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Assessment of Clarithromycin Susceptibility in Strains Belonging to the *Mycobacterium abscessus* Group by *erm*(41) and *rrl* Sequencing▽

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Clarithromycin was the drug of choice for *Mycobacterium abscessus* infections until inducible resistance due to *erm*(41) was described. Because *M. abscessus* was split into *M. abscessus* sensu stricto, *Mycobacterium massiliense*, and *Mycobacterium bolletii*, we looked for *erm*(41) in the three species and determined their clarithromycin susceptibility levels. Ninety strains were included: 87 clinical strains from cystic fibrosis patients (61%) and others (39%), representing 43 *M. abscessus*, 30 *M. massiliense*, and 14 *M. bolletii* strains identified on a molecular basis, and 3 reference strains. Clarithromycin and azithromycin MICs were determined by broth microdilution and Etest with a 14-day incubation period. Mutations in *rrl* (23S rRNA gene) known to confer acquired clarithromycin resistance were also sought. *erm*(41) was detected in all strains but with two deletions in all *M. massiliense* strains. These strains were indeed susceptible to clarithromycin (MIC_{90} of 1 μg/ml) except for four strains with *rrl* mutations. *M. abscessus* strains harbored an intact *erm*(41) but had a T/C polymorphism at the 28th nucleotide: T28 strains (Trp10 codon) demonstrated inducible clarithromycin resistance (MIC_{90} of >16 μg/ml), while C28 strains (Arg10) were susceptible (MIC_{90} of 2 μg/ml) except for two strains with *rrl* mutations. *M. bolletii* strains had *erm*(41) sequences similar to the sequence of the T28 *M. abscessus* group, associated with inducible clarithromycin resistance (MIC_{90} of >16 μg/ml). *erm*(41) sequences appeared species specific within the *M. abscessus* group and were fully concordant with clarithromycin susceptibility when *erm*(41) sequencing was associated with detection of *rrl* mutations. Clarithromycin-resistant strains, including the six *rrl* mutants, were more often isolated in cystic fibrosis patients, but this was not significantly associated with a previous treatment.

Respiratory infections due to rapidly growing mycobacteria were first attributed to the *Mycobacterium chelonae* complex (45). Some years later, it appeared that they were mostly due to *Mycobacterium abscessus* (16), a newly described species that was distinguished from *M. chelonae* by DNA-DNA hybridization (23, 25). Indeed, the two species were difficult to distinguish by cultural and biochemical features and even by 16S rRNA gene sequencing, which is widely used for bacterial identification (8, 21). Routine *M. abscessus* identification became possible mostly with the wide use of PCR sequencing of housekeeping genes other than ribosomal genes (12, 13, 18). Recently, the *M. abscessus* species has been subclassified into three new species on the basis of *rpoB* sequences: *M. abscessus* (sensu stricto), *Mycobacterium massiliense*, and *Mycobacterium bolletii* (1, 3). Further taxonomic studies showed that differentiation of the three species was not trivial; they still shared ribosomal sequences, and even multilocus sequencing approaches cannot clearly assign clinical strains to one of the three species (24, 27, 43, 49).

The *M. abscessus* group (also called *M. abscessus* sensu lato and comprising the three species described above) is now considered the prominent mycobacteria, along with *Mycobacterium avium*, involved in bronchopulmonary infection in patients with cystic fibrosis or chronic pulmonary diseases (14, 16, 37, 40). Several outbreaks of skin and soft tissue disease were also recently reported, demonstrating the importance of this group in health care-associated infections, as well as in infections associated with surgical tourism (22, 24, 43). Infections due to the *M. abscessus* group are difficult to treat (5, 17) because these mycobacteria are intrinsically resistant not only to the classical antituberculous drugs but also to most of the antibiotics that are currently available (8). In the 1990s, clarithromycin became the drug of choice for *M. abscessus* infections, and therapeutic successes were reported (16, 30, 32). Even the observation of the *in vivo* selection of mutants with acquired resistance to clarithromycin reinforced the idea that clarithromycin was highly active against *M. abscessus* (44). Acquired resistance was associated with point mutations (at positions A2058 and A2059) in a region of the *rrl* gene encoding the peptidyltransferase domain of the 23S rRNA (44), as has also been described in other bacteria (10, 31). However, primary failures with no selection of *rrl* mutants and low efficacy of the clarithromycin regimen were also reported without clear explanations for the failures (15, 17). A second mechanism of resistance to macrolides has been described in the *M. abscessus* complex as inducible clarithromycin resistance conferred by

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the *erm*(41) gene (33). Nash and colleagues showed that although a strain might appear susceptible after three days of *in vitro* incubation (35), due to induction of the synthesis of a methyltransferase (33), the strain was clarithromycin resistant if incubation was extended to 14 days or if the strain was preincubated with clarithromycin. Consequently, the use of clarithromycin in the treatment of *M. abscess*us complex infections was discussed, and because only rare drugs (amikacin or cefoxitin) can be proposed as alternatives, the treatment of these infections is nearly impossible (15, 16).

Nash et al. (33) and recently Kim et al. (19) had sought *erm*(41) in several strains of *M. abscessus* and *M. massiliense*, but only two strains of *M. bolletii* strains have been studied so far. From their results, it appeared that most of the *M. massiliense* strains are susceptible to clarithromycin, whereas susceptibility was less clear for *M. abscessus*, with some strains being susceptible and others resistant. In addition, a polymorphism in *erm*(41) (T or C) was described at position 28 and was associated with susceptibility in nine *M. abscessus* strains harboring C28 (19, 33). However, since identification was not done on the molecular basis described above and because in Kim et al. susceptibility testing did not show results with extended incubation, more data are needed on the correlation between the *erm*(41) genotype and the clarithromycin phenotype. In order to provide advice for the treatment of *M. abscessus* infections, we need to know whether detection of a complete *erm*(41) sequence in strains is associated with clarithromycin resistance and whether such resistance relies on a species-dependent intrinsic resistance characteristic.

Our objective was to seek the presence of *erm*(41) in a large collection of clinical strains precisely identified as *M. abscessus*, *M. massiliense*, and *M. bolletii* and to correlate these results with clarithromycin susceptibility. We showed that, apart from the strains with acquired resistance to clarithromycin that harbored expected *rrl* mutations, the *erm*(41) sequence differentiates the three species, and its specific features (presence of deletions and nature of the amino acid at position 10) are predictive for clarithromycin susceptibility or resistance. In our study, clarithromycin-resistant strains were more often isolated in cystic fibrosis than in other underlying diseases, but we could not show that it was statistically significantly associated with a previous treatment.

MATERIALS AND METHODS

Strains. Clinical strains were isolated from two clinical microbiology laboratories: the Centre National de Référence des Mycobactéries et Résistance des Mycobactéries aux Antituberculeux, or National Reference Laboratory for Mycobacteria and Resistance to Antituberculous Drugs [NRL]) and the Laboratory of Bacteriology at Ambroise Paré Hospital (APH). All isolates were harvested from patients who met the criteria for diagnosis of respiratory disease (15). Some of the APH isolates were harvested from patients in a previously described cystic fibrosis cohort (27, 39). The other strains have been consecutively received by the NRL for identification and susceptibility testing from 2006 to 2009. Overall, 87 clinical strains belonging to the *M. abscessus* complex were studied. Reference strains were *M. abscessus* CIP 104536^T (ATCC 19977^T), *M. bolletii* CIP 108541^T, and *M. massiliense* CIP 108297^T.

Clinical strains were identified molecularly using a first screening by the GenoType Mycobacteria CM assay (Hain Lifescience), followed by PCR sequencing of *hsp*, *rpoB*, and *sodA* as described by Macheras et al. (27), and were subsequently assigned to the species *M. abscessus*, *M. bolletii*, or *M. massiliense*.

Susceptibility testing. MICs of clarithromycin were determined in Mueller-Hinton medium by the broth microdilution method, as recommended (15), using Sensititer RGMYCO plates (Trek Diagnosis Systems/Biocentric, France). Plates were submitted to an extended incubation as described by Nash et al. (33), with successive readings after 5, 7, 9, and 14 days of incubation at 30°C. MICs of clarithromycin were also determined using the Etest method (AB Biodisk) with similar successive readings. In addition, MICs of azithromycin and quinupristin-dalfopristin were determined by the Etest method because they are not part of the commercially available Sensititer plate.

***erm*(41) and *rrl* PCR sequencing.** *erm*(41) detection was performed using three sets of primers: MC8-22f (5′-GAGCGCCGTCACAAGATGCACA-3′) and MC8-27r (5′-GTGCTGGTGATCAGGCGGCGC-3′) for PCR1, ERM1f (5′-CGCCAACGACGAGCAGCTCG-3′) and ERM2r (5′-GCCGAATCCGGTGTTCGCTC-3′) for PCR2, and ERM3f (5′-GCCGAATCCGGTGTTCGCTC-3′) and MC8-27r (5′-GTGCTGGTGATCAGGCGGCGC-3′) for PCR3. The first set (MC8-22 and MC8-27), described by Nash et al. (33), encompasses the *erm*(41) gene and was used for *erm*(41) detection. The two other sets were designed on the basis of the GenBank sequence of strain MAB30 (GenBank EU590129.1) in order to amplify the 5′ and 3′ ends of the gene, respectively, and were used for sequencing. PCRs were carried out as described previously (33) except that the hybridization temperature was 57°C for PCR1 and PCR2.

Mutations in the 23S rRNA gene (*rrl*) were sought by PCR sequencing as described by Wallace et al. (44). Sequences were compared to the *M. abscessus* genome reference sequence NC_010397 (38).

Amplified DNA fragments were sequenced using the same primers as in the PCR and as described by Brossier et al. (7). Briefly, unincorporated nucleotides and primers were removed by filtration with Microcon 100 microconcentrators (Amicon Inc., Beverly, MA), and the gene targets were sequenced using a Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems Inc., Foster City, CA) in an ABI Prism 310DNA sequencer (Applied Biosystems).

Nucleotide sequence accession numbers. Sequences of the *erm*(41) gene in *M. massiliense* CIP 108297, *M. bolletii* CIP 108541, the T28 *M. abscessus* sequevar, and the C28 *M. abscessus* sequevar have been deposited in the GenBank database under accession numbers HQ127368, HQ127367, HQ127365, and HQ127366, respectively.

RESULTS

Presence of the *erm*(41) gene in clinical isolates and *erm*(41) sequences. For all strains of the three species, PCR1 generated an amplicon showing the presence of the *erm*(41)gene at the expected size (892 bps) for *M. abscessus* and *M. bolletii* strains and at a size approximately 250 bp smaller for *M. massiliense* strains.

erm(41) sequences were compared first within the strains of the same species and then to the published sequence of the strain MAB30 (33). This double comparison elucidated features of the *erm*(41) sequences that appeared species specific: (i) deletions, (ii) promoter sequences, and (iii) the 28th nucleotide belonging to the 10th codon. These features are detailed in Table 1.

All *M. massiliense* strains share 100% homology for *erm*(41), with two deletions observed in comparison to the reference sequence (33) and specific features which are described in Table 1. There were also some additional single nucleotide differences (GenBank accession number HQ127368) in comparison with the MAB30 sequence.

We could distribute the *M. abscessus* strains into two groups that differ only with regard to the nucleotide at position 28 in *erm*(41) (i.e., the 10th codon in the amino acid sequence). The first group has a thymine 28 (T28 *M. abscessus* sequevar; GenBank accession number HQ127366) corresponding to a tryptophan codon. The *M. abscessus* type strain (ATCC 19977^T) belongs to this T28 *M. abscessus* sequevar. The second group has a cytosine 28 (C28 *M. abscessus* sequevar; GenBank accession number HQ127365) corresponding to an arginine codon. The MAB30 strain belongs to this C28 *M. abscessus* sequevar, as described by Nash (33).

erm(41) sequences for *M. bolletii* strains were 98% homolo-

TABLE 1. Sequence features of the *erm*(41) gene and upstream region that were species specific for *M. abscessus* sensu stricto, *M. bolletii*, and *M. massiliense* strains[a]

erm(41) gene feature	*M. abscessus*	*M. bolletii*	*M. massiliense*
Size of PCR1 amplicon (bp)	892	892	616
Promoter sequence at position −35	TATCGA	TGTCGA	TGTCGA
Nucleotide at position 28	T or C	T	T
Deletion	No	No	Nucleotides 64 and 65; 276 bp after nucleotide 158[b]
Positions of nucleotide substitutions[b]			
171	A	A or G	−[c]
231	T	T or C	−
249	G	G or A	−
253	C	C or A	−
255	G	G or T	−
258	T	T or C	−
312	A	C	−
336	T	C	−
414	A	A or G	−

[a] The reference sequence is that of MAB30 (GenBank EU590129.1) (33).
[b] Numbering system of *erm*(41), with the GTG start codon as 1.
[c] −, no nucleotide at this position because of the 276-bp deletion.

gous to the sequence of MAB30 and other *M. abscessus* sensu stricto sequences. Among *M. bolletii* strains, the sequence varied with regard to the strain considered (GenBank accession number HQ127367). The −35 sequence was the same as that of *M. massiliense*, and the nucleotide at position 28 was also a thymine. Two differences were consistently observed among *M. bolletii* strains at nucleotide positions 312 and 336, but polymorphisms described in Table 1 were observed equally among *M. bolletii* strains.

On the basis of *erm*(41) sequences, we reclassified six isolates (6/87 clinical strains, or 7%): three *M. abscessus* strains were reclassified to the *M. massiliense* group, one *M. abscessus* strain was classified as *M. bolletii*, and one *M. massiliense* strain was reclassified in the *M. abscessus* group. Overall, the strain distribution changed from 46 *M. abscessus* sensu stricto, 27 *M. massiliense*, and 14 *M. bolletii* strains to 43 *M. abscessus* sensu stricto, 30 *M. massiliense*, and 14 *M. bolletii* strains. The latter distribution has been used throughout the manuscript.

Clarithromycin susceptibility with regard to species. Susceptibility to clarithromycin was determined for the 90 strains (87 clinical strains and 3 reference strains) by the broth microdilution method and Etest. Results are presented with regard to species and *erm*(41) sequence (Table 2 for *M. massiliense* and *M. bolletii* strains and Table 3 for *M. abscessus*). MICs for strains harboring *rrl* mutations are presented separately in Table 4.

After repeated readings at days 5, 7, 9, and 14, we clearly distinguished three distinct patterns of susceptibility. Pattern 1 included 36 strains (26 *M. massiliense* and 10 *M. abscessus* strains) that remained susceptible to clarithromycin until day 14, with a MIC of ≤ 2μg/ml. Pattern 2 included 45 strains (31 *M. abscessus* and 14 *M. bolletii*) whose MICs increased from day 5 to day 14, with a final MIC of >16 μg/ml by microdilution determination and of >256 μg/ml by Etest. Pattern 3 included six strains (four *M. massiliense* and two *M. abscessus*) that were resistant to clarithromycin with a MIC of >16 μg/ml from day 7.

The MICs for the reference strains are listed below. For *M. abscessus* ATCC 19977, the clarithromycin MIC was 8 μg/ml at day 5 and >16 μg/ml at days 7, 9, and 14 by microdilution; it was 1 μg/ml at day 5 and 4 μg/ml at day 7 by Etest. The azithromycin MIC for *M. abscessus* ATCC 19977 was 8 μg/ml at day 5 and >256 μg/ml at day 7. This strain was classified as susceptibility pattern 2 and in the T28 *M. abscessus* group. For *M. bolletii* CIP 108541^T, the clarithromycin MIC was 4 μg/ml at day 5 and >16 μg/ml at days 7, 9, and 14 by microdilution; by Etest, it was 4 μg/ml at day 5 and 4 μg/ml at day 7. The

TABLE 2. MICs of clarithromycin and azithromycin for *M. massiliense* and *M. bolletii* clinical and reference strains devoid of *rrl* mutations, with regard to the method of susceptibility testing and the time of incubation

Species (*n*)[a]	Antibiotic	Testing method	Incubation time (days)[b]	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	MIC range (μg/ml)
M. massiliense (26)	Clarithromycin	Etest	5	0.125	0.5	0.016–0.5
			7	0.125	0.5	0.016–0.5
		Microdilution	5	0.12	0.5	<0.12–0.5
			7	0.25	0.5	<0.12–2
			9	0.25	1	<0.12–2
			14	0.5	1	<0.12–2
	Azithromycin	Etest	5	0.38	2	0.047–3
			7	1	2	1–2
M. bolletii (14)	Clarithromycin	Etest	5	16	48	0.25–>256
			7	>256	>256	1–>256
		Microdilution	5	2	16	0.25–16
			7	4	16	0.5–16
			9	>16	>16	4–>16
			14	>16	>16	>16–>16
	Azithromycin	Etest	5	6	256	4–>256
			7	12	>256	4–>256

[a] *n*, number of strains.
[b] Day of testing.

TABLE 3. MICs of clarithromycin and azithromycin against 41 clinical strains of *M. abscessus* with regard to the *erm*(41) genotype (nucleotide at position 28) and the incubation time

erm(41) genotype (*n*)[a]	Antibiotic	Testing method	Incubation time (days)[b]	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	MIC range (μg/ml)
T 28 sequevar (31)	Clarithromycin	Etest	5	1.5	8	0.064–>256
			7	6	>256	0.19–>256
		Microdilution	5	2	16	<0.06–>16
			7	16	>16	0.5–>16
			9	>16	>16	4–>16
			14	>16	>16	>16
	Azithromycin	Etest	5	8	>256	0.25–>256
			7	256	>256	1.5–>256
C 28 sequevar (10)	Clarithromycin	Etest	5	0.064	0.125	<0.016–0.125
			7	0.125	0.25	0.064–0.38
		Microdilution	5	0.25	0.5	<0.06–0.5
			7	0.25	1	0.25–1
			9	0.5	1	0.25–1
			14	1	2	0.25–2
	Azithromycin	Etest	5	0.25	1.5	0.064–1.5
			7	1	1.5	0.25–1.5

[a] *n*, number of strains.
[b] Day of reading.

azithromycin MIC for *M. bolletii* CIP 108541^T was >256 μg/ml at days 5 and 7. This strain was classified as susceptibility pattern 2 with the other *M. bolletii* strains. For *M. massiliense* CIP 108297^T, the clarithromycin MIC was 0.25 μg/ml at day 5, 0.5 μg/ml at days 7 and 9, and 1 μg/ml at day 14 by microdilution; by Etest, it was 0.094 μg/ml at days 5 and 7. The azithromycin MIC for *M. massiliense* CIP 108297^T was 2 μg/ml at days 5 and 7. This strain was classified as susceptibility pattern 1 with the other *M. massiliense* strains.

For all the strains resistant to clarithromycin (MIC of >2 μg/ml), the *rrl* region involved in clarithromycin resistance was sequenced. Mutations in the peptidyltransferase region of the 23S rRNA gene were found for the six strains with clarithromycin susceptibility pattern 3 (i.e., resistant to clarithromycin beginning at the day 5 reading). Strains and mutations are described in Table 4. One or two mutations were observed at the expected nucleotide position of 2058 or 2059, with a similarly high level of resistance (MIC of >256 μg/ml).

For the strains devoid of *rrl* mutations, we easily matched the *erm*(41) sequence results with those of clarithromycin susceptibility. On one hand, strains harboring *erm*(41) deletions were fully susceptible to clarithromycin along with the strains harboring an intact *erm*(41) but with a C28 sequevar. On the other hand, all strains with a T28 sequevar, either *M. abscessus* or *M. bolletii*, were clearly resistant to clarithromycin when the incubation lasted for 14 days (MIC_{90} of >256 μg/ml).

Azithromycin MICs are presented in Tables 2 and 3 for strains with no *rrl* mutation. For strains with an *rrl* mutation, MICs were >256 μg/ml at day 5. Overall, the MIC values for azithromycin were higher than those for clarithromycin but varied in the same way. At the day 5 reading, the differences between the MICs for the strains that appeared susceptible and for strains that appeared resistant after the extended incubation did not differ between azithromycin and clarithromycin: the MIC_{50} ratio ranged from 16 to 32 (0.25 and 0.38 versus 8 and 6) for azithromycin and from 12 to 266 (0.06 and 0.12 versus 1.5 and 16) for clarithromycin; the MIC_{90} ratio ranged from 256 to 341 (1.5 and 2 versus >256) for azithromycin and from 166 to 400 for clarithromycin (0.12 and 0.5 versus 8 and 48).

MICs of quinupristin-dalfopristin were >32 μg/ml for all strains regardless of species, showing that clarithromycin and azithromycin are the only compounds of the macrolides-lincos-

TABLE 4. Clarithromycin MICs and *erm*(41) genotype for strains with *rrl* mutation

Strain	Species	Clinical context (previous treatment)	*erm41* genotype[a]	*rrl* mutation[b]	Clarithromycin MIC (μg/ml) by method and date of testing					
					Microdilution				Etest	
					Day 5	Day 7	Day 9	Day 14	Day 5	Day 7
CR5093	*M. massiliense*	Cystic fibrosis (yes)	Deletion	A2058G	>16	>16	>16	>16	>256	>256
20031177	*M. massiliense*	Cystic fibrosis (yes)	Deletion	A2058C	>16	>16	>16	>16	>256	>256
RPC11	*M. massiliense*	Unknown	Deletion	A2059T	8	>16	>16	>16	32	>256
20021158	*M. massiliense*	Cystic fibrosis (yes)	Deletion	A2058C	>16	>16	>16	>16	>256	>256
20031307	*M. abscessus*	Cystic fibrosis (no)	C28 sequevar	A2058G A2059C	>16	>16	>16	>16	>256	>256
RPC160	*M. abscessus*	Cystic fibrosis (no)	C28 sequevar	A2058T A2059C	>16	>16	>16	>16	>256	>256

[a] Deletions are those described for *M. massiliense* strains in Table 1.
[b] Numbering system in the *rrl* gene of *Escherichia coli*.

TABLE 5. Distribution of the clinical strains studied of the *M. abscessus* complex according to clinical features (underlying disease or origin of infection) and speciation attributed on the basis of *erm*(41) gene sequences

Clinical feature	Strain distribution (no.) by species				
	M. abscessus T28	*M. abscessus* C28	*M. massiliense*	*M. bolletii*	Total
Cystic fibrosis	20	6	17	10	53
Chronic obstructive pulmonary disorder	1	3	1	0	5
Bronchiectasis	1	1	5	0	7
Lung cancer		1	0	0	1
Tuberculosis sequelae	2	1	1	1	5
Immunodeficiency[a]	4		1	1	6
Other	2[b]		4[c]	1[d]	7
Unknown	1		1	1	3
Total	31	12	30	14	87

[a] HIV infection (one *M. abscessus* and one *M. bolletti* strain), pulmonary or renal transplantation (one *M. abscessus* and one *M. massiliense* strain, respectively), rheumatoid arthritis (*M. abscessus*), and Wegener's granulomatosis (*M. abscessus*).
[b] Synovitis and skin nodule.
[c] Asthma, Ondine syndrome, trauma of the cornea, and hepatitis C.
[d] Bursitis of the knee.

TABLE 6. Distribution of the clinical strains according to treatment data

Susceptibility group and strain	Strain distribution (no.) by treatment			
	New case	Treated case	Unknown	Total
Clarithromycin-resistant strains[a]				
M. abscessus T28 sequevar	19	11	1	31
M. bolletii	5	6	3	14
M. massiliense with *rrl* mutation	0	3	1	4
M. abscessus C28 sequevar with *rrl* mutation	2	0	0	2
Total for group	26	20	5	51
Clarithromycin susceptible strains				
M. abscessus C28 sequevar without *rrl* mutation	6	2	2	10
M. massiliense without *rrl* mutation	17	7	2	26
Total for group	23	9	4	36
Total for both groups	49	29	9	87

[a] Inducible resistance or high-level resistance.

amides-streptogramin B (MLS) family that may be active since other compounds have already been tested (33).

Clarithromycin resistance with regard to clinical features, species, and antibiotic treatment. The description of the clinical isolates with regard to clinical features appears in Table 5, and distribution according to treatment is given in Table 6.

The majority (73/87, or 84%) of diseases associated with *M. abscessus* infection were respiratory diseases, with only three inoculation infections, which occurred in immunocompetent patients. Strains were isolated in 61% (53/87) of respiratory samples from cystic fibrosis patients. Distribution with regard to species was not statistically different ($P = 0.5$ to 1) between cystic fibrosis patients (26 *M. abscessus*, 17 *M. massiliense*, and 10 *M. bolletii* strains) and other patients (17 *M. abscessus*, 13 *M. massiliense*, and 4 *M. bolletii* strains). After an assessment of the T28/C28 genotype of the *M. abscessus* strains, it appeared that 72% (31/43) of the *M. abscessus* strains were of the T28 sequevar, with no difference between cystic fibrosis patients and other patients ($P = 0.6$).

Clarithromycin resistance (inducible or acquired resistance due to *rrl* mutations) was calculated according to the species: 77% (33/43) of *M. abscessus*, 100% (14/14) of *M. bolletii*, and 13% (4/30) of *M. massiliense* strains were resistant. Overall, 59% (51/87) of the strains were clarithromycin resistant, with more resistant strains in cystic fibrosis patients than in other patients (36/51 resistant cystic fibrosis patients versus 15/36 resistant other patients; $P = 0.04$). Moreover, all six strains with acquired resistance to clarithromycin and *rrl* mutations were from cystic fibrosis patients. The six strains with *rrl* mutations were significantly more often isolated in the *M. massiliense* and *M. abscessus* C28 sequevar than in the *M. bolletii* and *M. abscessus* T28 sequevar (6/42 versus 0/45; $P = 0.02$).

We distributed the strains with regard to previous antibiotic treatment based on what we knew of the clinical data and history of the patients. This distribution resulted in a total of 49 new cases or first isolations (56%), 29 previously treated cases (33%), and 9 cases for which we did not have information (10%). Although clarithromycin-resistant strains were more frequent in treated cases than in new cases (20/29, or 69%, versus 26/49, or 53%), the difference was not statistically significant ($P = 0.23$).

DISCUSSION

M. abscessus infections are emerging infections in cystic fibrosis (14, 37, 40) and other (primarily chronic respiratory) diseases (5, 17, 42). Clarithromycin had been the drug of choice for these infections for the past 20 years (8, 15) before a resistance gene, *erm*(41), was described in some strains (33). Because therapeutic success was reported with clarithromycin (15, 17, 44), we hypothesized that some strains might not harbor *erm*(41) or might not express clarithromycin resistance.

The data produced by our study of 87 *M. abscessus* group strains involved in respiratory or other infections, added to data obtained in the studies of Nash et al. and in Kim et al., demonstrated that the results of *erm*(41) and *rrl* sequencing are fully concordant with phenotypic clarithromycin susceptibility: resistance was observed when *erm*(41) was a T28 sequevar or when an *rrl* mutation previously associated with acquired resistance was observed; sensitivity was observed when *erm*(41) showed a deletion or was a C28 sequevar, with the strain also being devoid of *rrl* mutations. Moreover, *erm*(41) sequences appeared species specific with regard to the two new species, *M. bolletii* and *M. massiliense*, and we observed two sequevars (T28 and C28) among the *M. abscessus* sensu stricto species.

Clarithromycin susceptibility was assessed according to testing recommendations (33). Incubation for 14 days, which has been shown to give similar results to preincubation with clarithromycin (33), was easy to implement using microdilution plates. At day 14, the MIC results were clearly different between susceptible and resistant strains. Antibiotic susceptibility

of nontuberculous mycobacteria has been difficult to standardize in the past, especially for rapidly growing mycobacteria, where agar diffusion, Etest, and microdilution methods were used successively (47, 48). Although microdilution was considered the most reproducible method and was therefore recommended by CLSI (35, 46), we missed the resistance character conferred by the *erm*(41) gene by taking a reading after 72 h of incubation. This is similar to what was described in *Mycobacterium fortuitum*, which harbors *erm*(39) (34), and *M. tuberculosis*, which harbors *erm*(37) (9). These species appeared to be falsely susceptible to clarithromycin when tested with incubations of short duration (6, 41, 48). In our hands, the Etest method (with a reading at 7 days) gave results comparable to those of the microdilution method (with a reading at 14 days).

We found that among 87 strains of the *M. abscessus* group, 59% were resistant to clarithromycin by a mechanism of structure modification of the target (23S rRNA mutation) or its methylation (Erm41). In the remaining strains which appeared susceptible to clarithromycin, these mechanisms are probably lacking. In the *M. massiliense* strains, deletions in *erm*(41) (2-bp deletion resulting in a shift in the code and a large 3′-end deletion) explain why these strains were not resistant. As yet, there is no explanation of why the Erm41 protein with Arg10 is associated with susceptibility while Trp10 is associated with resistance. Nash et al. showed that these differences had no effect at the level of RNA transcription (33) and concluded that the protein harboring an Arg10 was nonfunctional. *In vitro* experiences targeting RNA-Erm interaction would be necessary in order to show whether this protein cannot bind to the 23S rRNA cleft or cannot methylate A2058 (29).

With the progress of mycobacterial taxonomy and speciation, it appeared that antibiotic susceptibility of nontuberculous mycobacteria is tightly related to mycobacterial species and, conversely, that a homogenous intrinsic susceptibility pattern may reflect the homogeneity of the species (4, 8, 15). Since many new species have been described recently with the assistance of molecular biology and genomics, we need to assess their intrinsic susceptibility patterns species by species. The *M. abscessus* group has been reorganized into three species on the basis of the sequences of housekeeping genes (1, 24, 49), and we have demonstrated that these species also differ with respect to specific *erm*(41) features and intrinsic clarithromycin susceptibility patterns: all *M. massiliense* strains were intrinsically susceptible, whereas all *M. bolletii* were resistant. For the *M. abscessus* sensu stricto isolates, two sequevars were identified: the C28 sequevar associated with clarithromycin susceptibility and the T28 sequevar associated with clarithromycin resistance. The C28 genotype was reported by Nash (33) and Kim (19) in two and seven strains of *M. abscessus*, respectively, which showed susceptibility. In all of the studies so far, most variations were reported in the clarithromycin susceptibility results within the *M. abscessus* group even with strains precisely identified and even with reference strains (2, 11, 20, 24, 36). For example, *M. bolletii* was shown to be clarithromycin resistant by Adekambi et al. (2) and susceptible by Leao et al. (24); in addition, one strain was susceptible, and one was resistant in Kim et al. (19). However, since the 14-day protocol was not applied in the previous studies, we hypothesize that most often false susceptibility was reported.

The six strains with acquired resistance to clarithromycin and mutation in *rrl* were consisted of four *M. massiliense* and two *M. abscessus* C28 sequevar strains. Conversely, no strains of the *M. bolletii* or *M. abscessus* T28 sequevar group had mutations in *rrl*. These data show that there might be less selection of resistant *rrl* mutants for the strains expressing inducible resistance (*M. bolletii* and *M. abscessus* sequevar T28) than for strains not expressing inducible resistance (*M. massiliense* and *M. abscessus* sequevar C28). This fact, as in other mycobacterial diseases, explains the poor clarithromycin efficacy against strains expressing inducible resistance as the selection of a more resistant mutant would not bring any selective advantage in the presence of clarithromycin. This difference was also reported by Leao et al. but not by Nash et al. (24, 33). Although our study was retrospective, we looked to see if resistant strains were more frequently isolated in treated cases and susceptible strains in new cases. There were no statistically significant differences, but we will need to conduct a prospective clinical study since cystic fibrosis patients likely receive treatment with multiple antibiotics of which we are unaware.

Still, the clinical impact of inducible resistance remains to be determined. In other bacterial species such as *Staphylococcus aureus*, the presence of an *erm* gene has been linked to clinical failure in case of clindamycin monotherapy (26). As the number of antibiotics active against the *M. abscessus* group is very limited, we need to know whether clarithromycin has any activity, even in combined regimens, for the treatment of strains displaying inducible resistance. Comparison of *in vivo* efficacy and *in vitro* susceptibility data needs to be done. This is why we would like to propose that clarithromycin susceptibility be assessed on a molecular basis for all new cases of *M. abscessus* group infections and that the outcomes of clarithromycin therapy be observed, as follows. First, we will perform a PCR encompassing *erm*(41) to detect the deletion observed in *M. massiliense*. Second, we will sequence a DNA fragment containing the *erm*(41) promoter sequences (so as to be able to distinguish *M. bolletii* from *M. abscessus*) and the T/C polymorphism at nucleotide 28. In addition, for the relapse cases, we will seek the *rrl* region involved in resistance for specific mutations. The resulting data will be used to determine species (*M. abscessus*, *M. bolletii*, and *M. massiliense*) within the *M. abscessus* group, assess clarithromycin susceptibility, and guide treatment.

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