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Original Article

Antimicrobial susceptibility testing of *Mycobacteroides* (*Mycobacterium*) *abscessus* complex, *Mycolicibacterium* (*Mycobacterium*) *fortuitum*, and *Mycobacteroides (Mycobacterium) chelonae*☆

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ABSTRACT

The drug susceptibility of rapidly growing mycobacteria (RGM) varies among isolates. Treatment strategies similarly differ depending on the isolate, and for some, no clear strategy has been identified. This complicates clinical management of RGM. Following Clinical and Laboratory Standards Institute standard M24-A2, we assessed the susceptibility of 140 RGM isolates to 14 different antimicrobial drugs by measuring their minimal inhibitory concentrations (MICs). We also investigated the correlation of clarithromycin (CAM) MICs with the *erm*(41) and *rrl* gene mutations in the *Mycobacteroides* (*Mycobacterium*) *abscessus* complex, the *rrl* mutation in *Mycobacteroides* (*Mycobacterium*) *chelonae*, and the *erm*(39) mutation in *Mycolicibacterium* (*Mycobacterium*) *fortuitum* to determine the contribution of these mutations to CAM susceptibility. The five species and subspecies examined included 48 *M. abscessus* subsp. *abscessus* isolates (34.3%), 35 (25.0%) being *M. abscessus* subsp. *massiliense*, and two (1.4%) being *M. abscessus* subsp. *bolletii*. The *M. abscessus* complex accounted for 85 isolates (60.7%) in total, whereas 43 isolates (30.7%) were *M. fortuitum*, and 12 (8.6%) were *M. chelonae*. Our results demonstrated species-specific susceptibility to antimicrobials. In most cases, susceptibility to CAM could be predicted based on genetic pattern, but since one isolate did not fit that pattern, MIC values needed to be measured. Some isolates also exhibited rates of resistance to other drugs that differed from those previously reported in other locations, indicating that accurate identification of the bacterial isolate and use of the correct method for determining MIC are both important for the diagnosis of RGM.

1. Introduction

Rapidly growing mycobacteria (RGM) are a clinical concern because they cause a wide variety of infections in the lungs, skin, soft tissues, and other parts of the body. In particular, non-tuberculosis mycobacteria (NTM) infections are on the rise in countries with rapidly aging populations [1], and RGM infections may also be increasing [2]. Because the susceptibility of RGM to drugs varies among isolates, treatment strategies similarly differ depending on the isolate concerned, and there remain some isolates for which no clear treatment strategy has been identified [3]. This complicates the clinical management of RGM.

Drug susceptibility tests for antimicrobials are essential in the treatment of infections, and they play an important role in determining the course of treatment. The *Mycobacteroides* (*Mycobacterium*) *abscessus* complex is the most difficult of the RGM to treat, and drug susceptibility data are required before treatment can be started. The *M. abscessus* complex includes *M. abscessus* subsp. *abscessus* (*M. abscessus*), *M. abscessus* subsp. *bolletii* (*M. bolletii*), and *M. abscessus* subsp. *massiliense* (*M. massiliense*) [4]. It has been

Abbreviations: rapidly growing mycobacteria, RGM; minimal inhibitory concentration, MIC; *Mycobacteroides* (*Mycobacterium*) *abscessus* subspecies *abscessus*, *M. abscessus*; *Mycobacteroides* (*Mycobacterium*) *abscessus* subspecies *bolletii*, *M. bolletii*; *Mycobacteroides* (*Mycobacterium*) *abscessus* subspecies *massiliense*, *M. massiliense*; amikacin, AMK; cefoxitin, CFX; levofloxacin, LVFX; clarithromycin, CAM; doxycycline, DOXY; minocycline, MINO; imipenem, IPM; linezolid, LZD; meropenem, MEPM; moxifloxacin, MFLX; sulfamethoxazole-trimethoprim, ST; tobramycin, TOB; clofazimine, CLF; sitafloxacin, STFX.

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recently suggested that these bacteria should be dealt with separately owing to the differences in their responses to treatment [5]. In this study, we analyzed data from *M. abscessus*, *M. bolletii*, and *M. massiliense* both separately and also together as the *M. abscessus* complex. United States Clinical and Laboratory Standards Institute (CLSI) standard M24-A2 [6] describes several drug susceptibility tests for RGM. We measured the minimal inhibitory concentrations (MICs) for RGM following standard M24-A2 to identify the susceptibility of RGM to different antimicrobial drugs. We also investigated the correlation of clarithromycin (CAM) MICs with the *erm*(41) and *rrl* gene mutations in the *M. abscessus* complex [7–9], the *rrl* mutation in *Mycobacteroides* (*Mycobacterium*) *chelonae* [9], and the *erm*(39) mutation in *Mycolicibacterium* (*Mycobacterium*) *fortuitum* [10] to determine the contribution of these mutations to CAM susceptibility.

2. Materials and methods

RGM strains used in the study consisted of 140 isolates of five species and subspecies isolated from clinical respiratory samples in the Japan Anti-Tuberculosis Association Fukujuji Hospital between 2004 and 2014. All isolates were the first detections of the patients, but the treatment status was uncertain. The species were identified by DNA-DNA hybridization kits (Kyokuto Pharmaceutical Industrial, Tokyo, Japan) following the manufacturer's instructions. Subspecies and species unidentifiable by DNA-DNA hybridization were identified by gene homology analysis using 16S rRNA, *hsp65*, and *rpoB*. Total DNA was extracted from bacteria grown on solid culture medium using ISOPANT (Nippon Gene, Tokyo, Japan). Direct sequencing of the 16S rRNA, *hsp65*, and *rpoB* genes was carried out following the method of Nakanaga et al. [11]. The primers used are shown in Table 1. AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA) was used to amplify the target genes. Polymerase chain reaction (PCR) products were purified using a MagExtractor (Toyobo, Osaka, Japan) prior to sequencing with a BigDye Terminator cycle sequencing kit ver. 3.1 (Applied Biosystems) on an ABI 3500 Genetic Analyzer (Applied Biosystems). The sequences thus obtained were subjected to homology analysis using Basic Local Alignment Tool (BLAST; http://www.ncbi.nlm.nih.gov/BLAST).

MIC values were measured following USA CLSI standard M24-A2 using a broth microdilution method and cation-adjusted Mueller-Hinton broth (CAMHB; BD Biosciences, Franklin Lakes, NJ, USA). The following 14 drugs were tested: amikacin (AMK), cefoxitin (CFX), levofloxacin (LVFX), clarithromycin (CAM), doxycycline (DOXY), minocycline (MINO), imipenem (IPM), linezolid (LZD), meropenem (MEPM), moxifloxacin (MFLX), sulfamethoxazole-trimethoprim (ST), tobramycin (TOB), clofazimine (CLF), and sitafloxacin (STFX). The ranges for the concentrations of these 14 drugs prepared by two-fold dilution were selected according to the resistance cutoff points given in CLSI standard M24-A2 and values cited in the literature (Table 1) [12–17].

Pyrosequencing following the method described by Yoshida et al. [18] was used to analyze *erm*(41), *erm*(39), and *rrl* mutations using the primers listed in Table 2. In brief, pyrosequencing was performed by carrying out PCR using a PyroMark PCR Kit (QIAGEN GmbH, Hilden, Germany), purifying the PCR products with a PyroMark Workstation (QIAGEN), and analyzing single-nucleotide polymorphisms with a PyroMark Q24 sequencer (QIAGEN) using PyroMark Gold Q24 Reagents (QIAGEN). The sequences obtained were identified by comparison of point mutations at position 28 in *erm*(41), position 1 in *erm*(39), and positions T2057, T2058, and T2059 in *rrl* (*Escherichia coli* numbering) with sequences of standard strains (*M. abscessus* ATCC 19977, *M. chelonae* ATCC 35752, and *M. fortuitum* ATCC 06841).

3. Results

Of the 140 isolates, 48 (34.3%) were of *M. abscessus* subsp. *abscessus* (*M. abscessus*), 35 (25.0%) of *M. abscessus* subsp. *massiliense* (*M. massiliense*), and two (1.4%) of *M. abscessus* subsp. *bolletii*

Table 2
Oligonucleotide primers used in PCR and sequencing.

Target gene	Primer	Nucleotide sequence (5′–3′)
For PCR		
16S rRNA	285 F	GAGAGTTTGATGCTGGCTCAG
	264 R	TGCACACAGGCCACAAGGGA
hsp65	TB11	ACCAACGATGGTGTGTCCAT
	TB12	CTTGTCGAACCGCATACCCT
rpoB	MabrpoF	GAGGGTCAGACCACGATGAC
	MabrpoR	AGCCGATCAGACCGATGTT
erm(41)	ERM F1	CCTAAGCGCACGTTCTGAC
	ERM R1	(biotin)-CCGCCCAGTCATCAGTGAG
erm(39)	erm39F	AGTGACCTACATCCGCTTG
	erm39R	(biotin)-ATCACCTCGGCAGTGGTTC
rrl	rrl F	CGAAATTCCTTGTCGGGTAA
	rrl R	(biotin)-ACGGTCCGAGGTTAGAGGTT
For Sequencing		
16S rRNA	259 R	TTTCACGAACAACGCGACAA
	244 R	CCCACTGCTGCCTCCCGTAG
hsp65	TB12	CTTGTCGAACCGCATACCCT
rpoB	MabrpoR	AGCCGATCAGACCGATGTT
erm(41)	AQ 1	CAACGGTCGCGACGC
erm(39)	erm39 SQ2	TCCCTGCGACTGCGGAGCTC
rrl	rrl SQ	TGCACTACGAGTAAAGATGCTC

Table 1
Breakpoints used for rapidly growing mycobacteria drug susceptibility testing by broth dilution.

Antimicrobial agent	MIC (μg/mL)			Broth dilution range (μg/mL)
	Susceptible	Intermediate	Resistant	
Amikacin	≤16	32	≥64	1–32
Cefoxitin	≤16	32–64	≥128	2–64
Levofloxacin	≤1	2	≥4	0.25–8
Clarithromycin	≤2	4	≥8	0.06–32
Doxycycline	≤1	2–4	≥8	1–16
Minocycline	≤1	2–4	≥8	1–16
Imipenem	≤4	8–16	≥32	2–64
Linezolid	≤8	16	≥32	1–32
Meropenem	≤4	8–16	≥32	1–64
Moxifloxacin	≤1	2	≥4	0.25–8
Sulfamethoxazole-Trimethoprim	≤38/2	–	≥76/4	4.8/0.25–152/8
Tobramycin	≤2	4	≥8	1–16
Clofazimine	–	–	–	0.13–4
Sitafloxacin	–	–	–	0.25–8

(*M. bolletii*), indicating that the *M. abscessus* complex accounted for 85 isolates (60.7%) in total, whereas 43 isolates (30.7%) were of *M. fortuitum* and 12 (8.6%) of *M. chelonae*.

Depending on the MIC values obtained, the *M. abscessus* complex (*M. abscessus* and *M. massiliense*, in total and separately) *M. fortuitum*, and *M. chelonae* isolates were classified as susceptible, intermediate, or resistant according to the CLSI criteria. Resistance rate between *M. abscessus* and *M. massiliense* were analyzed by Fisher's exact test. The distribution of the corresponding degrees of susceptibility is shown in Table 3. Graphs of the cumulative distributions of MICs for each drug are shown in Fig. 1; details are provided in the supplemental material (Figs. S1–S15).

Table 4 shows the correlation between the distributions of CAM MICs for *M. abscessus*, *M. massiliense*, and *M. bolletii* on the one hand, and *erm*(41) and *rrl* gene mutations on the other hand. In 44 of the 48 *M. abscessus* isolates, the base at position 28 in *erm*(41) was T (T28) and the bases at positions 2057, 2058, and 2059 of *rrl* were A (A2057), A (A2058), and A (A2059), respectively. In 43 of those isolates, the MIC value was >32 μg/mL, and it was 0.5 μg/mL in only one isolate. In that isolate, the *erm*(41) mutation contained T28; the *rrl* mutation contained A2057, A2058, and G2059; and the CAM MIC value was >32 μg/mL. In two isolates in which *erm*(41) contained C28 and *rrl* contained A2057, A2058, and A2059, CAM MIC values were 1 μg/mL and 2 μg/mL. In 34 of 35 *M. massiliense* isolates, *erm*(41) contained T28; *rrl* contained A2057, A2058, and A2059; and CAM MIC values ranged from ≤0.06 μg/mL to 2 μg/mL. In one isolate, *erm*(41) contained T28; *rrl* contained T2057, A2058, and A2059; and the CAM MIC value was >32 μg/mL. In the two *M. bolletii* isolates, *erm*(41) contained T28; *rrl* contained A2057, A2058, and A2059; and these isolates were resistant to CAM with MIC values ≥ 32 μg/mL. The same association between the distribution of CAM MICs and *rrl* mutations was also evident for *M. chelonae*. In 11 of 12 *M. chelonae* isolates, the nucleotide bases at positions 2057, 2058, and 2059 of *rrl* were A2057, A2058, and A2059, respectively, and these isolates were susceptible to CAM with MIC values of 0.25–0.5 μg/mL. However, in one isolate, *rrl* contained A2057, A2058, and G2059, and that isolate was resistant to CAM with a MIC value of >32 μg/mL. An association between the distribution of CAM MICs and *erm*(39) was evident in all 43 isolates of *M. fortuitum*. The base at position 1 of *erm*(39) was G (G1) in all 43 isolates, all of which were resistant to CAM with MIC values of ≥32 μg/mL.

4. Discussion

We evaluated the susceptibility of the common RGM isolated in Japan by measuring the MIC values of antimicrobials for these bacteria following the method set out in CLSI standard M24-A2. Fig. 2 shows the yearly changes in the rates of isolation of these RGM in the Japan Anti-tuberculosis Association Fukujuji Hospital. The most common isolates were of *M. abscessus* complex and *M. fortuitum*. In 2004, *M. fortuitum* was the most commonly isolated species, but the number of *M. abscessus* complex isolates started rising in 2006 and rose further from 2013 to 2014. Namkoong et al. [19] reported that the incidence of NTM disease in Japan increased rapidly to 14.7 cases per 10^5 person-years in 2014. The main causative organism was the *M. avium-intracellulare* complex, but in the same study, they also reported that the incidence of *M. abscessus* infection had risen five-fold in seven years, which is consistent with our findings. Morimoto et al. [20] collected and analyzed Japanese acid-fast bacteria test data and found that the incidence of RGM infections was 3.5%, of which the *M. abscessus* complex accounted for a high rate. The Sensititer kit (Thermo Fisher Scientific, Waltham, MA, USA) for measuring MICs, which is compliant with CLSI standard M24-A2, is commercially available in parts of the world, and data on the MIC values for RGM have been published. In Japan, however, no such simple MIC measurement kit is available, despite the rise in the number of patients with RGM infections. Although the standard therapeutic regimen indicated by the Japanese Society for Tuberculosis is usually followed in all cases, information on drug susceptibility of isolates is still lacking. We carried out this study because we believe that information on the status of resistance is essential to providing clinically relevant treatment.

Following the criteria set out in CLSI standard M24-A2, we evaluated the susceptibility of the *M. abscessus* complex, *M. fortuitum*, and *M. chelonae*, which are the most common clinical RGM isolates. Susceptibility to the macrolide antimicrobial CAM varied widely depending on the species or subspecies. Macrolide-induced resistance in the *M. abscessus* complex isolates has been reported, but resistance-inducing activity varied in different gene sequence variants (sequevars). Macrolide methylase is encoded by the *erm*(41) gene and isolates with base T at position 28 (T28 sequevars) possess this activity, whereas those in which this base is mutated do not possess it [7]. The isolates evaluated in this study reflected this finding; of those in the *M. abscessus* complex, most isolates of *M. abscessus* and *M. bolletii* that possessed methylase against macrolide antimicrobials exhibited high rates of resistance, whereas the rate was lower for *M. massiliense*, which has a mutation in the methylase-encoding gene. Two isolates of *M. abscessus* (4.2%) belonged to the C28 sequevar, and both were susceptible to CAM. The proportion of the C28 sequevar has previously been reported as 10.7–28.6% [18,21–23], but in our study the value was

Table 3
Susceptibility of the *M. abscessus* complex, *M. abscessus*, *M. massiliense*, *M. fortuitum*, and *M. chelonae* to 12 antimicrobial agents determined by the broth dilution method.

	M. abscessus complex (85)			*M. abscessus* (48)			*M. massiliense* (35)			*M. fortuitum* (43)			*M. chelonae* (12)		
	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)
Amikacin	28.2	67.1	4.7	20.8	72.9	6.3	40.0	57.1	2.9	100	0	0	0	58.3	41.7
Cefoxitin	4.7	75.3	20.0	4.2	79.1	16.7	2.9	71.4	25.7	9.3	90.7	0	0	0	100
Levofloxacin	0	0	100	0	0	100	0	0	100	95.3	2.3	2.3	0	0	100
Clarithromycin	42.4	1.2	56.5	4.2	2.1	93.8[a]	97.1	0	2.9[a]	0	0	100	91.7	0	8.3
Doxycycline	1.2	0	98.8	2.1	0	97.9	0	0	100	37.2	2.3	60.5	25.0	0	75.0
Minocycline	1.2	2.4	96.5	2.1	0	97.9	0	2.9	97.1	39.5	4.7	55.8	25.0	0	75.0
Imipenem	0	10.6	89.4	0	14.6	85.4	0	2.9	97.1	30.2	55.8	14.0	0	8.3	91.7
Linezolid	1.2	14.1	84.7	0	2.1	97.9[a]	0	34.2	65.7[a]	44.2	16.3	39.5	16.7	50.0	33.3
Meropenem	0	3.5	96.5	0	4.2	95.8	0	0	100	11.6	86.0	2.3	0	0	100
Moxifloxacin	0	0	100	0	0	100	0	0	100	97.7	0	2.3	0	0	100
Sulfamethoxazole-Trimethoprim	5.9	–	94.1	2.9	–	97.1	6.3	–	93.8	67.4	–	32.6	8.3	–	91.7
Tobramycin	0	0	100	0	0	100	0	0	100	0	0	100	16.7	66.7	16.7

[a] Significant difference between *M. abscessus* and *M. massiliense* was observed in resistance rate to clarithromycin and linezolid (Fisher's exact tests $P < 0.001$).

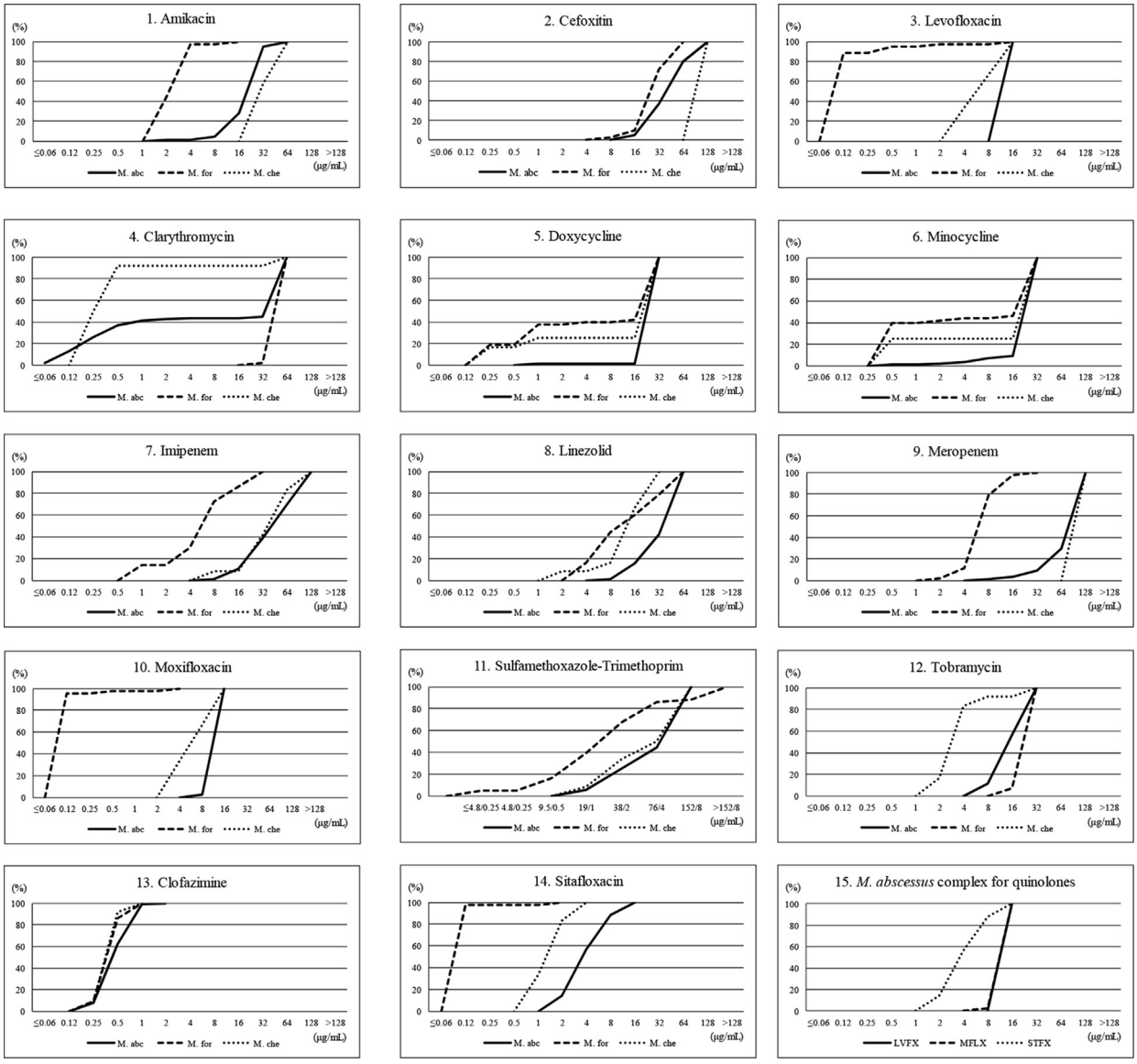

Fig. 1. Cumulative percentages of isolates inhibited by each drug tested in this study. Cumulative distribution of minimal inhibitory concentrations (MICs) of 14 antimicrobial agents (panels 1–14) and of quinolones (panel 15) for *M. abscessus* complex, *M. fortuitum*, and *M. chelonae* isolates revealed by the broth dilution method. Abbreviations: M. abc, *M. abscessus* complex; M. for, *M. fortuitum*; M. che, *M. chelonae*; LVFX, levofloxacin; MFLX, moxifloxacin; STFX, sitafloxacin.

Table 4
Correlation of clarithromycin MICs with the presence of mutations in *erm*(41), *erm*(39), and *rrl* in corresponding strains.

	Clarithromycin MIC (μg/mL)											*erm* (41)	*erm* (39)	*rrl*		
														A	A	A
	≤0.06	0.125	0.25	0.5	1	2	4	8	16	32	>32	T28	G1	2057	2058	2059
M. abscessus (48)				1						1	42	T		A	A	A
											1	T		A	A	G
					1	1						C		A	A	A
M. massiliense (35)	2	9	11	8	3	1						T		A	A	A
											1	T		T	A	A
M. bolletii (2)											2	T		A	A	A
M. chelonae (12)			6	5										A	A	A
											1			A	A	G
M. fortuitum (43)										1	42		G			

lower. Because the *erm*(41) gene is incomplete in *M. massiliense*, the main mutations contributing to CAM resistance are mutations of the bases at positions 2058 and 2059 of the *rrl* gene, which codes for 23S rRNA [7]. One of the *M. massiliense* isolates in this study exhibited resistance to CAM. In that isolate, *rrl* contained A2058 and A2059, and the T2057 mutation was present. The contribution of *rrl* A2057 mutation to CAM resistance in the *M. abscessus* complex was the same as that previously reported by Luo et al. [24].

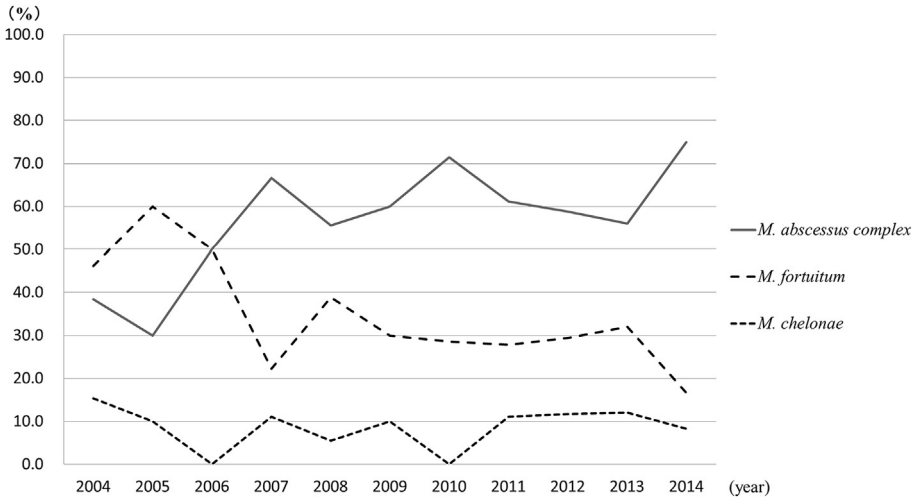

Fig. 2. Annual changes in the isolation rates of rapidly growing mycobacteria in Fukujuji Hospital from 2004 to 2014.

CAM susceptibility was evident in 91.7% of the 12 isolates of *M. chelonae*, with one isolate (8.3%) exhibiting some resistance. According to Wallace et al. [9], CAM resistance in *M. chelonae* is due to 23S rRNA mutations, with 38% and 62% of CAM-resistant isolates exhibiting A2058 and A2059 mutations, respectively. In our study, the 23S rRNA gene mutation identified in the single isolate that displayed resistance was A2059G.

Like *M. abscessus*, *M. fortuitum* exhibited 100% natural resistance to CAM, with no susceptible isolates identified. According to Nash et al. [10], CAM resistance in *M. fortuitum* is caused by *erm*(39) gene activity. Macrolide susceptibility is conferred by a mutation at the starting point of the *erm*(39) gene. All 43 isolates of *M. fortuitum* were resistant to CAM, and none had a mutation at the starting point of the *erm*(39) gene. This result supported the findings of Nash et al. [10].

The aminoglycoside antimicrobials used in this study were AMK and TOB. Regarding RGM resistance to aminoglycosides, Nessar et al. [12] reported that T1406A, A1408G, C1409T, and G1491T mutations in the 16S rRNA gene (*E. coli* numbering) contribute to high levels of resistance to kanamycin, AMK, and gentamicin. In our experiments with AMK, the best results were for *M. fortuitum*, with no resistance, followed by the *M. abscessus* complex isolates, of which only 4.7% were resistant to AMK. The highest proportion of resistant isolates, 41.7%, was observed in *M. chelonae* isolates. The fact that the levels of AMK resistance differed significantly among these three species (Fisher's exact test, $P < 0.01$) indicates that it is important to distinguish between these bacteria. The American Thoracic Society/Infectious Diseases Society of America (ATS/IDSA) guidelines [3] include AMK among the treatment options for *M. fortuitum*, and our results also suggest that AMK may have an important role in treating infections caused by this species. The lowest rate of resistance to TOB was seen in *M. chelonae* at 16.7% of the isolates, whereas 100% of the *M. abscessus* complex and *M. fortuitum* isolates were resistant. This supports the statement in standard M24-A2 that TOB should mainly be used for the treatment of *M. chelonae,* but not for the treatment of the *M. abscessus* complex or *M. fortuitum.* However, we found that 16.7% of *M. chelonae* isolates did display resistance, suggesting that evaluation by MIC determination may be essential.

The distribution of CFX MICs was the lowest for *M. fortuitum* isolates, followed by the *M. abscessus* complex isolates, whereas 100% of *M. chelonae* isolates exhibited resistance. According to the ATS/IDSA guidelines, there is currently no chemotherapy regimen that can reliably cure *M. abscessus* infection. Additionally, although we observed that *M. abscessus* complex isolates exhibited susceptibility or intermediate resistance to CFX, the link between this finding and clinical efficacy is uncertain.

Almost all isolates of *M. fortuitum* exhibited susceptibility to the fluoroquinolone antimicrobials LVFX (95.3%) and MFLX (97.7%). In contrast, all *M. abscessus* complex and *M. chelonae* isolates were resistant to both. Fluoroquinolones are included in the ATS/IDSA guidelines as a treatment option for *M. fortuitum*, and our result was highly consistent with that recommendation. Although no CLSI categories have been established for the quinolone antimicrobial STFX, our results showed that like LVFX and MFLX, the distribution of STFX MICs was low for *M. fortuitum* and high for the *M. abscessus* complex and *M. chelonae* isolates. Fig. 1 (panel 15) shows the cumulative distributions of MICs of the three quinolone antimicrobials for the *M. abscessus* complex isolates, for which STFX MIC distribution was the lowest. There have been no reports of the clinical efficacy of STFX, and whether the results corresponding to the observed MICs can be achieved in the clinical setting is uncertain.

According to the ATS/IDSA guidelines, 90% of all *M. chelonae* isolates exhibit susceptibility or intermediate resistance to LZD. Broda et al. [25] found that 83% of *M. chelonae* isolates were susceptible, which was a higher proportion than that for the *M. abscessus* complex (37%). In our study, however, even after the fractions with susceptibility and intermediate resistance were pooled together, the rates were 66.7% for *M. chelonae*, 60.5% for *M. fortuitum*, and 15.3% for the *M. abscessus* complex. This suggested that Japanese isolates may exhibit high levels of resistance to LZD. In comparison of resistance rate to LZD between *M. abscessus* (97.9%) and *M. massiliense* (65.7%), a significant difference was observed ($p < 0.001$).

The distributions of MICs of the tetracycline antibiotics DOXY and MINO were similar in all three species. Those of the carbapenem antimicrobials IPM and MEPM also tended to be similar in all three species, with the *M. abscessus* complex and *M. chelonae* isolates exhibiting a high rate of resistance of more than 89.4%. This tendency was similar to that reported by Broda et al. [25]. Although the ATS/IDSA guidelines state that *M. fortuitum* has 100% susceptibility to IPM, 14.0% of isolates in our study were resistant. Standard M24-A2 states that if the MIC value for *M. fortuitum* is > 8 μg/mL, it should be retested within 3 days of the date of the result, but in this study, retesting was not performed, and this may have increased the observed resistance rate of *M. fortuitum*. Koh et al. [5] reported that resistance rates of *M. abscessus* and *M. massiliense* to

IPM were 67% and 44%, respectively, and the resistance rate was significantly high in *M. massiliense*. In our study, resistance rates of *M. abscessus* and *M. massiliense* to IPM were 85.4% and 97.1%, respectively, and the resistance rate was also high in *M. massiliense* though significant difference was not observed.

CLF is not in the list of recommended drugs in CLSI standard M24-A2. However, we showed that its MIC_{50} and MIC_{90} were 0.5 μg/mL and 1.0 μg/mL, respectively, for the *M. abscessus* complex, and that they were both 0.5 μg/mL for *M. fortuitum* and *M. chelonae* isolates. In a study of the synergistic effect of CLF and AMK against NTM, Van Ingen et al. [13] showed that CLF MIC_{90} for RGM was $\leq$1 μg/mL, and in another trial of its synergistic effect with AMK, they also found that it reduced the AMK MIC value for NTM, including RGM, suggesting that a combination therapy using CLF and AMK may be promising.

We found that the characteristics of susceptibility to antimicrobials varied among different species and subspecies. CLSI standard M24-A2 stipulates that the time before the results of MIC measurements can be assessed is 3–5 days, and in light of the existence of induced resistance to CAM, the final determination should be made on day 14, whereas if *M. fortuitum* exhibits resistance to IPM, it should be retested within 3 days. In terms of the relationship between CAM MIC values and *erm*(41) and *rrl* gene mutations, in most cases, susceptibility to CAM could be predicted on the basis of the genetic pattern, but since one isolate did not fit that pattern, MIC values needed to be measured. Some isolates also exhibited rates of resistance to other drugs that differed from those previously reported in other parts of the world, indicating that accurate identification of bacterial isolates and the correct method of determining MIC are both important for the management of RGM infection.

The current study has some limitations. Though the study provided current drug susceptibilities of major RGM, the treatment history of tested isolates was uncertain, as described in the methodology section. It also should be noted that the isolate collection did not represent the whole NTM population in Japan. In addition, the MICs were tested one time only, so the reproducibility was not confirmed. Further prospective cohort study shall be considered to sort out these problems.

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Declarations of interest

None.

Appendix A. Supplementary data

Fig. S1. Cumulative distribution of MICs of amikacin for *M. abcsessus* complex, *M. fortuitum*, and *M. chelonae* isolates revealed by the broth dilution method. Abbreviations: M. abc, *M. abcsessus* complex; M. for, *M. fortuitum*; M. che, *M. chelonae*.

Fig. S2. Cumulative distribution of MICs of cefoxitin for *M. abcsessus* complex, *M. fortuitum*, and *M. chelonae* isolates revealed by the broth dilution method. Abbreviations: M. abc, *M. abcsessus* complex; M. for, *M. fortuitum*; M. che, *M. chelonae*.

Fig. S3. Cumulative distribution of MICs of levofloxacin for *M. abcsessus* complex, *M. fortuitum*, and *M. chelonae* isolates revealed by the broth dilution method. Abbreviations: M. abc, *M. abcsessus* complex; M. for, *M. fortuitum*; M. che, *M. chelonae*.

Fig. S4. Cumulative distribution of MICs of clarythromycin for *M. abcsessus* complex, *M. fortuitum*, and *M. chelonae* isolates revealed by the broth dilution method. Abbreviations: M. abc, *M. abcsessus* complex; M. for, *M. fortuitum*; M. che, *M. chelonae*.

Fig. S5. Cumulative distribution of MICs of doxycycline for *M. abcsessus* complex, *M. fortuitum*, and *M. chelonae* isolates revealed by the broth dilution method. Abbreviations: M. abc, *M. abcsessus* complex; M. for, *M. fortuitum*; M. che, *M. chelonae*.

Fig. S6. Cumulative distribution of MICs of minocycline for *M. abcsessus* complex, *M. fortuitum*, and *M. chelonae* isolates revealed by the broth dilution method. Abbreviations: M. abc, *M. abcsessus* complex; M. for, *M. fortuitum*; M. che, *M. chelonae*.

Fig. S7. Cumulative distribution of MICs of imipenem for *M. abcsessus* complex, *M. fortuitum*, and *M. chelonae* isolates revealed by the broth dilution method. Abbreviations: M. abc, *M. abcsessus* complex; M. for, *M. fortuitum*; M. che, *M. chelonae*.

Fig. S8. Cumulative distribution of MICs of linezolid for *M. abcsessus* complex, *M. fortuitum*, and *M. chelonae* isolates revealed by the broth dilution method. Abbreviations: M. abc, *M. abcsessus* complex; M. for, *M. fortuitum*; M. che, *M. chelonae*.

Fig. S9. Cumulative distribution of MICs of meropenem for *M. abcsessus* complex, *M. fortuitum*, and *M. chelonae* isolates revealed by the broth dilution method. Abbreviations: M. abc, *M. abcsessus* complex; M. for, *M. fortuitum*; M. che, *M. chelonae*.

Fig. S10. Cumulative distribution of MICs of moxifloxacin for *M. abcsessus* complex, *M. fortuitum*, and *M. chelonae* isolates revealed by the broth dilution method. Abbreviations: M. abc, *M. abcsessus* complex; M. for, *M. fortuitum*; M. che, *M. chelonae*.

Fig. S11. Cumulative distribution of MICs of sulfamethoxazole-trimethoprim for *M. abcsessus* complex, *M. fortuitum*, and *M. chelonae* isolates revealed by the broth dilution method. Abbreviations: M. abc, *M. abcsessus* complex; M. for, *M. fortuitum*; M. che, *M. chelonae*.

Fig. S12. Cumulative distribution of MICs of tobramycin for *M. abcsessus* complex, *M. fortuitum*, and *M. chelonae* isolates revealed by the broth dilution method. Abbreviations: M. abc, *M. abcsessus* complex; M. for, *M. fortuitum*; M. che, *M. chelonae*.

Fig. S13. Cumulative distribution of MICs of clofazimine for *M. abcsessus* complex, *M. fortuitum*, and *M. chelonae* isolates revealed by the broth dilution method. Abbreviations: M. abc, *M. abcsessus* complex; M. for, *M. fortuitum*; M. che, *M. chelonae*.

Fig. S14. Cumulative distribution of MICs of sitafloxacin for *M. abcsessus* complex, *M. fortuitum*, and *M. chelonae* isolates revealed by the broth dilution method. Abbreviations: M. abc, *M. abcsessus* complex; M. for, *M. fortuitum*; M. che, *M. chelonae*.

Fig. S15. Cumulative distribution of MICs of *M. abcsessus* complex isolates for quinolons revealed by the broth dilution method. Abbreviations: LVFX, levofloxacin; MFLX, moxifloxacin; STFX, sitafloxacin.

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References

[1] Marras TK, Daley CL. Epidemiology of human pulmonary infection with nontuberculous mycobacteria. Clin Chest Med 2002;23:553–67.
[2] Brown-Elliott BA, Wallace Jr RJ. Clinical and taxonomic status of pathogenic nonpigmented or late-pigmenting rapidly growing mycobacteria. Clin Mycrobiol Rev 2002;15:716–46.
[3] Griffith DE, Aksamit T, Brown-Elliott BA, Catanzaro A, Daley C, Gordin F, et al. An official American Thoracic Society (ATS) and Infectious Diseases Society of America (IDSA) statement: diagnosis, treatment, and prevention of nontuberculous mycobacterial diseases. New York, NY: American Thoracic Society; 2007.
[4] Kim HY, Kook Y, Yun YJ, Park CG, Lee NY, Shim TS, et al. Proportion of *Mycobacterium massiliense* and *Mycobacterium bolletii* strains among Korean *Mycobacterium chelonae-Mycobacterium abscessus* group isolates. J Clin Microbiol 2008;46:3384–90.

[5] Koh WJ, Jeon K, Lee NY, Kim BJ, Kook YH, Lee SH, et al. Clinical significance of differentiation of *Mycobacterium massiliense* from *Mycobacterium abscessus*. Am J Respir Crit Care Med 2011;183:405–10.
[6] Clinical and Laboratory Standards Institute. Susceptibility testing of mycobacteria, Nocardiae and other aerobic actinomycetes. Approved standard. CLSI document M24-A2. Wayne, PA: CLSI; 2011.
[7] Nash KA, Brown-Elliott BA, Wallace Jr RJ. A novel gene, *erm*(41), confers inducible macrolide resistance to clinical isolates of *Mycobacterium abscessus* but is absent from *Mycobacterium chelonae*. Antimicrob Agents Chemother 2009;53:1367–76.
[8] Bastian S, Veziris N, Roux A-L, Brossier F, Gaillard J-L, Jarlier V, et al. Assessment of clarithromycin susceptibility in strains belonging to the *Mycobacterium abscessus* group by *erm*(41) and *rrl* sequencing. Antimicrob Agents Chemother 2011;55:775–81.
[9] Wallace Jr RJ, Meier A, Brown BA, Zhang Y, Sander P, Onyi GO, et al. Genetic basis for clarithromycin resistance among isolates of *Mycobacterium chelonae* and *Mycobacterium abscessus*. Antimicrob Agents Chemother 1996;40:1676–81.
[10] Nash KA, Zhang Y, Brown-Elliott BA, Wallace Jr RJ. Molecular basis of intrinsic macrolide resistance in clinical isolates of *Mycobacterium fortuitum*. J Antimicrob Chemother 2005;55:170–7.
[11] Nakanaga K, Sekizuka T, Fukano H, Sakakibara Y, Takeuti F, Wada S, et al. Discrimination of *Mycobacterium abscessus* subsp. *massiliense* from *Mycobacterium abscessus* subsp. *abscessus* in clinical isolates by multiplex PCR. J Clin Microbiol 2014;52:1–9.
[12] Nessar R, Cambau E, Reyrat JM, Murray A, Gicquel B. *Mycobacterium abscessus*: a new antibiotic nightmare. J Antimicrob Chemother 2012;67:810–8.
[13] van Ingen J, Totten SE, Helstrom NK, Heifets LB, Boeree MJ, Daley CL. *In vitro* synergy between clofazimine and amikacin in treatment of nontuberculous mycobacterial disease. Antimicrob Agents Chemother 2012;56:6324–7.
[14] Woods GL, Bergmann JS, Witebsky FG, Fahle GA, Wanger A, Boulet B, et al. Multisite reproducibility of results obtained by the broth microdilution method for susceptibility testing of *Mycobacterium abscessus, Mycobacterium chelonae*, and *Mycobacterium fortuitum*. J Clin Microbiol 1999;37:1676–82.
[15] Yang SC, Hsueh PR, Lai HC, Teng LJ, Huang LM, Chen JM, et al. High prevalence of antimicrobial resistance in rapidly growing mycobacteria in Taiwan. Antimicrob Agents Chemother 2003;47:1958–62.
[16] Fernández-Roblas R, Martín-de-Hijas NZ, Fernández-Martínez AI, García-Almeida D, Gadea I, Esteban J. In vitro activities of tigecycline and 10 other antimicrobials against nonpigmented rapidly growing mycobacteria. Antimicrob Agents Chemother 2008;52:4184–6.
[17] Brown-Elliott BA, Killingley J, Vasireddy S, Bridge L, Wallace Jr RJ. *In vitro* comparison of ertapenem, meropenem, and imipenem against isolates of rapidly growing *Mycobacteria* and *Nocardia* by use of broth microdilution and Etest. J Clin Microbiol 2016;54:1586–92.
[18] Yoshida S, Tsuyuguti K, Suzuki K, Tomita M, Odaka M, Shimada R, et al. Rapid identification of strains belonging to the *Mycobacterium abscessus* group through *erm* (41) gene pyrosequencing. Diagn Microbiol Infect Dis 2014;79:331–6.
[19] Namkoong H, Kurashima A, Morimoto K, Hoshino Y, Hasegawa N, Ato M, et al. Epidemiology of pulmonary nontuberculous mycobacterial disease. Japan Emerg Infect Dis 2016;22:1116–7.
[20] Morimoto K, Hasegawa N, Izumi K, Namkoong H, Uchimura K, Yoshiyama T, et al. A laboratory-based analysis of nontuberculous mycobacterial lung disease in Japan from 2012 to 2013. Ann Am Thorac Soc 2017;14:49–56.
[21] Kim HY, Kim BJ, Kook Y, Yun YJ, Shin JH, Kim BJ, et al. *Mycobacterium massiliense* is differentiated from *Mycobacterium abscessus* and *Mycobacterium bolletii* by erythromycin ribosome methyltransferase gene (*erm*) and clarithromycin susceptibility patterns. Microbiol Immunol 2010;54:347–53.
[22] Brown-Elliott BA, Vasireddy S, Vasireddy R, Iakhiaeva E, Howard ST, Nash K, et al. Utility of sequencing the *erm* (41) gene in isolates of *Mycobacterium abscessus* subsp. *abscessus* with low and intermediate clarithromycin MICs. J Clin Microbiol 2015;53:1211–5.
[23] Kehrmann J, Kurt N, Rueger K, Bande F-C, Buer J. GenoType NTM-DR for identifying *Mycobacterium abscessus* subspecies and determining molecular resistance. J Clin Microbiol 2016;54:1653–5.
[24] Luo RF, Curry C, Taylor N, Budvytiene I, Banaei N. Rapid detection of acquired and inducible clarithromycin resistance in *Mycobacterium abscessus* group by a simple real-time PCR assay. J Clin Microbiol 2015;53:2337–9.
[25] Broda A, Jebbari H, Beaton K, Mitchell S, Drobniewski F. Comparative drug resistance of *Mycobacterium abscessus* and *M. chelonae* isolates from patients with and without cystic fibrosis in the United Kingdom. J Clin Microbiol 2013;51:217–23.