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Genetic correlates of clarithromycin susceptibility among isolates of the *Mycobacterium abscessus* group and the potential clinical applicability of a PCR-based analysis of *erm*(41)

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Objectives: To define the genetic basis of clarithromycin resistance among isolates of the *Mycobacterium abscessus* group (MAG).

Methods: We analysed 133 isolates identified as MAG. Species identification was confirmed by sequencing the *rpoB* gene. Clarithromycin susceptibility testing was performed according to CLSI recommendations, with an extended 14 day incubation. Known resistance genotypes of *erm*(41) and *rrl* were identified by sequencing; the presence of deletions in *erm*(41) was detected by PCR.

Results: The 133 MAG isolates included 82 *M. abscessus*, 27 *Mycobacterium massiliense* and 24 *Mycobacterium bolletii*. After the 3 day incubation, only five isolates demonstrated clarithromycin resistance (R); after 14 days of extended incubation, an additional 92 exhibited inducible resistance (IR), with the remaining being susceptible (S). The distribution of susceptibility phenotypes varied among the species. Among *M. abscessus* isolates, 11% were S, 84% IR and 5% R; among *M. bolletii* isolates, 96% were IR and 4% R; and among *M. massiliense* isolates 100% were S. Sequencing of *rrl* identified only a single isolate with the A2058G mutation. Deletions in *erm*(41) were present in 30 susceptible isolates; among the remaining 103 isolates, 97 were R or IR (sensitivity, 83%; specificity, 100%; positive predictive value, 100%; negative predictive value, 94%). Among the six susceptible isolates without deletions, all carried the *erm*(41) T28C point mutation.

Conclusions: A significant proportion of MAG isolates demonstrate inducible resistance to clarithromycin that is only detectable with an extended 14 day incubation. Further, the majority of clarithromycin-susceptible MAG isolates have characteristic deletions in *erm*(41) that can rapidly and reliably be detected by a simple PCR.

Introduction

Isolates of the *Mycobacterium abscessus* group (MAG) are the most common rapidly growing mycobacteria isolated from patients with pulmonary infection. These environmental organisms are often opportunistic pathogens and have been associated with nosocomial infections, including outbreaks.[1–8] Species classification of MAG isolates remains a controversial issue owing to considerable similarity between *Mycobacterium bolletii* and *Mycobacterium massiliense*. Based on DNA-DNA hybridization studies, Leão *et al.*[9,10] have proposed that MAG be classified as a single species with two subspecies: *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *bolletii*, the latter including both *M. bolletii* and *M. massiliense*. Other studies applying whole-genome analysis support the separation into three distinct species.[11–14] Recently, Tortoli *et al.*[15] proposed that the species *M. abscessus* encompasses *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *bolletii* and a third subspecies named *M. abscessus* subsp. *massiliense* comb. nov.

Treatment of MAG infections is complicated by the high frequency of antibiotic resistance among the isolates.[16–18] Current guidelines identify clarithromycin and azithromycin as the primary agents used, but emphasize the need for multidrug treatment regimens to ensure effective therapy until susceptibility results are available.[19] Clarithromycin resistance in MAG can be mediated by several mechanisms. The *erm*(41) gene, which is widely prevalent among MAG, transfers one or two methyl groups to an adenine in the peptidyl region of 23S rRNA, and thereby prevents clarithromycin binding.[20–23] Two alterations of *erm*(41) are independently associated with reversion to clarithromycin susceptibility: a 274 bp deletion[7] and a T28C point mutation.[8,24–28] The *rrl* gene encodes

the 23S rRNA peptidyl transferase; the A2058G mutation in *rrl* confers high levels of resistance to clarithromycin.[19–21]

This study examined a collection of pulmonary MAG isolates for which species identification was resolved by *rpoB* sequencing; clarithromycin resistance phenotype was based on *in vitro* susceptibility testing with extended incubation; *rrl* and *erm*(41) genotypes were determined by sequence analysis; and the presence of deletions in *erm*(41) was assessed using PCR.

Materials and methods

Isolates

Among 635 pulmonary isolates submitted to the Tuberculosis and Mycobacteriosis Branch of Adolfo Lutz Institute from January 2010 to December 2011 for species identification, 153 (24.1%) were classified as MAG by PRA-*hsp65* typing.[29] Of those, 134 were viable for analysis, including one isolate that by *rpoB* sequencing proved to be *Mycobacterium porcinum*, leaving 133 MAG isolates in the final study set.

Species identification

Definitive species identification was obtained by sequencing a fragment of the *rpoB* gene using Myco F (5′-GGCAAGGTCACCCCGAAGGG-3′) and Myco R (5′-AGCGGCTGCTGGGTGATCATC-3′) as primers.[30] The sequences were analysed with the software BioNumerics version 7.1 (Applied Maths, TX, USA) and the BLAST tool (http://blast.ncbi.nlm.nih.gov) was used to determine similarity with the following reference strains: *M. abscessus* ATCC 19977 (GenBank accession number JF346872), *M. bolletii* CIP 108541 (AY859692.1) and *M. massiliense* CIP 108297 (JX041985.1). The species classification reported for each study isolate represents the highest similarity score with the indicated reference strain. For simplification, the species *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *bolletii* and *M. abscessus* subsp. *massiliense* are referred to as *M. abscessus*, *M. bolletii* and *M. massiliense*, respectively.

Clarithromycin susceptibility testing with resazurin

The MIC test was performed according to CLSI[31] recommendations, with clarithromycin concentrations from 0.5 to 64 mg/L and using *Staphylococcus aureus* ATCC 29213 as quality control. Replicate plates were prepared to provide readings after 3, 5, 7, 10 and 14 days of incubation, where 3 days represents the current standard recommendation and 14 days represents a proposed extended incubation.[30] At each timepoint 30 μL of 0.01% resazurin was added to each well and the plate re-incubated overnight.[32] A change in colour from blue (oxidized state) to pink (reduced) indicates bacterial growth. The MIC value was defined as the lowest drug concentration that prevented growth, and isolates were classified as susceptible (S; MIC $\leq$2 mg/L) or resistant (R; MIC >2 mg/L). Isolates that met the criteria for susceptibility on day 3, but were classified as resistant subsequently, are reported as 'inducible resistance' (IR).[7,27,28]

erm(41) and rrl gene sequencing

The primers for amplifying *rrl* were SP1 (CCTGCACGAATGCGGTAACG) and SP2 (CACCAGAGGTTCGTCCGTC).[7] The primers erm41f2 and erm41r2, described by Maurer *et al.*,[7] were tested for amplification of the *erm*(41) gene. However, the amplified region did not contain the region where the mutation occurs. Consequently, the sequence to be amplified was analysed and the primer reverse was designed. The amplification and sequence analysis of *erm*(41) was performed using the following primers Forward (5′-GACCGGGGCCTTCTTCGTGAT-3′)[7] and Reverse (5′-GGAGTTCGTTGTGGATCT GG-3′). The resulting product, which includes 114 bp of the N-terminal coding region covering the T28C mutation[28] and the deletion at 64–65 bp,[33] was sequenced for all isolates. Sequence data were analysed with BioNumerics version 7.1 (Applied Maths, Sint-Martens-Latem, Belgium), using *M. abscessus* MAB30 (GenBank accession number EU590129) as the reference sequence for *erm*(41), and *M. abscessus* (GenBank accession number NC_010397.1) for *rrl*.

PCR analysis of erm(41)

The entire *erm*(41) coding region was amplified using the protocol of Brown-Elliott *et al.*[34] and primers *erm*F (5′-GACCGGGGCCTTCTTCGTGAT-3′) and *erm*R1 (5′-GACTTCCCCGCACCGATTCC-3′). The presence of the characteristic deletions in *erm*(41) (2 bp, nucleotides 64–65; 274 bp, nucleotides 159–432) was identified from the reduced size of the PCR product (expected, 673 bp; deletions present, 397 bp) and confirmed for selected isolates by sequencing the entire coding region.

Results

Identification

Among the 133 MAG isolates in the study set, all 78 isolates designated *M. abscessus* type 1 by PRA-*hsp65* typing were identified as *M. abscessus*. Of the 55 isolates of *M. abscessus* type 2, 27 were identified *as M. massiliense*, 24 as *M. bolletii* and 4 as *M. abscessus*.

Clarithromycin susceptibility testing

The proportion of isolates showing clarithromycin inducible resistance increased over the course of the extended incubation, with the sharpest increase observed between days 7 and 10 (Figure 1). At day 14, inducible resistance was similarly prevalent among isolates of both *M. abscessus* (84%) and *M. bolletii* (96%), but absent among *M. massiliense* (0%).

erm(41) and rrl gene sequencing

For all 133 isolates, the genotypes at *erm*(41) and *rrl* were compared with the results of clarithromycin susceptibility testing using the day 14 reading and the results of *rpoB* species identification (Table 1). Sequencing of *rrl* identified only a single isolate with the A2058G mutation; that isolate also had intact *erm*(41) and was resistant.

PCR analysis indicated the presence of deletions in *erm*(41) among 30 (83%) of the 36 clarithromycin-susceptible isolates, including all 27 *M. massiliense*. Of the six susceptible isolates without deletions, all were *M. abscessus* that carried the *erm*(41) T28C point mutation. Among the 97 resistant isolates, PCR indicated that all had an intact WT *erm*(41). Based on these results, PCR detection of deletions in the *erm*(41) gene had excellent performance characteristics as a presumptive test for clarithromycin susceptibility: with sensitivity, 83%; specificity, 100%; positive predictive value, 100%; and negative predictive value, 94%.

The sequence analysis of the N-terminal region of *erm*(41) was compared with the subspecies assignment determined by *rpoB*. All *M. bolletii* isolates were clustered (>99.2% homology) as a distinct subset within *M. abscessus*, and both of those subspecies were readily distinguished from *M. massiliense* (<95% homology; data not shown). As expected, all the *M. massiliense* isolates were clustered (>98% homology) and all had the smaller *erm*(41) PCR product consistent with the characteristic deletions.

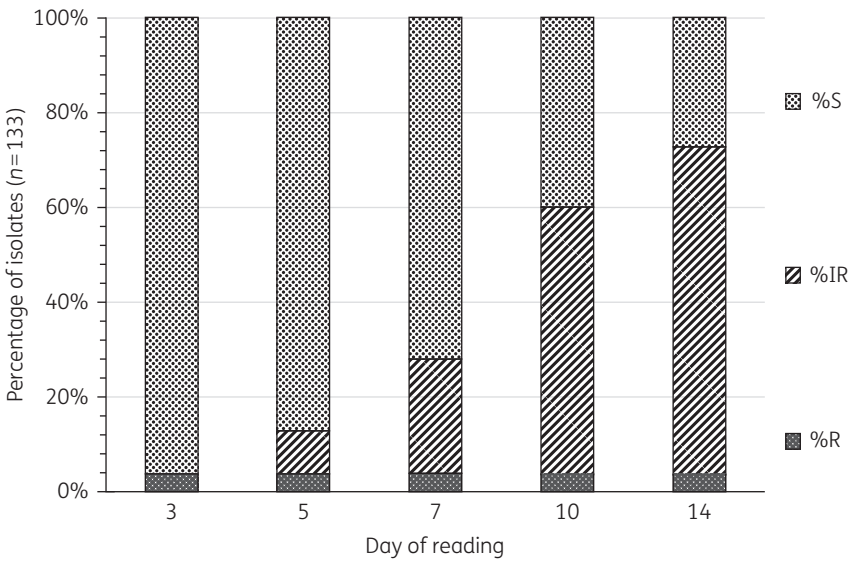

Figure 1. Percentage of 133 MAG isolates meeting the criteria for clarithromycin susceptible (S), resistant (R) or inducible resistance (IR) by day of reading of test.

Table 1. Genetic profile of the *erm*(41) and *rrl* genes and MIC results after 14 days of incubation

Genotype			Susceptibility to clarithromycin[a]			Species within MAG		
rrl	*erm*(41)	*n*	S (*n* = 36)	IR (*n* = 92)	R (*n* = 5)	*M. abscessus* (*n* = 82)	*M. bolletii* (*n* = 24)	*M. massiliense* (*n* = 27)
WT	WT	96	–	92	4	72	24	–
A2058G	WT	1	–	–	1	1	–	–
WT	deletions[b]	30	30	–	–	3	–	27
WT	T28C	6	6	–	–	6	–	–
A2058G	T28C	0	–	–	–	–	–	–

[a]S, susceptible after 14 days of incubation; R, resistant after 3 days of incubation; IR, inducible resistant based on change from 3 to 14 days of incubation. See text for details.
[b]Two deletions in *erm*(41) of 2 bp (nucleotides 64–65) and 274 bp (nucleotides 159–432).

The *M. massiliense* cluster included three *M. abscessus* isolates (isolates 640, 979 and 1189), which also demonstrated the smaller PCR product. The sequences of the complete coding region of *erm*(41) for these isolates were identical to each other and to *M. massiliense* (isolate 2883). It is unclear whether the three isolates acquired the altered gene from *M. massiliense* or whether the same deletion process occurred independently.

Discussion

Among pulmonary infections associated with rapidly growing mycobacteria species, ~80% are caused by MAG.[7,17,35,36] The *erm*(41) gene, which is essentially universal among these organisms, mediates resistance to the macrolide clarithromycin and thereby greatly complicates effective treatment.[26,28] Choi *et al.*[37] proposed that a large accumulation of *erm*(41) protein is required to effectively block the drug binding site and forms the basis for the high frequency of inducible resistance that is missed using the routine 3 day incubation and only detected with an extended 14 day incubation in the presence of the drug.

In this study of 133 pulmonary isolates of MAG, we performed replicate susceptibility tests with varying incubation periods ranging from 3 to 14 days to determine if IR could be reliably detected with shorter incubation, thereby providing a faster clinical result. The frequency of resistant phenotype after 3 days was <5% and increased steadily through 14 days of incubation to >70%, indicating the need for that entire period. This high rate of IR is consistent with other studies[38–40] and supports the need to modify the clarithromycin susceptibility testing protocol currently recommended by CLSI.[31] An incubation period of >14 days was not evaluated in this study; however, Christianson *et al.*[41] tested a prolonged incubation of 21 days for 14 *M. abscessus* isolates that were susceptible at day 14. Of these isolates, only one presented an MIC = 8 mg/L, but the authors concluded that there is no additional value to adding a 21 day reading to the test. They also tested *Mycobacterium chelonae* isolates that have no inducible resistance to clarithromycin to verify the stability of this drug. The isolates were incubated for 21 days, and all of them were susceptible. The authors concluded from these results that the clarithromycin was sufficiently active over the entire 21 day incubation period.

We further analysed the genotypes of the isolates using sequencing of *rpoB* to identify the species within MAG, sequencing of *erm*(41) to detect both a point mutation (T28C) and deletions associated with gene inactivation, and sequencing of *rrl* to detect the A2056G mutation associated with clarithromycin resistance.

The species classification reported for each study isolate represents the highest similarity score compared with the indicated reference strain. Despite the cut-off value ≥97% for species differentiation proposed by McNabb *et al.*,[42] we considered the similarity score ≥99%, as species belonging to MAG have only 1.46% divergence in *rpoB* sequences.[43]

Species identification indicated that both *M. abscessus* and *M. bolletii* isolates demonstrated high frequency of inducible resistance. In contrast, *M. massiliense* isolates were uniformly susceptible even after extended incubation and this was consistently associated with previously defined inactivating deletions. These observations are consistent those of Maurer *et al.*[44] and Mougari *et al.*[45]

Analysis of *erm*(41) and *rrl* genotypes with susceptibility phenotypes was also revealing. Of the 133 isolates studied, 36 (27%) were susceptible. Among those, 30 (83%) had *erm*(41) deletions, including all 27 *M. massiliense* and three *M. abscessus*. The remaining six (17%) susceptible isolates were *M. abscessus* with the *erm*(41) T28C mutation. Of note, the A2058G mutation in *rrl* was detected in only a single isolate in the collection; that isolate was resistant, but also had WT *erm*(41).

Finally, we applied PCR to *erm*(41) and consistently detected a shortened PCR product in isolates documented to have the inactivating deletions. Specifically, all 30 isolates with sequence-confirmed deletions in *erm*(41) were identified by PCR. These represented >80% of the susceptible isolates as determined by the extended 14 day incubation. We conclude that PCR provides a highly reliable method to detect inactivating deletions of the *erm*(41) gene and thereby rapidly identify the substantial majority of clarithromycin-susceptible MAG isolates and facilitate effective treatment. Some authors[14,15,33] have suggested that detecting deletions of the *erm*(41) gene may effectively differentiate *M. massiliense* from *M. abscessus* and *M. bolletii*. Our results appear consistent with this possibility. Of the 30 isolates in this report with a deletion in the *erm*(41) gene, three (10%; 95% CI: 2.1%–26.5%) were identified as subspecies *M. abscessus*. We believe that a substantially larger study is needed to define more precisely the reliability of *erm*(41) deletions for identification of *M. massiliense* subspecies.

In conclusion, this study verified that the clarithromycin susceptibility test with extended incubation for up to 14 days identified inducible resistance in 69.2% of MAG isolates that appeared susceptible by the standard 3 day incubation currently recommended.[31] Together, *erm*(41) and mutations in *rrl* currently represent the best defined mechanisms of macrolide resistance among MAG isolates. Sequencing confirmed that the most frequent and consistent correlate of susceptibility was the presence of previously identified inactivating deletions in *erm*(41). Further, those deletions were reliably demonstrated by PCR, which yielded a smaller amplification product readily distinguished from that of intact WT genes. These results suggest that in the management of infections due to MAG, a simple, rapid PCR assay might be more efficient than *in vitro* susceptibility testing.

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Transparency declarations

None to declare.

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