

RESEARCH ARTICLE

Inducible and Acquired Clarithromycin Resistance in the *Mycobacterium abscessus* Complex

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Abstract

Purpose

Clarithromycin was considered the cornerstone for the treatment of *Mycobacterium abscessus* complex infections. Genetic resistance mechanisms have been described and many experts propose amikacin as an alternative. Nevertheless, clarithromycin has several advantages; therefore, it is necessary to identify the non-functional *erm(41)* allele to determine the most suitable treatment. The aims of this study were to characterize the molecular mechanisms of clarithromycin resistance in a collection of *Mycobacterium abscessus* complex isolates and to verify the relationship between these mechanisms and the antibiogram.

Materials and Methods

Clinical isolates of *M. abscessus* complex ($n = 22$) from 16 patients were identified using four housekeeping genes (*rpoB*, *secA1*, *sodA* and *hsp65*), and their genetic resistance was characterized by studying *erm(41)* and *rrl* genes. Nine strains were recovered from the clinical isolates and subjected to E-test and microdilution clarithromycin susceptibility tests, with readings at 3, 7 and 14 days.

Results

We classified 11/16 (68.8%) *M. abscessus* subsp. *abscessus*, 4/16 (25.0%) *M. abscessus* subsp. *bolletii*, and 1/16 (6.3%) *M. abscessus* subsp. *massiliense*. T28 *erm(41)* allele was observed in 8 *Mycobacterium abscessus* subsp. *abscessus* and 3 *Mycobacterium abscessus* subsp. *bolletii*. One strain of *M. abscessus* subsp. *bolletii* had an *erm(41)* gene truncated and was susceptible to clarithromycin. No mutations were observed in *rrl* gene first isolates. In three patients, follow-up of initial *rrl* wild-type strains showed acquired resistance.

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Conclusions

Most clinical isolates of *M. abscessus* complex had inducible resistance to clarithromycin and total absence of constitutive resistance. Our findings showed that the acquisition of resistance mutations in *rrl* gene was associated with functional and non-functional *erm(41)* gene. Caution is needed when using *erm(41)* sequencing alone to identify *M. abscessus* subspecies. This study reports an acquired mutation at position 2057 of *rrl* gene, conferring medium-low clarithromycin constitutive resistance.

Introduction

Mycobacterium abscessus complex subspecies are rapid-growing mycobacteria (RGM) responsible for chronic pulmonary infections, cutaneous infections and, in some cases, bacteraemia [1,2]. These subspecies are considered as the most drug-resistant of all the RGM [3–5], which complicates treatment of the infections they cause. Macrolides such as clarithromycin and azithromycin were considered the cornerstone for the treatment of *Mycobacterium abscessus* complex infections [5]. Molecular resistance mechanisms have been described and many experts propose amikacin as an alternative [6]. Nevertheless, clarithromycin has several advantages; therefore, it is necessary to identify the non-functional *erm(41)* allele to determine the most suitable treatment.

Clarithromycin resistance in the *M. abscessus* complex can be constitutive or inducible, depending on two mechanisms. Constitutive resistance involves spontaneous point mutations, selected during macrolide-based chemotherapy, at positions 2058 and 2059 of the *rrl* gene, which encodes for the 23S rRNA [7]. The second mechanism is associated with inducible resistance [8], involving the presence of a functional *erm(41)* gene and following methylation of the ribosome. Constitutive resistance is characterized by *in vitro* high level resistance values to clarithromycin at 3 days of culture [4,7], while inducible resistance associated with the *erm(41)* gene is characterized by *in vitro* MIC resistant values only at 7 and even 14 days [9].

The taxonomy of the *M. abscessus* group is complex and still unresolved. In 2011, *M. bolletii* and *M. massiliense* were classified as a single subspecies (*M. abscessus* subsp. *bolletii*) due to their low genetic diversity [10]. In 2013, whole genome sequencing data analysis supported the differentiation of the *M. abscessus* complex into 3 subspecies: *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *bolletii* and *M. abscessus* subsp. *massiliense* [11]. In the present study, we use this nomenclature for the three subspecies.

It is widely accepted that several housekeeping genes are needed to identify the three subspecies in the complex [12,13]. These three subspecies are closely related, and recombination and gene transfer has been described [11]. The genes most commonly used to classify *M. abscessus* subspecies are the *rpoB*, *secA1*, *sodA*, *recA* and *hsp65* genes [12,13]. Even when powerful new techniques such as matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) [14] are used, identification of *M. abscessus* complex isolates at the subspecies level is not 100% accurate.

The *erm(41)* gene differs slightly between *M. abscessus* complex subspecies. A complete *erm(41)* gene with 10 sequevars is described for *M. abscessus* subsp. *abscessus* [15]: sequevars with nucleotide T28 are associated with inducible clarithromycin resistance, and the ones with nucleotide C28 are linked to clarithromycin susceptibility. In *M. abscessus* subsp. *bolletii*, the *erm(41)* gene is similar to T28 *M. abscessus* subsp. *abscessus* [16]. In *M. abscessus* subsp.

massiliense, the *erm(41)* gene is known to have 2 deletions, making it non-functional. Sequence analysis of the *erm(41)* gene has been used to classify *M. abscessus* complex subspecies [17].

We collected *M. abscessus* complex clinical isolates from 1995 to 2014 at a university hospital in Spain. The aims of this study were to characterize the molecular mechanisms of clarithromycin resistance in a collection of *Mycobacterium abscessus* complex initial isolates, identified at the subspecies level, and to verify the relationship between the genetic resistance mechanisms and the antibiogram results. We also studied 10 isolates in the follow-up of 4 patients by molecular typing and phenotypic and genotypic antibiograms.

Materials and Methods

Ethics statement

This study was retrospective without interaction with patients and all the patients' information was de-identified prior to analysis. Therefore the Fundació de Gestió Sanitaria del Hospital de la Santa Creu i Sant Pau ethics committees waived the need for informed consent and approved this research study (IISBP-CLA-2014-23).

Samples

Twenty-two clinical isolates were obtained between 1995 and 2014 from 16 patients: 3 from skin and 19 from lung infections. Ten isolates from 4 patients were used for follow-up: 3 from patient 1, 3 from patient 2, 2 from patient 3, and 2 from patient 4. DNA from all isolates was recovered for genotypic identification and molecular clarithromycin resistance analysis. Nine first recovered isolates from 9 individual patients were subjected to clarithromycin susceptibility tests.

Subspecies classification

Total DNA was extracted from mycobacterial clinical isolates by thermal shock [18] or InstaGene matrix (Bio-Rad Laboratories, Hercules, CA, USA). Analysis of *rpoB*, *secA1*, *sodA* and *hsp65* genes was performed for identification at the subspecies level, as described previously [12,13]. PCR products were sent to MacroGen for sequencing (Meibergdeef, 1105AZ Amsterdam, The Netherlands). The sequences obtained were analyzed using the BLAST algorithm (NCBI).

Assessing the genetic basis of resistance

The *erm(41)* and *rml* genes were analysed for clarithromycin resistance. The *erm(41)* gene was amplified using primers *ermF* (5' -GACCGGGCCTTCTTCGTGAT-3') and *ermR* (5' -GACTTCCCCGCACCGATTCC-3') [7,8]. The *rml* gene was amplified using primers 19F (5' -GTAGCGAAATTCCCTTGTCGG-3') and 21R (5' -TTCCCGCTTAGATGCTTTTCAG-3') [7]. For the *erm(41)* gene, we analysed the complete gene sequence of 673 bp and T28 polymorphism. For the *rml* gene, we analysed a fragment of 836 bp that included nucleotides 2058–2059, whose mutations cause clarithromycin resistance.

For *erm(41)* and *rml* amplification, the PCR conditions were 5 min at 95°C, then 35 cycles at 95°C for 60 s, 62°C (for *erm(41)*) but 55°C for *rml*) for 60 s, and 72°C for 90 s, followed by 72°C for 10 min in the final extension.

Reference sequences

The reference sequences used for subspecies classification were *M. abscessus* subsp. *abscessus* ATCC 19977T, *M. abscessus* subsp. *bolletii* reference strain CCUG 50184 (GenBank accession

numbers AY859692 for *rpoB* and FJ442839.1 for *secA1*), *M. abscessus* subsp. *bolletii* reference strain CIP 108541 (GenBank accession numbers EU266576.1 for *hsp65* and AY862403 for *sodA*), and *Mycobacterium abscessus* subsp. *massiliense* CCUG 48898 (GenBank accession numbers AY593981 for *rpoB*, AY596465.1 for *hsp65*, NZ_AKVF01000005.1 for *secA1* and AY593975 for *sodA*).

The reference sequence used for the analysis of *erm(41)* and *rrl* genes was the *M. abscessus* subsp. *abscessus* ATCC 19977T (GenBank accession number NC_010397).

Clarithromycin susceptibility testing

Clarithromycin MIC determination was performed in 9/16 initial isolates by the E-test method (bioMérieux, bioMérieux España S.A., Madrid, Spain) in Mueller-Hinton agar plates at 30°C [19]. The clarithromycin susceptibility test was also done in cation-adjusted Mueller-Hinton medium using the broth microdilution method [5] in Sensititre RAPMYCOI plates (Sensititre, Trek Diagnostic Systems, East Grinstead, United Kingdom) according to CLSI guidelines [9]. Strains were incubated for 14 days, and clarithromycin MIC was read at 3, 7 and 14 days. Clarithromycin MIC cut-off for microdilution ranges from ≤ 2 susceptible to ≥ 8 resistant, and ≤ 1 susceptible to ≥ 4 resistant for the E-test [19].

Patient follow-up

Strain typing in the patient follow-up was performed by variable-number of tandem-repeat analysis (VNTR). The primers used were TR45, TR109, TR116, TR150, TR155 and TR172. The six pairs of primers and loci were the same as used by Wong *et al.* [20]. The PCR conditions for VNTR were as follows: a initial denaturalization step of 15 min at 95°C; 30 cycles of denaturalization at 95°C for 60 s, annealing at 59°C for 60 s and an extension at 72°C for 90 s, with a final elongation step at 72°C for 10 min. PCR amplicons were analyzed using the EPOCH capillary electrophoresis system (BioTek, Germany). Results were analyzed using GeneMapper (Applied Biosystems).

Sequences accession numbers

The obtained sequences for each gene were submitted to GenBank and given the following accession numbers: KT185536 to KT185557 for *rpoB*, KT185514 to KT185535 for *hsp65*, KT185603 to KT185624 for *sodA*, KT185581 to KT185602 for *secA1*, KT185492 to KT185513 for *erm41* and KT185558 to KT185580 for *rrl*.

Results

Multiple gene-based subspecies classification

Analysis of the initial isolate from each patient identified 11/16 *M. abscessus* subsp. *abscessus* (68.8%), 4/16 *M. abscessus* subsp. *bolletii* (25.0%) and 1/16 *M. abscessus* subsp. *massiliense* (6.3%). For all isolates except one, we observed agreement between *rpoB*, *secA1*, *sodA* and *hsp65* genes sequences. One strain (isolate 08I004 from Table 1) was *M. abscessus* subsp. *abscessus* according to *rpoB*, *sodA* and *hsp65* analysis but *M. abscessus* subsp. *bolletii* according to *secA1* gene. Table 1 shows subspecies classification of the 16 first clinical isolates included in the study.

Genetic basis of resistance to clarithromycin in *M. abscessus* complex

Table 1 also shows *rrl* and *erm(41)* results and the type of resistance in initial isolates. No mutations were observed in the *rrl* gene in any of these isolates. For the *erm(41)* T28 polymorphism,

Table 1. Clarithromycin resistance molecular mechanisms, clarithromycin susceptibility testing and subspecies identification of *M. abscessus* complex strains.

Patient	Strain	Date	Identification of the strain	VNTR profile ^a	Source	Ern nt ^b 28 (Functionality)	rrf ^c	MIC ^{d,e,f,g,h}					
								Microdilution			E-test		
								3D	7D	14D	3D	7D	14D
1	071011	05/06/2007	<i>M. abscessus</i> subsp. <i>abscessus</i>	Profile I	Lung	C (Not functional)	Wt	<0.06	0.25	<2	<0.016	0.19	1.5
1	101281	24/10/2010	<i>M. abscessus</i> subsp. <i>abscessus</i>	Profile I	Lung	C (Not functional)	A2058G	>16			>256		
1	131330	08/10/2013	<i>M. abscessus</i> subsp. <i>abscessus</i>	Profile I	Lung	C (Not functional)	A2058G	>16			>256		
2	091152	24/03/2009	<i>M. abscessus</i> subsp. <i>abscessus</i>	Profile II	Lung	T (functional)	Wt	<0.06	4	>16	0.38	>256	>256
2	111110	23/02/2011	<i>M. abscessus</i> subsp. <i>abscessus</i>	Profile II	Lung	T (functional)	Wt	<2			0.75		
2	111441	12/12/2011	<i>M. abscessus</i> subsp. <i>abscessus</i>	Profile II	Lung	T (functional)	A2057G/A2057 ⁱ	8			64		
3	091380	17/09/2009	<i>M. abscessus</i> subsp. <i>abscessus</i>	Profile III	Lung	T (functional)	Wt	0.12	8	>16	0.094	4	>256
3	111289	05/07/2011	<i>M. abscessus</i> subsp. <i>abscessus</i>	Profile III	Lung	T (functional)	A2059G	>16			>256		
4	041462	22/12/2004	<i>M. abscessus</i> subsp. <i>bolletii</i>	Profile IV	Lung	T (functional)	Wt	-	-	-	-	-	-
4	051586	24/11/2005	<i>M. abscessus</i> subsp. <i>bolletii</i>	Profile IV	Lung	T (functional)	Wt	-	-	-	-	-	-
5	001031	01/12/1995	<i>M. abscessus</i> subsp. <i>bolletii</i>	ND	Lung	T (functional)	Wt	0.12	16	>16	0.38	16	>256
6	141016	17/01/2014	<i>M. abscessus</i> subsp. <i>bolletii</i>	ND	Lung	T (functional)	Wt	0.12	8	>16	<0.016	0.38	>256
7	071004	03/04/2007	<i>M. abscessus</i> subsp. <i>bolletii</i>	ND	Skin	Gen truncated (Not functional)	Wt	-	-	-	-	-	-
8	051284	15/07/2005	<i>M. abscessus</i> subsp. <i>massiliense</i>	ND	Skin	Gen truncated (Not functional)	Wt	-	-	-	-	-	-
9	081420	31/10/2008	<i>M. abscessus</i> subsp. <i>abscessus</i>	ND	Lung	T (functional)	Wt	0.12	16	>16	0.25	>256	>256
10	101050	18/02/2010	<i>M. abscessus</i> subsp. <i>abscessus</i>	ND	Lung	T (functional)	Wt	-	-	-	-	-	-
11	061323	20/08/2006	<i>M. abscessus</i> subsp. <i>abscessus</i>	ND	Lung	T (functional)	Wt	-	-	-	-	-	-
12	091456	04/12/2009	<i>M. abscessus</i> subsp. <i>abscessus</i>	ND	Lung	T (functional)	Wt	-	-	-	-	-	-
13	081004	28/02/2008	<i>M. abscessus</i> subsp. <i>abscessus</i>	ND	Skin	T (functional)	Wt	0.25	8	>16	0.125	8	>256
14	011019	03/10/1997	<i>M. abscessus</i> subsp. <i>abscessus</i>	ND	Lung	T (functional)	Wt	0.12	>16	>16	0.125	192	>256
15	141019	23/01/2014	<i>M. abscessus</i> subsp. <i>abscessus</i>	ND	Lung	C (Not functional)	Wt	0.06	0.12	<2	<0.016	<0.016	<1
16	141069	13/03/2014	<i>M. abscessus</i> subsp. <i>abscessus</i>	ND	Skin	C (Not functional)	Wt	-	-	-	-	-	-

^a ND, Not determined

^b nt, Nucleotide

^c Wt, Wild type

^d D, days

^e MIC values in µg/mL

^f MIC ranges for microdilution broth: ≤ 2, Susceptible; ≥ 8, Resistant

^g MIC ranges for E-test: ≤ 1, Susceptible; ≥ 4, Resistant

^h The hyphen indicates that phenotypic techniques were not performed due to strain non-recovery.

ⁱ Double peak in DNA sequence

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we identified 8 *M. abscessus* subsp. *abscessus* and 3 *M. abscessus* subsp. *bolletii*. For the *erm(41)* C28 polymorphism, we identified 3 *M. abscessus* subsp. *abscessus*. We found an *erm(41)* truncated gene in 1 *M. abscessus* subsp. *massiliense* and in 1 *M. abscessus* subsp. *bolletii*, in each case with two deletions at positions 64 and 65 and a final deletion of 276 bp, as described in the literature for *M. abscessus* subsp. *massiliense* [16].

For the first time, to our knowledge, in this study was found an *erm(41)* gene truncated in 1 *M. abscessus* subsp. *bolletii*. Data of the similarity of the *rpoB*, *secA1*, *sodA* and *hsp65* gene sequences of this strain and the reference strains of *M. abscessus* complex is shown in the Table 2.

Clarithromycin susceptibility testing

We recovered 9 strains of the *M. abscessus* group for phenotypic analysis. E-test and microdilution methods were performed with recovered strains to compare the two methods and relate the *in vitro* MIC results with our genotypic analysis. Table 1 shows clarithromycin MIC for the E-test and microdilution. All strains were clarithromycin-susceptible with both techniques at 3 days. Five out of nine strains were resistant at 7 days for both methods and two strains were susceptible. One strain was resistant for E-test and susceptible for microdilution, the remaining strain was susceptible for E-test and resistant for microdilution (Table 1). At day 14, 7/9 strains were resistant with both methods. In both methods, susceptible and resistant strains agreed at day 14.

Patient follow-up

Table 1 also describes the follow-up results for 4 patients: *M. abscessus* subsp. *abscessus* strains obtained from 3 patients (Patients 1–3) showed the acquisition of a point mutation in the *rrl* gene. Patient 1, *erm(41)* C28 sequevar, showed an acquired resistance at position A2058G. Patient 3, *erm(41)* T28 sequevar, showed an acquired resistance at position A2059G. The *erm(41)* T28 sequevar from patient 2 showed a double peak at position A2057G/A in the DNA sequence, indicating a new selected mutation for clarithromycin constitutive resistance. In contrast, the *M. abscessus* subsp. *bolletii* strain from patient 4 showed no acquired mutation in the *rrl* gene.

All the strains studied in the follow-up were identical to the initial isolates for each patient. Each patient had a different strain. Table 1 also shows the profile of the variable-number of tandem-repeat assay.

Table 2. Comparison between *Mycobacterium abscessus* subsp. *bolletii* 071004 strain and *Mycobacterium abscessus* complex reference strains.

Strain	Reference Strains	Percentage of identity			
		<i>rpoB</i>	<i>hsp65</i>	<i>secA1</i>	<i>sodA</i>
71004 <i>M. abscessus</i> subsp. <i>bolletii</i>	<i>M. abscessus</i> subsp. <i>abscessus</i> ATCC 19977T	95.75	98.6	98.02	98.64
	<i>M. abscessus</i> subsp. <i>bolletii</i> CCGU 50184 ^b and CIP 108541 ^c	99.45	100	100	99.79
	<i>M. abscessus</i> subsp. <i>massiliense</i> CCUG48898	98.27	99.3	98.71	98.46

^a Values shown in bold indicate the highest percentage of identity

^b *M. abscessus* subsp. *bolletii* CCGU 50184 used for *rpoB* and *secA1* genes.

^c *M. abscessus* subsp. *bolletii* CIP 108541 used for *hsp65* and *sodA* genes.

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Discussion

Clarithromycin is a key agent in the treatment of *M. abscessus* complex infections [9]. The main cause of treatment failure is inducible resistance [8,21]. Significant differences in the *erm(41)* gene associated with inducible resistance are found among the *M. abscessus* complex [15,22], suggesting that an accurate *erm(41)* allele identification is important to predict treatment outcomes. As the three *M. abscessus* complex subspecies are closely related and show horizontal gene transfer [11,23], more than one housekeeping gene is needed for subspecies identification [13]. Nevertheless, the genes to be used are not defined [24]. This work aimed to characterize the molecular mechanisms of clarithromycin resistance in the *M. abscessus* complex and to verify the relationship between these mechanisms and the clarithromycin susceptibility test.

In contrast with some authors [12,13] and in agreement with Nie *et al.* [25], we found each gene (*rpoB*, *secA1*, *sodA* and *hsp65*) was able to classify *M. abscessus* subspecies, with the exception of one strain, which was identified as *M. abscessus* subsp. *abscessus* by *rpoB*, *sodA* and *hsp65*, and *M. abscessus* subsp. *bolletii* by *secA1* gene. In contrast with our results, Tan *et al.* [26] were able to correctly classify all their strains by sequencing the *secA1* gene. *M. abscessus* subsp. *abscessus* was the most predominant subspecies of the complex (68.8%), in keeping with findings in the literature [12,21,27], where it ranges from 51.2% to 78.5%. Next in predominance was *M. abscessus* subsp. *bolletii* (25.0%), in contrast with many papers where the second most frequent subspecies is *M. abscessus* subsp. *massiliense* [16,28].

Regarding genetic resistance mechanisms, *rrl* gene sequencing in the initial isolates showed an absence of mutations. This is in contrast with previous reports [21,29], where the presence of constitutive clarithromycin resistance ranges from 2.7% to 28.6%. In agreement with the literature, however, we observed inducible resistance due to nucleotide T28 of *erm(41)* in 68.8% (11/16) of *M. abscessus* complex isolates [8,21].

As previously reported, *erm(41)* is a subspecies-specific gene in the *M. abscessus* group [7,16]. We found only one discrepancy with respect to the published criteria [7,17], attributed to an *M. abscessus* subsp. *bolletii* strain with a truncated *erm(41)* gene. To our knowledge, this is the first description of *M. abscessus* subsp. *bolletii* with a non-functional truncated *erm(41)* gene. The strain (07I004) matched *M. abscessus* subsp. *bolletii* reference strains CCUG 50184 and CIP108541 for the four housekeeping genes studied but not for the *erm(41)* gene. This finding supports other reports suggesting that gene transfer and recombination occurs between subspecies [11,28]. Several cases of *M. abscessus* subsp. *massiliense* with the functional *erm(41)* gene have been reported [30]. Our data thus support the notion that *erm(41)* is not a subspecies-specific gene, and that *erm(41)* gene sequencing should not be used as the only technique to classify *M. abscessus* complex subspecies.

The data produced by our study revealed the acquisition of constitutive clarithromycin resistance. Constitutive resistance to clarithromycin due to mutations in *rrl* was selected in both *erm(41)* C28 (as shown in Patient 1) [7] and *erm(41)* T28 strains (as shown in Patients 2 and 3) [21]. With the advantage of having the initial *rrl* wild-type strains, our results support the idea, in opposition to Bastian *et al.* [7], that selection of *rrl* mutants is similar in T28 and C28 strains.

We described the acquisition of a 2057 point mutation in a T28 *M. abscessus* subsp. *abscessus* strain with an initial *rrl* wild-type isolate. A double peak in the 2057 DNA sequence was observed, which suggests the coexistence of two mixed DNA populations (an *rrl* wild-type and a 2057 mutated population), since these mycobacteria possess only a single copy of the *rrl* gene in their genome [5]. The amplification and DNA sequencing was repeated three independent times to discard amplification or sequencing errors. Further work should include cloning to determine whether two variants exist in this strain.

It is important to emphasize that mutations at position 2057 of *rml* in other microorganisms are related to medium-low levels of clarithromycin resistance *in vitro* [31], as seen in this study. Vester *et al.* [31] also reported that mutations at positions 2057 and 2611 of the *rml* gene caused resistance to macrolides and ketolides due to their proximity to the action centre. Mutations at these positions obstruct the link between the antibiotic and its target, and cause low-level resistance to 14-membered-ring macrolides and no resistance to 16-membered-ring macrolides. In contrast, recently, Luo *et al.* [32] reported a 2057 point mutation in a *M. abscessus* subsp. *abscessus* which showed a high level resistance (MIC >256 µg/mL).

In our study we found complete agreement between *erm(41)*, *rml* sequencing and clarithromycin susceptibility testing. After a 3-day culture, all strains were susceptible as was observed with both methods (E-test and microdilution). Following a 7-day culture, 85% (6/7) of inducible resistance [*erm(41)* T28 sequevar] was detected, and after a 14-day culture, we detected all inducible resistance strains. Although we did not find significant differences between the two methods, one strain with the E-test and one strain with the microdilution method took 14 days to reveal resistance. This is in contrast with other authors who report differences between E-test and microdilution performance [33]. Although the microdilution is the standard method according to the CLSI [9], in our study, the E-test showed the same results as the microdilution, and MIC reading at early stages was easier.

We would like to emphasize that one of the limitations of our study is the relatively small amount of isolates included. This is important to consider when drawing conclusions on epidemiology, but it does not invalidate the molecular observations.

Conclusion

In conclusion, our findings identified one strain of *M. abscessus* subsp. *bolletii* with a truncated and non-functional *erm(41)* gene. Caution is needed if *erm(41)* gene sequencing is the only technique used for subspecies identification. Although clarithromycin constitutive resistance is not detected in the initial isolates, it can be acquired in T28 and C28 *erm(41)* strains of the *M. abscessus* complex. Finally, the acquisition of a mutation at position 2057 in the *rml* gene confers medium-low level resistance to clarithromycin.

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Author Contributions

Conceived and designed the experiments: PC. Performed the experiments: MR FM MG CM ME. Analyzed the data: MR FM PC. Contributed reagents/materials/analysis tools: MR FM MG CM ME PC. Wrote the paper: MR PC.

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