCC 19977^T, were tested for the sequence of the *erm*(41) gene.

Clarithromycin broth microdilution MIC testing. Susceptibility testing was performed using the CLSI-recommended broth microdilution MIC method (9). Clarithromycin MICs were determined initially after 3 days and then daily (except on weekends) for up to 14 days of incubation at 30°C. Current CLSI MIC breakpoints for clarithromycin were utilized, i.e., ≤2 μg/ml (susceptible), 4 μg/ml (intermediate), and ≥8 μg/ml (resistant), to assess the susceptibility of the isolates. Isolates with macrolide resistance at the initial reading were considered resistant on the basis of a 23S rRNA gene mutation, not due to an inducible *erm* gene, and were excluded from the study (4, 10).

Nucleotide sequence accession numbers. Representative sequences of each *erm* gene sequevar (1 to 10) have been deposited in GenBank under consecutive accession numbers KP676133 through KP676142.

RESULTS

Isolates. A total of 357 isolates of *M. abscessus* subsp. *abscessus* collected between February 2009 and September 2012, plus one isolate collected in November 2014, were identified by *hsp65* PRA and/or *rpoB* gene sequencing and underwent antimicrobial susceptibility testing.

***erm*(41) gene sequence.** Nine *erm* gene sequevars closely related to the sequence of the *M. abscessus* subsp. *abscessus* type strain, ATCC 19977 (10 total), were identified and numbered sequevars 2 to 10 (Table 1). No *erm* gene sequevars consistent with the other *M. abscessus* subspecies were identified among these isolates. Multiple single nucleotide polymorphisms (SNPs) (range of 2 to 8) were noted in all but one sequevar (type 8, which has a single SNP). The SNPs are listed in Table 2.

Isolates with MICs of >16 μg/ml were associated with seven of the sequevars (sequevars 1, 4, and 6 to 10), although the majority of these macrolide-resistant isolates (approximately two-thirds) had a sequevar or type 1 *erm*(41) gene sequence identical to that of the type strain (ATCC 19977). The seven macrolide-resistant sequevars all had a thymine (T) at position 28.

Of the total of 358 isolates, 85 had clarithromycin MICs of ≤8 μg/ml (Table 1). Sixty-two of the 85 (73%) isolates with clarithromycin MICs of ≤8 μg/ml belonged to the three sequevars (i.e., 2, 3, and 5) with the characteristic T28C substitution (i.e., MAB30 subgroup isolates). This substitution was previously associated with functional loss of the *erm* gene and susceptibility to clarithromycin. No isolates with an MIC of >8 μg/ml had this base substitution. Sixty of the 62 (97%) isolates that had the T28C substitution had a sequevar 2 *erm* gene that matched the MAB30 sequence previously described by Nash et al. (4). The type 2 *erm* sequence exhibited a 4-bp difference from the sequevar 1 sequence, but the 3 bp other than bp 28 (C) were found in other sequevars that consisted of macrolide-resistant isolates. No base pair difference from the type 1 sequevar, other than T28C, was associated with loss of function of the gene.

TABLE 1 Eighty-five isolates of *Mycobacterium abscessus* subsp. *abscessus* with 14-day clarithromycin MICs of ≤8 μg/ml on initial testing, by *erm* gene sequevar type

erm gene sequevar (type)	Position 28		No. of isolates	Extended-incubation clarithromycin MICs (μg/ml) (range)[b]	No. of isolates with extended-incubation clarithromycin MIC (μg/ml)		
	Base	Amino acid			≤2	4	8
1[a]	T	Trp	16	1–8	4	3	9
2	C	Arg	60	≤0.06–8	48	6	6
3	C	Arg	1	≤0.006	1	0	0
4	T	Trp	0	NA	0	0	0
5	C	Arg	1	≤0.12	1	0	0
6	T	Trp	5	8–>16	0	0	5
7	T	Trp	1	8–>16	0	0	1
8	T	Trp	0	NA	0	0	0
9	T	Trp	0	NA	0	0	0
10	T	Trp	1	2	1	0	0
Total			85	≤0.006–8	55	9	21

[a] Sequevar present in the type strain, ATCC 19977.
[b] NA, not applicable.

Clarithromycin broth microdilution MIC testing. Nine of the 358 isolates (2.5%) had MICs of >16 μg/ml at 3 to 5 days of incubation, were presumed to have mutational macrolide resistance, and were excluded. As stated above, 85 of the remaining 349 wild-type isolates (24%) had MICs of ≤8 μg/ml. Table 1 shows the MIC ranges for these 85 isolates and their *erm* sequevar types. Of the remaining 264 isolates, with MICs of >16 μg/ml, the modal time for detection of clarithromycin resistance was 14 days, with a range of 8 to 14 days.

Susceptibilities of the 60 type 2 *erm* gene sequevar (MAB30) isolates revealed 48 (80%) with MICs of ≤2 μg/ml, 6 (10%) with an MIC of 4 μg/ml, and 6 (10%) with an MIC of 8 μg/ml (Table 1). Repeat testing was performed on the six isolates with an MIC of 8 μg/ml, and all had clarithromycin MICs of ≤2 μg/ml. Of the six isolates of *M. abscessus* subsp. *abscessus* with an MIC of 4 μg/ml, five showed repeat clarithromycin MICs of ≤2 μg/ml (one isolate still had an MIC of 4 μg/ml after testing was repeated twice).

Sixteen of 85 (19%) isolates with MICs of ≤8 μg/ml (4 with MICs of ≤2 μg/ml, 2 with an MIC of 4 μg/ml, and 9 with an MIC of 8 μg/ml) had a type 1 *erm* sequevar (lacked the T28C substitution). On repeat testing, 11 had MICs of ≥16 μg/ml, 1 still had a

TABLE 2 SNPs associated with the 10 *erm* gene sequevar types identified in *M. abscessus* subsp. *abscessus*

Sequevar (type)	SNP at sequevar 1 base (with codon and corresponding amino acid indicated)[a]												
	G8 GGC Gly	T28 TGG Trp	G66 CGG Arg	A120 GCA Ala	T159 GGT Gly	G168 GTG Val	A238 ATA Ile	G255 CTG Leu	G279 CGG Arg	A330 ATA Ile	T336 AGT Ser	A380[c] CAG Gln	C419 CCT Pro
1[b]	●	●	●	●	●	●	●	●	●	●	●	●	●
2	●	C (Arg)	●	●	C	●	G (Val)	●	●	C	●	●	●
3	●	C (Arg)	●	●	C	●	●	●	●	●	●	C (Pro)	●
4	●	●	●	●	C	●	G (Val)	●	●	C	●	●	●
5	C (Ala)	C (Arg)	●	●	C	●	●	●	●	●	●	C (Pro)	●
6	●	●	●	●	C	●	G (Val)	A	T	C	C	●	●
7	●	●	●	●	C	●	G (Val)	A	●	C	●	●	●
8	●	●	●	●	●	C	●	●	●	●	●	●	●
9	●	●	●	●	C	●	●	●	●	C	●	●	T (Leu)
10	●	●	●	G	C	●	G (Val)	A	T	C	C	●	●

[a] The underlined base in each codon sequence indicates the base substitution site. Bullets indicate that the bases are the same as in the type strain sequence (type 1 sequevar).
[b] The type 1 sequevar includes the *M. abscessus* subsp. *abscessus* type strain, ATCC 19977.
[c] Sequencing of the type strain, ATCC 19977, as part of this study indicated an A at position 380, whereas the *erm*(41) sequence in the whole-genome data previously deposited in GenBank (accession no. CU458896; corresponding base position 2,346,334) had a T for this base.

clarithromycin MIC of 8 μg/ml, and 1 had an MIC of 2 μg/ml (3 other isolates were not available for repeat testing).

DISCUSSION

Macrolides have remained the cornerstone of therapy for most nontuberculous mycobacterial (NTM) infections (11). Isolates of RGM and slowly growing mycobacteria, which generally contain only a single copy of the ribosome, develop acquired macrolide resistance during or after treatment due to a mutation in the 23S rRNA gene which causes a base change at position 2058 or 2059 (*Escherichia coli* numbering) (10). This type of gene mutation in NTM during or after therapy with macrolides has been reported for several species, including *Mycobacterium avium*, *Mycobacterium intracellulare*, *Mycobacterium chelonae*, *Mycobacterium kansasii*, and *M. abscessus* (11–14). Several isolates of mutationally resistant *M. abscessus* have been studied, but the specific subspecies and *erm*(41) gene sequence have been established for only a few strains, which were strains of *M. abscessus* subsp. *massiliense* (3). Clinically acquired mutational resistance in *M. abscessus* subsp. *abscessus* is relatively unusual [presumably because it is already resistant on the basis of the inducible *erm*(41) gene] and is distinguished from inducible resistance by the finding of initial (3 to 4 days) resistance based on clarithromycin MICs (10).

Intrinsic inducible macrolide resistance conferred by (functional) *erm* genes is common among most nonpigmented pathogenic RGM, including *Mycobacterium goodii* and *Mycobacterium smegmatis* [*erm*(38)], *Mycobacterium fortuitum* [*erm*(39)], *Mycobacterium mageritense* and *Mycobacterium wolinskyi* [*erm*(40)], and *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *bolletii* [*erm*(41)] (i.e., isolates that match the type strain [CIP 108541; CCUG50184] of the former species *M. bolletii*) (4, 11). Species not found to have an *erm* gene include *Mycobacterium peregrinum* and *Mycobacterium senegalense*. Taxonomic groups with a nonfunctional *erm* gene include *M. abscessus* subsp. *massiliense* and some isolates of *M. abscessus* subsp. *abscessus*.

The finding of the *erm*(41) gene in *M. abscessus* subsp. *abscessus* isolates has evoked major questions about the utility of macrolides against these isolates and may explain the lack of efficacy of macrolide-based regimens against this group (especially for chronic lung disease) (15). Using the current CLSI clarithromycin breakpoints of ≤2 μg/ml (susceptible), 4 μg/ml (intermediate), and ≥8 μg/ml (resistant), based on 3 days of incubation (9), 73% of the 358 clinical isolates from the United States demonstrated inducible clarithromycin resistance (14-day MICs of ≥16 μg/ml) (the majority of which appeared to result from the presence of the *erm* type 1 sequevar, including in ATCC 19977^T) (5, 9). In contrast, 85 of the isolates of *M. abscessus* subsp. *abscessus* had MICs of ≤8 μg/ml after extended incubation. Sequencing of the *erm* gene in isolates of *M. abscessus* subsp. *abscessus* (24%) revealed 10 sequevars, although only those associated with a T28C substitution (primarily of the type 2 sequevar) were found to be nonfunctional and showed extended-incubation clarithromycin MICs of ≤4 μg/ml. The type 2 sequevar corresponded to the MAB30 subgroup isolates previously described by Nash et al. (4).

In accordance with the findings in this study, wild-type (untreated) isolates of *M. abscessus* subsp. *abscessus* with a type 1 *erm* sequevar matching that of ATCC 19977^T or one of the six other, less frequently encountered *erm* gene sequences of sequevar types 4 and 6 to 10, associated with clarithromycin MICs of >16 μg/ml, should be considered macrolide resistant. Isolates with the type 2 *erm* sequevar (MAB30), as well as types 3 and 5 (all three of which contain the T28C substitution), should be considered macrolide susceptible. Sequencing of the *erm* gene is an especially important diagnostic tool for assessment of clarithromycin susceptibility in isolates of *M. abscessus* subsp. *abscessus* which have equivocal MICs of 4 to 8 μg/ml. In the current study, 73% of the isolates of *M. abscessus* subsp. *abscessus* with clarithromycin MICs of ≤8 μg/ml had a T28C mutation (previously associated with loss of function of the *erm* gene), and 60/62 (97%) isolates matched the previously described MAB30 strain (type 2 *erm* sequevar) (4).

The T28C mutation leads to a Trp (hydrophobic aromatic side chain)-to-Arg (positively charged side chain) amino acid substitution. This change would be expected to have a significant impact on protein structure and function and thus is consistent with the absence of resistance in the type 2 sequevar isolates. Although there are several other missense polymorphisms (i.e., exchanging one amino acid for another), all but one represent changes to

comparable amino acids, mostly replacing one aliphatic residue with another. The remaining change of Gln to Pro in sequevars 3 and 5 would most likely also have a minimal impact on the protein, unless this was in a catalytic site, since both of these amino acids have hydrophobic regions in their side chains.

Intriguingly, the 12 type 2 *erm* sequevar isolates with an MIC of 4 or 8 μg/ml exhibited MICs of ≤2 μg/ml on repeat testing, for all but 1 isolate. The interpretation of MICs after extended incubation is not without pitfalls. This difference in MICs with repeat testing is thought to be due partially to the difficulty in interpreting macrolide MICs after extended incubation and, in some cases, to the poor growth of some isolates in broth. Previously treated isolates can sometimes produce suboptimal growth in the current susceptibility broth (i.e., Mueller-Hinton broth). However, a more enriched broth, such as Middlebrook 7H9 broth, may alter the susceptibility, most likely due to the decrease in pH. A more enriched broth that does not result in altered macrolide MICs has yet to be developed. The use of *erm* gene sequencing as a backup for indeterminate MIC results is probably the best current solution to improve phenotypic results.

We recommend that the CLSI should evaluate the current clarithromycin susceptibility breakpoints for *M. abscessus* subsp. *abscessus*, and we further propose that isolates with a clarithromycin MIC of 8 μg/ml (currently considered resistant) (9) should have repeat MIC testing and/or *erm* gene sequencing performed. Sequencing of the *erm*(41) gene is preferred, as sequencing can produce an answer more quickly than repeating the MIC tests.

Current subspecies identification of *M. abscessus* isolates requires multigene sequencing, because the three subspecies have identical complete 16S rRNA genes and the presence of gene recombination for commonly studied gene targets, such as the *hsp65* gene and the *rpoB* gene. Interestingly, to date, no recombination of the *erm* gene between subspecies has been identified, and recombination was not observed among the >100 clinical isolates of *M. abscessus* subsp. *abscessus* with *erm* gene sequences in the current study. Further support for the genetic stability of this gene comes from the growing body of data for *M. abscessus* subsp. *massiliense* (2, 4, 16), indicating that most isolates of this subspecies carry exactly the same deletion mutant *erm*(41) allele irrespective of the source patients' geographic locations. This makes the *erm* gene a good candidate for subspecies identification. An added benefit would be preliminary determination of *erm* gene functionality pending the results of the slower susceptibility testing method.

Recently, Shallom et al. (16) described two strains of *M. abscessus* subsp. *massiliense* (C12040 and C182) with full-length functional *erm*(41) genes. However, these genes were highly unusual in that they were >1% mismatches to those of the type strains of *M. abscessus* subsp. *abscessus* and/or *M. abscessus* subsp. *bolletii*. To our knowledge, these are the only two isolates of *M. abscessus* subsp. *massiliense* with a functional *erm* gene which have been described in the literature to date (16). Since the sequevars of *erm* in *M. abscessus* subsp. *abscessus* are recognized, one should be suspicious of the taxonomic status of an isolate with this degree of mismatch from the type strain *erm* gene. Sequencing of other genes would seem to indicate a horizontal gene transfer, and the isolate would be identified as a "subspecies *abscessus*/subspecies *massiliense* cross strain."

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