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Prevalence of *erm* methylase genes in clinical isolates of non-pigmented, rapidly growing mycobacteria

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Abstract

The aim of this study was to determine the frequency of *erm* genes coding for macrolide resistance among clinical isolates of non-pigmented rapidly growing mycobacteria (NPRGM) and to evaluate their importance in phenotypic resistance. Broth microdilution susceptibility testing was performed for all NPRGM tested. A PCR assay with consensus primers was used to evaluate the presence of *erm* genes among the 167 clinical isolates studied, which belonged to nine species of NPRGM; *erm* genes were detected in all nine species and 109 strains were *erm*-positive. The highest percentage of *erm*-positive isolates was found among *Mycobacterium mageritense* (100%) and the lowest among *Mycobacterium mucogenicum* (14%). The MICs of macrolides were found to be lower for *erm*-negative isolates (MIC_{90}: 2 mg/L) than for *erm*-positive isolates (MIC_{90}: 16 mg/L), although in some cases high MICs were found for *erm*-negative isolates. The finding that *erm* methylases are present in the majority of the species of NPRGM analysed in this study is not in agreement with conventional susceptibility studies. It therefore appears necessary to use a combination therapy to treat infections caused by NPRGM.

Keywords: Clarithromycin, erithromycin, inducible, methylases, PCR
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Introduction

Treatment of infections caused by non-tuberculous mycobacteria is complicated because each species requires a different therapeutic approach [1]. Among these organisms, non-pigmented rapidly growing mycobacteria (NPRGM) have specific susceptibility characteristics. Treatment modalities for infections with NPRGM include antimicrobials different from those commonly used to treat infections caused by other bacteria (i.e. quinolones, β-lactams or aminoglycosides other than streptomycin) [2–4]. Macrolides are among the antibiotics most commonly used for NPRGM infections [4,5]. These drugs have been used either alone or in combination with other drugs (especially amikacin). However, resistance against these drugs has been described and, in some cases, resistance can develop during monotherapy [6–8].

A recent report examined the molecular mechanisms involved in macrolide resistance among these mycobacteria, in which different *erm* genes appear to be common and methylase production appears to be inducible [9]; the lack of correlation between phenotypic resistance and molecular detection of *erm* was also described.

Here, we report the evaluation of the presence of *erm* genes among clinical isolates of NPRGM and the correlation between the presence of these determinants and the susceptibility phenotype.

Materials and Methods

Clinical isolates of NPRGM were studied. Species identification was achieved using several biochemical tests (nitrate reduction, 3-day arylsulphatase, growth on McConkey agar without crystal violet, use of citrate, mannitol, inositol, sorbitol and rhamnose as carbon sources, and growth in the presence of NaCl (5%)) and PCR-restriction enzyme analysis (PRA) of the *hsp*65 gene. Collection strains *Mycobacterium fortuitum* ATCC 6841^T, *Mycobacterium chelonae* ATCC 35752^T, *Mycobacterium abscessus* DSM 44196^T, *Mycobacterium peregrinum* ATCC 14467^T, *Mycobacterium mucogenicum* DSM 44124, *Mycobacterium septicum* ATCC 700731^T, *Mycobacterium mageritense* ATCC 700351^T, *Mycobacterium porcinum*

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ATCC 33776^T and *Mycobacterium alvei* ATCC 51304^T were included in the study as controls. The results of all biochemical tests as well as of the PRA profiles were considered necessary to definitive identification. If results of identification were considered doubtful or atypical, or if an isolate was of an uncommon species (all species represented by less than three isolates), the isolate was sent to the Mycobacteria Reference Laboratory at the Centro Nacional de Microbiología (Majadahonda, Spain) to confirm the identification.

Susceptibility tests were performed according to Clinical Laboratory Standards Institute (CLSI) standards. Clarithromycin was acquired from Abbott Laboratories, Inc. (Abbott Park, IL, USA), erithromycin from Sigma-Aldrich, Inc. (St Louis, MO, USA) and azithromycin from Pfizer, Inc. (Groton, CT, USA). Concentration ranges were 0.03–64 mg/L. The CLSI breakpoints for clarithromycin are: ≤ 2 mg/L for susceptible; 4 mg/L for intermediate, and ≥ 8 mg/L for resistant.

PCR analysis was performed using the consensus primers CME-1Y (ACG TGG TGG TGG GCA AYCC TG) and CME-2 (AAT TCG AAC CAC GGC CAC CAC T), as described by Nash *et al.* [9]. The PCR protocol included the following steps: 94 °C for 2 min, followed by 25 cycles at 94 °C for 30 seconds, 60 °C for 30 seconds and 72 °C for 30 seconds, followed by 5 min at 72 °C. The reaction gave an amplification product of 175 bp. A negative control, with PCR reaction mix and DNA-free sterile distilled water (Sigma-Aldrich, Inc.), was included for every 20 reactions.

The amplified fragments were purified using the Montage Genomics PCR Cleanup Kit (Millipore Corp., Billerica, MA, USA). The purified fragments were sequenced in an automated sequencer (Secugen SL, Madrid, Spain). Results were analysed using National Center for Biotechnology Information (NCBI) nucleotide BLAST.

To calculate the correlation between phenotypic resistance and the presence of *erm* genes we used Pearson's correlation coefficient. Calculations were performed with SPSS Version 15.0 for Windows (SPSS, Inc., Chicago, IL, USA).

Results

A total of 167 clinical isolates belonging to nine species were included in the study (*M. abscessus* (9), *M. alvei* (2), *M. chelonae* (30), *M. fortuitum* (89), *M. mageritense* (5), *M. mucogenicum* (7), *M. peregrinum* (23), *M. porcinum* (1), *M. septicum* (1)). The MICs for, and *erm* status of, all isolates are shown in Table 1. The highest percentage of *erm*-positive isolates was found among *M. mageritense* (100%) and the lowest among *M. mucogenicum* (14%). The results for the control strains were as follows: *erm* genes were detected in

TABLE 1. MICs (mg/L) for clinical isolates of non-pigmented rapidly growing mycobacteria and *erm* gene status

Strain	Species	ERY	CLA	AZY	*erm*
F193	*M. abscessus*	0.5	≤ 0.03	0.12	neg
M70	*M. abscessus*	2	≤ 0.03	0.5	neg
M83	*M. abscessus*	1	≤ 0.03	≤ 0.03	neg
M86	*M. abscessus*	2	≤ 0.03	2	neg
M25	*M. abscessus*	0.06	≤ 0.03	2	pos
M24	*M. abscessus*	0.12	≤ 0.03	0.5	pos
F95	*M. abscessus*	> 64	32	16	pos
M1	*M. abscessus*	0.5	≤ 0.03	0.25	pos
F64	*M. abscessus*	8	0.25	1	pos
M40	*M. alvei*	> 64	4	16	neg
F108	*M. alvei*	32	16	> 64	pos
F172	*M. chelonae*	0.25	0.06	0.06	pos
F25	*M. chelonae*	≤ 0.03	0.06	0.06	neg
F43	*M. chelonae*	0.06	≤ 0.03	0.06	neg
F176	*M. chelonae*	0.06	≤ 0.03	0.06	neg
F82	*M. chelonae*	1	0.12	0.12	neg
F109	*M. chelonae*	32	4	1	pos
M32	*M. chelonae*	1	0.25	0.12	neg
M4	*M. chelonae*	0.25	0.06	0.25	neg
M61	*M. chelonae*	0.25	0.06	0.25	neg
M52	*M. chelonae*	0.5	0.06	0.25	neg
F51	*M. chelonae*	0.25	≤ 0.03	0.25	neg
F44	*M. chelonae*	0,12	0.06	0.5	neg
F29	*M. chelonae*	2	0.25	0.5	neg
F83	*M. chelonae*	0.06	≤ 0.03	0.5	neg
M16	*M. chelonae*	0.5	≤ 0.03	2	pos
F184	*M. chelonae*	4	4	16	pos
M59	*M. chelonae*	> 64	8	16	pos
F63	*M. chelonae*	≤ 0.03	≤ 0.03	0.5	neg
F237	*M. chelonae*	1	0.06	1	neg
F224	*M. chelonae*	1	0.12	1	neg
M31	*M. chelonae*	2	0.25	1	neg
F211	*M. chelonae*	0.25	< 0.03	4	neg
M80	*M. chelonae*	0.25	≤ 0.03	≤ 0.03	neg
F45	*M. chelonae*	> 64	32	> 64	pos
M94	*M. chelonae*	4	≤ 0.03	4	pos
M30	*M. chelonae*	4	2	32	pos
M93	*M. chelonae*	16	0.25	0.5	pos
F4	*M. chelonae*	16	2	16	pos
F5	*M. chelonae*	64	2	4	pos
M14	*M. chelonae*	≤ 0.03	≤ 0.03	≤ 0.03	pos
M27	*M. fortuitum*	0.06	≤ 0.03	0.5	pos
M33	*M. fortuitum*	0.12	0.06	1	pos
M90	*M. fortuitum*	0.25	≤ 0.03	0.5	pos
M35	*M. fortuitum*	0.25	≤ 0.03	1	pos
F206	*M. fortuitum*	1	0.12	0.25	pos
M92	*M. fortuitum*	1	0.06	4	pos
F207	*M. fortuitum*	1	0.25	≤ 0.03	pos
F22	*M. fortuitum*	4	0.12	0.06	neg
M36	*M. fortuitum*	2	1	32	pos
F36	*M. fortuitum*	4	2	0.06	pos
F191	*M. fortuitum*	8	0.5	0.06	pos
M50	*M. fortuitum*	8	1	8	pos
F236	*M. fortuitum*	1	0.06	0.5	neg
M42	*M. fortuitum*	16	0.06	2	pos
M41	*M. fortuitum*	16	0.06	4	pos
M43	*M. fortuitum*	16	0.5	4	pos
M65	*M. fortuitum*	> 64	4	4	pos
F5A	*M. fortuitum*	64	16	4	pos
M75	*M. fortuitum*	0.5	0.12	0.5	neg
F156	*M. fortuitum*	32	2	0.5	neg
F233	*M. fortuitum*	1	≤ 0.03	0.5	neg
F239	*M. fortuitum*	16	2	32	pos
F232	*M. fortuitum*	4	0.12	1	neg
M28	*M. fortuitum*	16	0.5	≤ 0.03	pos
M23	*M. fortuitum*	32	2	0.12	pos
F221	*M. fortuitum*	32	1	0.25	pos
F250	*M. fortuitum*	32	2	2	pos
F295	*M. fortuitum*	32	2	2	pos
F24	*M. fortuitum*	64	4	8	pos
F92	*M. fortuitum*	64	4	8	pos
F257	*M. fortuitum*	64	4	8	pos
M51	*M. fortuitum*	64	4	8	pos
F8	*M. fortuitum*	> 64	4	8	pos
F69	*M. fortuitum*	64	8	8	pos
F84	*M. fortuitum*	64	8	8	pos
F259	*M. fortuitum*	64	8	8	pos

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TABLE 1. (Continued).

Strain	Species	ERY	CLA	AZY	*erm*
M20	*M. fortuitum*	32	2	2	pos
F240	*M. fortuitum*	4	0.25	1	neg
M17	*M. fortuitum*	32	1	4	pos
F242	*M. fortuitum*	32	4	16	pos
M15	*M. fortuitum*	> 64	4	16	pos
M53	*M. fortuitum*	> 64	4	16	pos
M55	*M. fortuitum*	> 64	4	16	pos
F58	*M. fortuitum*	> 64	8	16	pos
F255	*M. fortuitum*	> 64	8	16	pos
F258	*M. fortuitum*	> 64	8	16	pos
F3	*M. fortuitum*	> 64	16	16	pos
F256	*M. fortuitum*	32	2	8	pos
M29	*M. fortuitum*	32	2	8	pos
M57	*M. fortuitum*	8	4	32	pos
M39	*M. fortuitum*	4	0.5	4	neg
M64	*M. fortuitum*	> 64	4	16	neg
M19	*M. fortuitum*	64	4	32	pos
M54	*M. fortuitum*	> 64	4	32	pos
F20	*M. fortuitum*	64	8	32	pos
M5	*M. fortuitum*	> 64	8	32	pos
M9	*M. fortuitum*	> 64	32	32	pos
F40P	*M. fortuitum*	32	4	64	pos
F175	*M. fortuitum*	> 64	16	64	pos
F241	*M. fortuitum*	> 64	16	64	pos
M2	*M. fortuitum*	> 64	16	64	pos
M66	*M. fortuitum*	> 64	2	32	neg
M11	*M. fortuitum*	> 64	64	32	neg
M7	*M. fortuitum*	> 64	32	64	pos
M60	*M. fortuitum*	> 64	4	64	neg
M8	*M. fortuitum*	> 64	32	64	pos
M89	*M. fortuitum*	64	0.5	4	pos
M74	*M. fortuitum*	64	1	8	pos
M38	*M. fortuitum*	64	8	> 64	neg
M22	*M. fortuitum*	64	2	8	pos
F61	*M. fortuitum*	32	8	> 64	pos
F260	*M. fortuitum*	> 64	8	> 64	pos
M78	*M. fortuitum*	> 64	8	> 64	pos
M82	*M. fortuitum*	> 64	8	> 64	pos
F56	*M. fortuitum*	32	16	> 64	pos
M87	*M. fortuitum*	0.5	≤ 0.03	≤ 0.03	neg
F55	*M. fortuitum*	64	16	> 64	pos
F194	*M. fortuitum*	> 64	32	> 64	pos
M13	*M. fortuitum*	> 64	32	> 64	pos
M77	*M. fortuitum*	64	2	8	pos
M76	*M. fortuitum*	64	2	16	pos
F230	*M. fortuitum*	64	2	32	pos
F247	*M. fortuitum*	64	2	64	pos
F65	*M. fortuitum*	64	2	> 64	pos
M91	*M. fortuitum*	> 64	1	8	pos
M72	*M. fortuitum*	> 64	2	16	pos
F37	*M. fortuitum*	> 64	2	32	pos
F267	*M. fortuitum*	> 64	2	> 64	pos
M79	*M. fortuitum*	> 64	2	> 64	pos
M58	*M. mageritense*	32	2	4	pos
M69	*M. mageritense*	32	2	8	pos
M21	*M. mageritense*	64	4	16	pos
M56	*M. mageritense*	64	8	16	pos
M45	*M. mageritense*	> 64	2	2	pos
M18	*M. mucogenicum*	0.06	≤ 0.03	0.06	neg
F19	*M. mucogenicum*	0.06	≤ 0.03	1	neg
F187	*M. mucogenicum*	0.06	≤ 0.03	1	neg
M71	*M. mucogenicum*	0.5	≤ 0.03	1	neg
F218	*M. mucogenicum*	≤ 0.03	≤ 0.03	≤ 0.03	neg
F155	*M. mucogenicum*	0.06	≤ 0.03	≤ 0.03	neg
F18	*M. mucogenicum*	32	16	> 64	pos
M6	*M. peregrinum*	0.25	≤ 0.03	0.25	pos
F46	*M. peregrinum*	1	0.06	0.25	pos
F15	*M. peregrinum*	2	0.25	0.25	pos
M44	*M. peregrinum*	8	0.12	0.06	pos
F81	*M. peregrinum*	8	2	> 64	pos
M10	*M. peregrinum*	16	0.12	0.5	pos
F48	*M. peregrinum*	0.12	0.06	0.25	neg
F26	*M. peregrinum*	0.06	0.25	0.25	neg
M81	*M. peregrinum*	8	≤ 0.03	0.25	neg
F272	*M. peregrinum*	2	0.12	0.5	neg
M12	*M. peregrinum*	32	0.12	0.5	neg
F53	*M. peregrinum*	0.5	0.06	1	neg
F213	*M. peregrinum*	1	0.06	1	neg
F223	*M. peregrinum*	2	0.25	1	neg
F274	*M. peregrinum*	2	0.25	1	neg
F273	*M. peregrinum*	0.12	≤ 0.03	1	neg
F271	*M. peregrinum*	0.06	0.06	2	neg
F94	*M. peregrinum*	4	2	64	neg
F85	*M. peregrinum*	0.25	0.06	≤ 0.03	neg
M3	*M. peregrinum*	> 64	16	> 64	pos
F10	*M. peregrinum*	0.06	≤ 0.03	≤ 0.03	neg
M49	*M. peregrinum*	1	≤ 0.03	≤ 0.03	neg
M46	*M. peregrinum*	≤ 0.03	≤ 0.03	≤ 0.03	pos
F40G	*M. porcinum*	> 64	8	32	pos
M95	*M. septicum*	2	≤ 0.03	0.5	pos

ERY, erithromycin; CLA, clarithromycin; AZY, azithromycin; pos, *erm*-positive; neg, *erm*-negative.

M. fortuitum ATCC 6841^T, *M. mageritense* ATCC 700351^T, *M. porcinum* ATCC 33776^T and *M. septicum* ATCC 700731^T, but not in *M. abscessus* DSM 44196^T, *M. chelonae* ATCC 35752^T, *M. peregrinum* ATCC 14467^T, *M. mucogenicum* DSM 44124^T or *M. alvei* ATCC 51304^T. As Table 1 shows, MICs were lower for the *erm*-negative isolates, although for some isolates high MICs were observed. When the CLSI breakpoints for clarithromycin are considered, five *erm*-negative isolates (one *M. alvei* and four *M. fortuitum*) were intermediate (three isolates) or resistant (two isolates). By contrast, 60 *erm*-positive isolates appeared to be clarithromycin-susceptible, according to the previously cited criteria (four *M. abscessus*, eight *M. chelonae*, 37 *M. fortuitum*, three *M. mageritense*, seven *M. peregrinum*, and one *M. septicum*). However, MICs of erythromycin were ≥ 8 mg/L for 38 of these isolates and MICs of azithromycin were ≥ 8 mg/L for 21 isolates. The MICs of all tested macrolides were < 4 mg/L for only 18 *erm*-positive isolates.

The bacterial resistance mechanisms of many bacteria have been known for a number of years, especially for *Streptococcus* spp. [10]. However, some resistance mechanisms among other genera, such as mycobacteria, have been described only recently. Although the intrinsic resistance of *Mycobacterium tuberculosis* against macrolides is well known, the corresponding molecular mechanism has been described in detail only in the last decade [11].

It was believed that this resistance was caused by permeability problems involving the special nature of the mycobacterial cell wall. However, the detection of an *erm* methylase (*erm*(37)) changes this view. Other *erm* methylases have been described among various species of mycobacteria, including NPRGM [9]. Development of resistance during monotherapy has been described in *M. chelonae*, in which it results from mutations in the peptidyltransferase region of 23S rRNA [7,8]. More recently, *erm* methylases have been described in many of the species of NPRGM, including *Mycobacterium boenickei*, *Mycobacterium houstonense*, *Mycobacterium*

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goodii, *Mycobacterium neworleansense*, *M. porcinum*, *Mycobacterium wolinskyi*, *M. fortuitum* and *Mycobacterium smegmatis* [9]. These enzymes appeared to be inducible [9,12] and, in some cases, as observed in this study, *in vitro* susceptibility tests gave low MICs for these strains.

A consensus PCR based on the published *erm* sequences was used. As no data are available in the literature about the sensitivity of the PCR using these primers, and as *erm* sequences differ by *c.* 70% [9], it is possible that other genes coding for this type of enzyme cannot be detected with this primer set. Apart from this uncertainty, this report includes a higher number of clinical isolates than other reports and presents some interesting findings.

Firstly, *erm* genes were detected in all species studied, including three that have been reported as *erm*-negative [9] (*M. abscessus*, *M. chelonae* and *M. peregrinum*) and two species not previously studied (*M. alvei* and *M. septicum*), a finding that may reflect several causes. The sequencing of the PCR fragments from *M. abscessus* revealed a nucleotide sequence identity of 90–100% with *erm*(39), of 83–84% with *erm*(38) and of 82–85% with *erm*(40). In *M. chelonae*, identity was 94–99% with *erm*(39), 73–99% with *erm*(38) and 84% with *erm*(40); in *M. peregrinum* it was 95–100% with *erm*(39) and 95% with *erm*(38), according to the data obtained from the NCBI website (http://www.ncbi.nlm.nih.gov/). This finding is especially relevant for *M. abscessus* isolates because no *erm*-positive isolate was reported by Nash *et al.* [9] and because no orthologue of NPRGM *erm* genes appeared in the recent genome sequence of the *M. abscessus* type strain (Genbank accession no. CU458896) (*Mycobacterium abscessus* chromosome: complete sequence; http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&val=NC_010397) and our *M. abscessus* collection strain was *erm*-negative.

Several explanations can be suggested for this discrepancy. There may be local or regional differences between strains, as there are in other aspects of the epidemiology of these mycobacteria. Another explanation may be that strains lose genetic information, even resistance genes, through common laboratory manipulations [13] (i.e. storage for long periods at low temperatures [14]), and collection strains (including type strains) may have fewer genes than clinical isolates. A low number of clinical isolates, as tested by Nash *et al.* [9], may also explain negative results. Finally, it may be possible that consensus primers lack specificity and yield false-positive PCR results. Further experiments will be needed to resolve the discrepant results.

The presence of *erm* genes varied among species; it was more frequent in the clinically relevant species *M. fortuitum*, *M. abscessus* and *M. chelonae* and in *M. mageritense*, a species in which enzyme production seems to be constitutive [9]. This finding may be extremely relevant because macrolides are recommended antibiotics for the therapy of infections caused by *M. chelonae* and *M. abscessus* [2,3,5,15], and because in some cases the presence of *erm* genes was not correlated with high MIC values. In these cases, because these enzymes are inducible, monotherapy with macrolides may be ineffective, despite *in vitro* susceptibility of the strains observed with standardized methods. This, combined with the possibility that resistance may develop through other mechanisms, makes it necessary to treat infections caused by *M. chelonae* and *M. abscessus* with a combination of antibiotics to which they have proved susceptible.

Although this assumption must be confirmed clinically, according to our data a high MIC of erythromycin correlates with the presence of *erm* methylases (Pearson's correlation coefficient 0.563) better than high MICs of either clarithromycin (Pearson's correlation coefficient 0.370) or azithromycin (Pearson's correlation coefficient 0.525) do.

Otherwise, high MICs for strains in which no *erm* genes are detected can be taken as unexplained as a result of low PCR sensitivity, but they can also reflect the presence of other resistance mechanisms, such as mutations in domain V of the 23S RNA or mutations in the ribosomal proteins L4 and L22 (i.e. the absence of *erm* genes does not necessarily imply macrolide susceptibility)[16].

In conclusion, *erm* methylases were present in all species of NPRGM analysed in our study. This finding does not agree with the results of conventional susceptibility studies. It is therefore of great therapeutic importance to use a combination of several antibiotics that have been shown to be active against the relevant clinical isolates in order to avoid the development of resistance *in vivo* as a result of the presence of these enzymes.

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Transparency Declaration

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