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Acquisition of clarithromycin resistance mutations in the 23S rRNA gene of *Mycobacterium abscessus* in the presence of inducible *erm*(41)

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Objectives: Antibiotic therapy of pulmonary *Mycobacterium abscessus* infection is based on a combination treatment including clarithromycin. Recent data demonstrated that *M. abscessus* may carry a chromosomal, inducible *erm* gene coding for the ribosomal methylase Erm(41). The purpose of this study was to investigate whether in patients with chronic *M. abscessus* infection undergoing clarithromycin therapy, *M. abscessus* acquires clarithromycin resistance mutations in the *rrl* gene in addition to the presence of an inducible Erm(41) methylase.

Methods: We determined clarithromycin MICs, *erm*(41) and *rrl* sequences for 29 clinical *M. abscessus* subsp. *abscessus* isolates of five different patients. The isolates were obtained between 2007 and 2011 covering a longitudinal observation period of 2–4 years for the individual patients.

Results: In three out of five patients with an initial *rrl* wild-type isolate, follow-up isolates demonstrated acquisition of resistance mutations in the *rrl* gene in addition to the presence of an inducible Erm methylase.

Conclusions: Our results show that in *M. abscessus*, clarithromycin resistance mutations in the 23S rRNA peptidyltransferase region provide an additional selective advantage independent of a functional *erm*(41) gene.

Keywords: rapidly growing mycobacteria, drug susceptibility, cystic fibrosis, methylase

Introduction

Chronic pulmonary infections caused by rapidly growing mycobacteria (RGM) primarily affect hosts suffering from underlying pulmonary diseases, e.g. bronchiectasis[1] and cystic fibrosis (CF).[2–4] Approximately 80% of these infections are related to *Mycobacterium abscessus*,[5] which is considered to be the most antibiotic-resistant species among all pathogenic RGM.[6] *M. abscessus* infections are a major complication following lung transplantation in CF patients.[7]

Antibiotic treatment of pulmonary *M. abscessus* infections remains problematic, and surgical resection of focal bronchiectasis and cavities is often required as part of the therapeutic regimen.[8,9] Recent studies concluded that although surgery may prolong microbiological response, actual cure of the disease can only be attained in a minority of cases.[10,11] The macrolide clarithromycin is considered a cornerstone in antimicrobial chemotherapy of pulmonary *M. abscessus* infections.[8,12,13] It is the only drug of demonstrated efficacy that can be administered orally. Two main mechanisms of clarithromycin resistance are well established. First, mutations in the drug-binding pocket, in particular at nucleotide positions 2058 and 2059, of the bacterial 23S rRNA gene.[14–17] Corresponding mutations confer high-level resistance to clarithromycin (MIC > 256 mg/L) in bacterial species with a limited number of chromosomal rRNA operons, including *Mycobacterium chelonae* and *M. abscessus*.[18–21] The second mechanism is conferred by a class of genes coding for inducible **e**rythromycin **r**ibosomal **m**ethylases (Erm), which mono- or di-methylate the adenine at position 2058 of the 23S rRNA. Erm genes have been described in many species of rapidly growing mycobacteria.[22] Recently, Nash *et al.*[23] demonstrated that a representative of this gene family, *erm*(41), is present in *M. abscessus* but not in the closely related *M. chelonae*.

Erm genes in subspecies of *M. abscessus* show characteristic differences. Compared with its homologues in *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *bolletii*, the Erm methylase in *M. abscessus* subsp. *massiliense* is dysfunctional due to a 2 bp deletion of nucleotides 64–65 and a 274 bp deletion of nucleotides 159–432.[23,24] In addition, the functionality of the methylase is dependent on the nucleotide at position 28 of the *erm*(41) gene. Wild-type T28 sequevars show inducible

Table 1. Clarithromycin MICs and sequencing results of *erm*(41) and *rrl* genes

			Date of isolation		Clarithromycin MIC (mg/L) by method				Genotype		
					microdilution			Etest			
Patient	Isolate	Source	month/year	months since first isolate	day 3	day 7	day 14	day 7	*erm*(41)[a]	23S peptidyltransferase region	*rpoB*
Control	MAZ01.1	left forearm	08/08	0	0.5	1	1	0.064	C28	wild-type	*M. abscessus* subsp. *abscessus*
MAZ02	MAZ02.1	respiratory tract	06/08	0	1	>128	>128	0.5	T28	wild-type	*M. abscessus* subsp.
	MAZ02.2	respiratory tract	10/08	4	2	>128	>128	0.125	T28	wild-type	*abscessus*
	MAZ02.3	respiratory tract	02/09	8	2	>128	>128	0.25	T28	wild-type	
	MAZ02.4	respiratory tract	10/09	16	1	>128	>128	1	T28	wild-type	
	MAZ02.5	respiratory tract	08/11	38	0.5	128	>128	4–256	T28	wild-type	
MAZ03	MAZ03.1	respiratory tract	01/09	0	0.5–8	64	128	0.25	T28	wild-type	*M. abscessus* subsp.
	MAZ03.2	respiratory tract	05/09	5	<0.25	4	128	0.5	ND	wild-type	*abscessus*
	MAZ03.3	respiratory tract	07/09	7	2	>128	>128	6–256	ND	wild-type	
	MAZ03.4	respiratory tract	09/09	9	<0.25	>128	>128	>256	T28	wild-type	
	MAZ03.5	respiratory tract	04/10	16	<0.25	>128	>128	8–256	ND	wild-type	
	MAZ03.6	respiratory tract	08/10	20	<0.25	>128	>128	>256	ND	wild-type	
	MAZ03.7	respiratory tract	12/10	23	<0.25	>128	>128	>256	ND	wild-type	
	MAZ03.8	respiratory tract	01/11	24	<0.25	>128	>128	>256	T28	wild-type	
	MAZ03.9	wound secretion	01/11	24	<0.25	>128	>128	>256	ND	wild-type	
MAZ04	MAZ04.1	sputum	04/07	0	<0.25	8	64	0.032–0.5	T28	wild-type	*M. abscessus* subsp.
	MAZ04.2	sputum	07/08	15	0.5	>128	>128	0.25–256	T28	wild-type/2058A→G[b]	*abscessus*
	MAZ04.3	sputum	08/09	28	32	>128	>128	>256	T28	2058A→G	
	MAZ04.4	sputum	10/11	54	128	>128	>128	>256	T28	2058A→G	
MAZ07	MAZ07.1	respiratory tract	06/08	0	0.5	16	>128	0.19	T28	wild-type	*M. abscessus* subsp.
	MAZ07.2	respiratory tract	02/09	8	0.5	>128	>128	0.125–256	T28	wild-type/2058A→G[b]	*abscessus*
	MAZ07.3	respiratory tract	04/09	10	1–128	>128	>128	1–256	T28	wild-type/2058A→C/2058A→G[b]	
	MAZ07.4	respiratory tract	09/09	15	2–128	>128	>128	1–256	T28	wild-type/2058A→C/2058A→G[b]	
	MAZ07.5	respiratory tract	12/09	18	>128	>128	>128	>256	T28	2058A→C/2058A→G[b]	
	MAZ07.6	respiratory tract	12/10	30	>128	>128	>128	>256	T28	2058A→C/2058A→G[b]	
	MAZ07.7	respiratory tract	01/11	31	>128	>128	>128	>256	T28	2058A→C/2058A→G[b]	
MAZ08	MAZ08.1	respiratory tract	08/08	0	<0.25	16	>128	1–256	T28	wild-type	*M. abscessus* subsp.
	MAZ08.2	respiratory tract	10/10	26	>128	>128	>128	>256	T28	wild-type/2058A→G[b]	*abscessus*
	MAZ08.3	respiratory tract	12/10	28	>128	>128	>128	>256	T28	wild-type/2058A→G[b]	
	MAZ08.4[c]	respiratory tract	08/11	36	1	>128	>128	1.5–256	T28	wild-type	
MC879[d]	850	sputum	06/91	0	0.5	4	32	ND	T28	wild-type	*M. abscessus* subsp.
	852	sputum	09/93	27	>128	>128	>128	ND	T28	2058A→C	*abscessus*
MC958[d]	855	BAL	11/91	0	0.5	16	>128	ND	T28	wild-type	*M. abscessus* subsp.
	858	BAL	09/92	10	>128	>128	>128	ND	T28	2059A→G	*abscessus*
MC1448[d]	868	sputum	08/94	0	>128	>128	>128	ND	T28	2058A→G	*M. abscessus* subsp. *abscessus*

Continued

Table 1. *Continued*

Patient	Isolate	Source	Date of isolation: month/year	Date of isolation: months since first isolate	Clarithromycin MIC (mg/L) by method: microdilution day 3	microdilution day 7	microdilution day 14	Etest day 7	Genotype: *erm*(41)[a]	Genotype: 23S peptidyltransferase region	Genotype: *rpoB*
MC1082[d]	859	lung	08/92	0	0.5	1	2	ND	T28[e]	wild-type	*M. abscessus* subsp. *massiliense*
	860	CSF	11/93	15	>32	>32	>32	ND	T28[e]	2059A→G	
MC1549[d]	889	sternum	03/95	0	>32	>32	>32	ND	T28[e]	2059A→G	*M. abscessus* subsp. *massiliense*
MC1568[d]	1033	sputum	05/95	0	>32	>32	>32	ND	T28[e]	2059A→C	*M. abscessus* subsp. *massiliense*

ND, not done.
[a]Numbering system of *erm*(41) with the GTG start codon as 1.
[b]Subpopulations carrying the indicated mutations.
[c]Recovered 8 months after administration of clarithromycin had been stopped.
[d]Previously analysed by Wallace *et al.*[21]
[e]Dysfunctional gene product due to deletions of nucleotides 64–65 and 159–432.

clarithromycin resistance, while C28 sequevars do not.[23] Bastian *et al.*[24] recently reported that clinically acquired *rrl* mutations conferring clarithromycin resistance were limited to *M. abscessus* subsp. *massiliense* and *M. abscessus* subsp. *abscessus* C28 sequevars, both of which lack a functional Erm methylase, while no such mutations were found in *M. abscessus* subsp. *abscessus* T28 sequevars. This finding suggested that selection for clarithromycin resistance mutations in the 23S rRNA gene is less likely to occur in strains harbouring intact Erm methylases.[24]

In this study, we analysed a patient cohort suffering from chronic *M. abscessus* infection for the development of constitutive clarithromycin resistance during treatment. Patient isolates were screened for the presence of the inducible *erm*(41) gene and the occurrence of *rrl* mutations.

Materials and methods

Strains

In total, 29 clinical isolates of strains from five patients (MAZ02, MAZ03, MAZ04, MAZ07 and MAZ08) were analysed in this study. As a control, the clarithromycin-susceptible isolate MAZ01.1 was included. An additional nine isolates of six clinical strains that had been described in a previous study[21] were subjected to re-evaluation. For all isolates, clarithromycin MICs were determined, and *rrl* and *erm*(41) sequences were obtained. Identification to subspecies level was performed by nucleic acid sequence analysis of the genes encoding 16S rRNA (*rrs*) and the β-subunit of bacterial RNA polymerase (*rpoB*).[25–27] In order to reflect the biological properties of the strains with respect to clarithromycin resistance, the terms *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *bolletii* and *M. abscessus* subsp. *massiliense* are used to refer to the respective organisms throughout this paper.

Antibiotic susceptibility testing (AST)

Clarithromycin susceptibility testing was done in cation-adjusted Mueller–Hinton medium using the broth microdilution method according to CLSI guidelines.[28] The clarithromycin breakpoints were ≤2 mg/L (susceptible), 4 mg/L (intermediate) and ≥8 mg/L (resistant). Incubation was continued for 14 days at 37°C, with readings after 3, 5, 7, 9 and 14 days. Additionally, clarithromycin MICs were determined by the Etest method (bioMérieux, Marcy l'Étoile, France).

PCR, nucleotide sequencing and strain typing

Total DNA was extracted from the bacterial isolates using InstaGene matrix (Bio-Rad Laboratories, Hercules, CA, USA). The *rrl* gene was amplified using primers SP1 (5′-CCTGCACGAATGGCGTAACG-3′) and SP2mod (5′-CACCAGAGGTTCGTCCGTC-3′). Analysis of the *erm*(41) gene was performed using primers erm41f2 (5′-TGGTATCCGCTCACTGATGA-3′; *erm* positions 34–53) and erm41r2 (5′-GCGGTGGATGATGGAAAG-3′; *erm* positions 484–467), which were designed using the published genome sequence of *M. abscessus* strain ATCC 19977 (GenBank accession number NC_010397.1) as the template. PCR conditions for both the *rrl* and *erm*(41) amplifications were 5 min at 95°C, followed by 40 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 60 s. PCR amplicons were purified using Performa DTR cartridges (EdgeBio, Gaithersburg, MD, USA) and sequenced with the corresponding forward and reverse primers on an Applied Biosystems 3130 Genetic Analyser using BigDye Terminator Cycle Sequencing chemistry (Applied Biosystems, Carlsbad, CA, USA).

For species identification, *rrs* and *rpoB* sequences were analysed using Lasergene SeqMan software (DNASTAR, Madison, WI, USA) and compared with reference sequences using the SmartGene database (SmartGene,

Zug, Switzerland) and the BLAST algorithm. To identify mutations in *rrl*, sequences were compared with the published *M. abscessus* genome (GenBank accession number NC_010397.1). Strains ATCC19977 (T28 sequevar, GenBank accession number FJ358483.1) and CR5701 (C28 sequevar, GenBank accession number HQ127366.1) were used as reference for *erm*(41) sequence analysis of all strains included in this study.

Strain typing was performed by randomly amplified polymorphic DNA PCR (RAPD-PCR) as described previously.[29] In brief, 100 ng of chromosomal DNA was used in four separate PCR reactions with 200 pmol of random primers: INS-2, IS986-FP, OPA2 and OPA18. PCR amplification used 40 cycles of 94°C for 60 s, 36°C for 60 s and 72°C for 120 s. Amplification products were separated by electrophoresis on a 2% agarose gel and detected using GelRed nucleic acid gel stain (Biotium Inc., Hayward, CA, USA). Strain typing was performed for the first and last isolate of each patient and for those isolates which showed a change in clarithromycin susceptibility in comparison with an earlier isolate.

Results and discussion

A total of 29 clinical *M. abscessus* subsp. *abscessus* isolates were obtained from five patients suffering from chronic infections. Isolates were recovered mainly from respiratory specimens obtained over a period of 2–4 years, during which all patients received antibiotic therapy including different clarithromycin regimens. Clarithromycin MICs were determined by broth microdilution and Etest assays. At the genetic level, isolates were genotyped for the *erm*(41) polymorphism at position 28 and for resistance mutations in *rrl* (the 23S rRNA gene) (Table 1). Strain typing was performed using RAPD-PCR and consistent results were obtained for all four primers. On the basis of these analyses, two different resistance patterns were observed.

(i) *M. abscessus* isolates from patients MAZ02 and MAZ03 showed an inducible *erm*(41) gene and no *rrl* mutation acquisition, during observation periods, 3 and 2 years, respectively (Table 1). Both patients had been treated with various antibiotic regimens containing clarithromycin, but exact details (dosages, treatment intervals) were not available to us. Isolates from patient MAZ03 produced identical RAPD-PCR patterns (Figure 1), indicating that the same strain had persisted. In contrast, strain typing of the MAZ02 isolates showed different patterns, which can be interpreted either as reinfection by a different strain or as a mixed infection with a transition from one dominant *M. abscessus* wild-type strain to another, with both strains

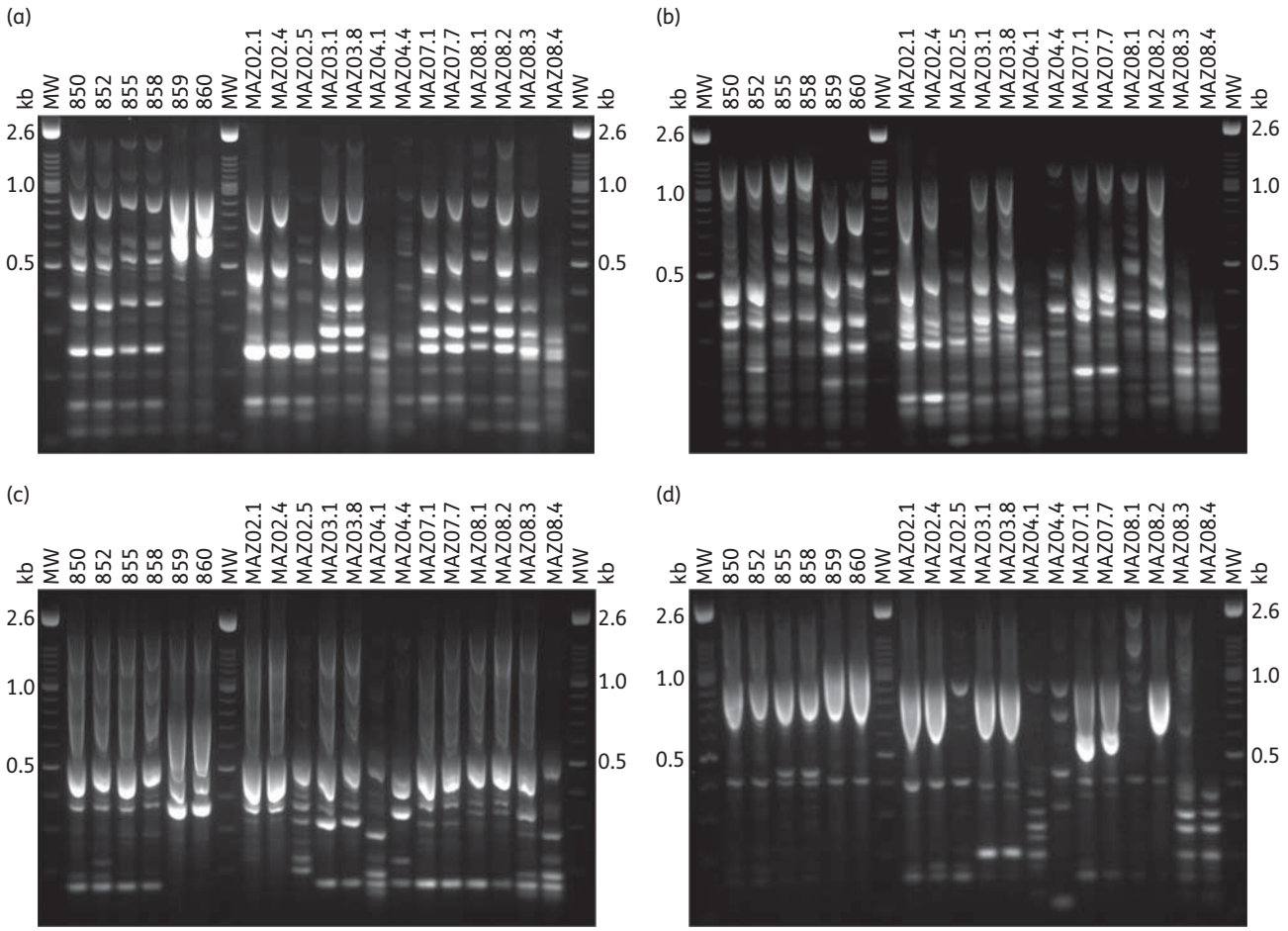

Figure 1. Genotyping of *M. abscessus* isolates using RAPD-PCR. The isolates analysed were: (i) subsequent isolates of five patients MAZ02, MAZ03, MAZ04, MAZ07 and MAZ08; and (ii) isolates published by Wallace *et al.*[21] (isolates 850, 852, 855, 858, 859 and 860). Primers used in RAPD-PCRs were: (a) INS-2, (b) IS986-FP, (c) OPA2 and (d) OPA18. MW, molecular weight standard.

present since the beginning of the observation period (Table 1 and Figure 1).

(ii) Initial isolates from patients MAZ04, MAZ07 and MAZ08 contained an inducible *erm*(41) allele (T28 sequevars), but additional *rrl* mutations resulting in constitutive clarithromycin resistance were detected in subsequent isolates. Electropherograms of the *rrl* genes of early follow-up isolates showed A/C or A/G double peaks at position 2058 (Table 1), indicating the emergence of a mutant population. At the phenotypic level, growth at up to 256 mg/L clarithromycin on the Etest plate was initially observed for single colonies only, but appeared as dense bacterial lawns in later isolates, reflecting the increasing dominance of the mutant (resistant) population. Strain typing for isolates of patients MAZ04 and MAZ07 revealed two different mechanisms for *rrl*-mediated acquisition of high-level resistance in *M. abscessus* subsp. *abscessus* populations with an intact Erm methylase (Figure 1). A wild-type *rrl* population may have been replaced by a mutant population of the same strain (patient MAZ07), or a wild-type *rrl* strain may have been replaced by a mutant population of a different strain (patient MAZ04).

Acquisition and subsequent loss of a 2058A→G mutation in the *rrl* gene was observed in patient MAZ08. The first isolate, MAZ08.1, showed a wild-type *rrl*. The second and third isolates, MAZ08.2 and MAZ08.3, consisted of both wild-type and mutated subpopulations. At this stage, administration of clarithromycin was stopped, and treatment was continued with amikacin, moxifloxacin and imipenem. Interestingly, the fourth isolate (MAZ08.4), which was obtained 8 months after clarithromycin was removed from the antibiotic regimen, showed a wild-type *rrl*. Strain typing the different isolates was consistent with a switch from one wild-type strain (MAZ08.1) to a different wild-type strain (MAZ08.4) with intermediate mixed populations containing both wild-type and mutant bacteria of different genotypes (MAZ08.2 and MAZ08.3) (Figure 1).

These findings prompted us to reanalyse clarithromycin-resistant *M. abscessus* isolates described in the 1996 study by Wallace *et al*.,[21] as at the time of the study *M. abscessus* had been regarded as a single taxonomic entity (without subspecies separation). The data further demonstrate that, regardless of a functional or dysfunctional *erm*(41), high-level resistance to clarithromycin emerging under drug therapy was associated with mutations in 23S rRNA (Table 1).

The data presented in this study show that both *erm*- and *rrl*-mediated resistance to clarithromycin can occur simultaneously in patient isolates, and that a functional Erm does not exclude selection for *rrl* mutations as suggested previously.[24]

Erm methylases are known to produce different MIC values of macrolides-lincosamides-streptogramin B (MLS_B) and ketolide antibiotics, depending on whether they act as mono- or dimethylases on position 2058 of the 23S rRNA.[30] Similar to other representatives of this class of enzymes that have been described in mycobacteria,[22,31–33] Erm(41) is presumed to act as a monomethylase,[23] although it might dimethylate a minor fraction of ribosomal RNA. The occurrence of *rrl* mutations in strains with intact *erm*(41) alleles may thus reflect a limited modification of 23S rRNA position 2058 by monomethylation.

Our data maintain the paradigm that point mutations in 23S rRNA constitute the main mechanism for acquired high-level resistance in mycobacterial species which carry only a single rRNA gene copy. The association of 23S rRNA mutations with clarithromycin resistance in *M. abscessus sensu lato* indicates that, despite an inducible *erm*(41), there is a selective advantage for the acquisition of a 23S rRNA mutation, most probably due to either increased mutation-associated antibiotic resistance or less resistance-associated biological cost.[17,34,35] It remains an unresolved question as to whether patients carrying a wild-type *rrl* strain with an inducible macrolide resistance determinant may still benefit from clarithromycin treatment.

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Transparency declarations

None to declare.

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