Nationwide surveillance of antimicrobial susceptibility of 509 rapidly growing mycobacteria strains isolated from clinical specimens in Japan

Keisuke Kamada1,2,3, Atsushi Yoshida3, Shigekazu Iguchi2, Yuko Arai1, Yutaka Uzawa1, Satoshi Konno2, Masahiro Shimojima4 & Ken Kikuchi1*

This study aimed to identify effective treatments against rapidly growing mycobacteria (RGM) infections by investigating the minimum inhibitory concentrations (MIC) of 24 antimicrobial agents and their molecular mechanisms of resistance. In total, 509 clinical RGM isolates were identified by analyzing the sequences of three housekeeping genes (hsp65, rpoB, and sodA), and their susceptibilities to 24 antimicrobial agents were tested. We also performed sequencing analysis of antimicrobial resistance genes (rrl, rrs, gyrA, and gyrB). To identify Mycobacteroides abscessus group subspecies, we performed PCR-based typing and determined the sequevar of erm(41). We identified 15 RGM species, most of which were susceptible to amikacin and linezolid. Among these species, arbekacin and sitafloxacin had the lowest MIC among the same class of antimicrobials. The MIC of rifabutin for M. abscessus subsp. abscessus (MAB) was lower than that for M. abscessus subsp. massiliense (MMA). The proportion of MAB isolates with MIC ≤ 2 mg/L for rifabutin was significantly higher than that of MMA [MAB: 50/178 (28.1%) vs. MMA: 23/130 (17.7%); p = 0.041]. In summary, our study revealed the antimicrobial susceptibility profile of 15 RGM species isolated in Japan and indicated that arbekacin, sitafloxacin, and rifabutin may be possible therapeutic options for RGM infections.

Rapidly growing mycobacteria (RGM) infections constitute a serious public health concern worldwide, particularly in East Asia, and the proportion of RGM among nontuberculous mycobacteria (NTM) is high. The prevalence of infections caused by the Mycobacteroides abscessus group (MAG), a major group of RGM, has increased in Japan. Several mycobacterial species causing RGM infections have a natural resistance to several antimicrobials, rendering standard treatment regimens inefficient. Several gene mutations related to drug susceptibility or resistance in RGM have been reported, including: *erm*(41) C28 sequevar, which is related to macrolide susceptibility; *rrl*, which is associated with acquired resistance to macrolides; *rrs*, which affects aminoglycoside resistance; and *gyrA* and *gyrB*, which encode a quinolone resistance-determining region (QRDR) related to emerging quinolone resistance. Of these genes, the most important is *erm*(41), which is involved in the macrolide-induced resistance of MAG. When MAG is exposed to a macrolide, the *erm*(41) gene is expressed, and the product of this gene methylates the macrolide binding site on 23S rRNA. This inhibits the action of the macrolide, resulting in drug resistance. To accurately evaluate this induced resistance, it is necessary to wait until the 14th day after the start of the drug susceptibility test.

There are differences in the *erm*(41) gene sequence among MAG subspecies.

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1Department of Infectious Diseases, Tokyo Women's Medical University, 8-1 Kawada-Cho, Shinjuku-ku, Tokyo 162-8666, Japan. 2Department of Respiratory Medicine, Faculty of Medicine and Graduate School of Medicine, Hokkaido University, Kita 14-jo Nishi 5-chome, Kita-ku, Sapporo-shi, Hokkaido 001-0014, Japan. 3Department of Mycobacterium Reference and Research, The Research Institute of Tuberculosis, Japan Anti-Tuberculosis Association, Tokyo 204-0022, Japan. 4BML, Inc, Matoba 1361-1, Kawagoe-shi, Saitama 350-1101, Japan. *email: kikuchi.ken@twmu.ac.jp
In *M. abscessus* subsp. *massiliense* (MMA), there is a deletion in the *erm*(41) gene, and its function is lost\(^9\). Therefore, MMA is macrolide-susceptible, whereas *M. abscessus* subsp. *abscessus* (MAB) and *M. abscessus* subsp. *bolletii* (MBO), which do not have this deletion, are often macrolide-induced resistant\(^9\). However, it is known that this macrolide-induced resistance is lost because of the T28C mutation in the *erm*(41) gene, even in the *erm*(41) gene without the deletion\(^4\).

Previous reports in the USA showed that there were 10 different sequevar types in the *erm*(41) gene, and that the sequevar types with C28 (type 2, 3, and 5) were macrolide-susceptible, whereas the other sequevar types with T28 (type 1, 4, 6, 7, 8, 9, and 10) were mostly macrolide-resistant\(^10\). Because differences in the sequence of the *erm*(41) gene are useful in predicting susceptibility to macrolides, the Clinical Laboratory Standards Institute (CLSI) recommends that the sequevar type of the *erm*(41) gene be evaluated in MAB\(^11\). However, in actual clinical practice in Japan, the *erm*(41) sequevar type is rarely determined, strictly due to the labor and cost required for testing, and these epidemiological data are unknown.

Susceptibility of RGM to antimicrobials remains controversial for multiple reasons. First, because of the high degree of phylogenetic similarity between different RGM, accurate species identification requires detailed genetic analysis. Previously reported large-scale antimicrobial susceptibility tests have not always identified RGM species with sufficient accuracy\(^12\). The minimum inhibitory concentration (MIC) breakpoints of only 11 antimicrobials are described in CLSI M24A-2\(^8\), and the MIC values of other antimicrobials that have been measured according to the CLSI method are not sufficiently evaluated. Second, recent reports demonstrate that susceptibility to macrolides correlates with the response rate\(^13,14\), and that the use of azithromycin, imipenem, and amikacin is associated with good therapeutic results\(^15\) for pulmonary infections caused by MAG. However, the correlation between breakpoints proposed by CLSI and treatment outcomes remains unclear for most antimicrobials in many settings of RGM infection. Gathering information regarding the MIC values of antimicrobials that can be used as therapeutic options for treating infections caused by RGM species is essential. Third, epidemiological information related to the gene mutations involved in antimicrobial resistance is scarce.

Therefore, the current study aimed to determine the MIC of 24 antimicrobial agents for clinically isolated RGM and record relevant epidemiological and genetic information to identify potential therapeutic agents.

### Results

The details of 15 species that were identified are shown in Table 1. Eleven isolates, *M. abscessus* subsp. *abscessus* (MAB) (5), *M. abscessus* subsp. *massiliense* (MMA) (3), *M. chelonae* (2), and *M. senegalense* (1) grew poorly in the culture medium at five days after the start of susceptibility test, and thus we could not obtain MIC data for these isolates.

### Characteristics of antimicrobial susceptibilities of RGM species.

Other than MMA, which was susceptible to both amikacin and clarithromycin, MAB and *M. abscessus* subsp. *bolletii* (MBO) were susceptible to only amikacin (Table 2). Although *M. fortuitum* was resistant to macrolides, it was susceptible to amikacin.
cin, imipenem, fluoroquinolones, and trimethoprim/sulfamethoxazole (Table 3). Only three isolates were not susceptible to fluoroquinolones. Most *M. chelonae* isolates were susceptible to clarithromycin. However, the proportion of isolates intermediate and resistant to aminoglycosides, imipenem, cefoxitin, and fluoroquinolones was high. Additionally, we found that 46% of *M. chelonae* strains were susceptible to tobramycin (Table 3). *M. mageritense* isolates showed remarkably high resistance to clarithromycin and amikacin, but were susceptible to fluoroquinolones, imipenem, and cefoxitin (Table 3). The results of the antimicrobial susceptibility test and

| Antimicrobial agents | M. abscessus group (MAG: n = 313<sup>a</sup>) | subsp. abscessus (MAB: n = 178) | subsp. massiliense (MMA: n = 130) |
|----------------------|-----------------------------------------------|---------------------------------|----------------------------------|
|                      | Susceptibility<sup>b</sup> % (n) | Susceptibility % (n) | Susceptibility % (n) |
| Linezolid            | 33 (104) | 19 (59) | 48 (150) | 30 (54) | 19 (34) | 51 (90) | 37 (48) | 19 (25) | 44 (57) |
| Clarithromycin (ERT) | 82 (256) | 6 (20) | 12 (37) | 74 (131) | 11 (19) | 15 (28) | 93 (121) | 1 (1) | 6 (8) |
| Clarithromycin (LRT) | 43 (135) | 3 (9) | 54 (169) | 11 (19) | 3 (6) | 86 (153) | 89 (116) | 2 (3) | 9 (11) |
| Amikacin             | 87 (271) | 12 (38) | 1 (4) | 90 (160) | 9 (16) | 1 (2) | 82 (106) | 17 (22) | 1 (2) |
| Tobramycin           | 2 (7) | 9 (27) | 89 (279) | 3 (5) | 12 (22) | 85 (151) | 1 (2) | 4 (5) | 95 (123) |
| Imipenem             | 25 (80) | 59 (184) | 16 (49) | 27 (48) | 58 (103) | 15 (27) | 25 (32) | 60 (78) | 15 (20) |
| Ciprofloxacin        | 2 (6) | 4 (12) | 94 (295) | 3 (5) | 6 (10) | 91 (163) | 1 (1) | 1 (2) | 98 (127) |
| Moxifloxacin         | 5 (16) | 6 (20) | 89 (277) | 11 (19) | 9 (16) | 85 (151) | 4 (5) | 3 (4) | 93 (121) |
| Cefoxitin            | 23 (72) | 69 (215) | 8 (26) | 25 (44) | 67 (120) | 8 (14) | 22 (28) | 69 (90) | 9 (12) |

Table 2. Antimicrobial susceptibility of *Mycobacteroides abscessus* group (MAG) strains. A) These strains included 5 M. abscessus subsp. bolletii (MBO). B) S: Susceptible; I: intermediate; R: resistant. C) Minimum inhibitory concentration (MIC) data for minocycline could not be determined for one strain. D) Trimethoprim/sulfamethoxazole. E) Amoxicillin/clavulanic acid.
MICs for other rare RGM species are shown in Table 4 and Table S2, respectively. Amikacin and linezolid were the most effective against the 15 isolated RGM species (Table 4).

We also investigated the MICs against RGM for antibacterial drugs for which CLSI did not set breakpoints. The MIC50 of sitafloxacin was the lowest among all the fluoroquinolones for all RGM species (Tables 2, 3).

### Table 3. Antimicrobial susceptibility of major rapidly growing mycobacteria (RGM) strains other than *M. abscessus* group (MAG). A) Minimum inhibitory concentration (MIC) for ciprofloxacin and moxifloxacin could not be determined for one strain. B) Trimethoprim/sulfamethoxazole. C) Amoxicillin/clavulanic acid.

| Antimicrobial agents | *M. fortuitum* (n = 85) | *M. chelonae* (n = 55) | *M. peregrinum* (n = 11) | *M. mageriense* (n = 10) |
|----------------------|-------------------------|------------------------|--------------------------|--------------------------|
|                      | Susceptibility % | Susceptibility % | Susceptibility % | Susceptibility % |
| Linezolid            | 71 | 14 | 15 | 49 | 33 | 18 | 82 | 18 | 0 | 80 | 10 | 10 |
| Clarithromycin (ERT) | 18 | 12 | 70 | 96 | 4 | 0 | 82 | 0 | 18 | 0 | 0 | 100 |
| Clarithromycin (LRT) | 0 | 1 | 99 | 89 | 4 | 7 | 82 | 0 | 18 | 0 | 0 | 100 |
| Amikacin             | 100 | 0 | 0 | 62 | 34 | 4 | 100 | 0 | 0 | 0 | 60 | 40 |
| Tobramycin           | 4 | 0 | 96 | 46 | 47 | 7 | 0 | 18 | 82 | 0 | 0 | 100 |
| Imipenem             | 94 | 5 | 1 | 82 | 53 | 29 | 82 | 0 | 18 | 70 | 20 | 10 |
| Ciprofloxacin        | 95 | 1 | 4 | 5 | 20 | 75 | 91 | 0 | 9 | 89 | 11 | 0 |
| Moxifloxacin         | 97 | 2 | 1 | 20 | 73 | 91 | 90 | 1 | 0 | 89 | 11 | 0 |

| **Table 3.** | **Antimicrobial agents** | **MIC (mg/L)** |
|---------------|--------------------------|----------------|
| **Linezolid** | <1 to >64 | 4 | 32 | <1 to >64 | 16 | 32 | <1 to 16 | 4 | 16 | 1 | 16 |
| **Clarithromycin (ERT)** | <0.12 to 4 | 8 | >32 | <0.12 to >32 | 0.25 | 1 | <0.12 to 16 | 1 | 8 | 32 | >32 |
| **Clarithromycin (LRT)** | <0.12 to >32 | >32 | >32 | <0.12 to >32 | 1 | 4 | <0.12 to 16 | 1 | >32 | >32 | >32 |
| **Azithromycin (ERT)** | 0.5 to >32 | >32 | >32 | >32 | 2 | 8 | 0.25 to >32 | 2 | >32 | >32 | >32 |
| **Azithromycin (LRT)** | 16 to >32 | >32 | >32 | 0.25 to >32 | 8 | 32 | 0.5 to >32 | 16 | >32 | >32 | >32 |
| **Arbekacin** | <1 to 16 | 4 | 4 | <1 to 16 | 4 | 8 | <1 to 4 | <1 | 4 | 8 to 64 | 32 | 64 |
| **Amikacin** | <1 to 16 | 2 | 4 | 2 to 64 | 16 | 32 | <1 to 8 | <1 | 2 | 32 to >128 | 64 | 128 |
| **Gentamicin** | <1 to 128 | 8 | 16 | 2 to 32 | 8 | 16 | <1 to 8 | 4 | 8 | 16 to >128 | 64 | 128 |
| **Tobramycin** | 2 to >64 | 32 | 64 | <0.5 to 32 | 4 | 4 | 4 to 16 | 8 | 8 | >64 to >64 | >64 | >64 |
| **Imipenem** | 1 to >64 | 4 | 4 | 1 to >64 | 16 | 32 | 1 to 8 | 4 | 8 | 2 to 32 | 2 | 16 |
| **Doripenem** | 2 to >128 | 8 | 16 | 4 to >128 | >128 | >128 | 2 to 16 | 8 | 16 | 2 to 64 | 4 | 32 |
| **Faropenicillin** | 2 to >128 | 16 | 32 | 32 to >128 | >128 | >128 | <1 to 32 | 8 | 16 | 2 to 128 | 4 | 32 |
| **Levofloxacin** | <0.5 to 32 | <0.5 | 2 | 2 to 32 | 16 | 32 | <0.5 to 32 | <0.5 | 4 | 16 to >32 | <0.5 | 2 |
| **Sitafloxacin** | <0.03 to 1 | 0.12 | 0.25 | 0.25 to >4 | 1 | 4 | <0.03 to 1 | 0.06 | 0.12 | <0.03 to 0.5 | 0.06 | 0.25 |
| **Ciprofloxacin** | <0.12 to >16 | 0.25 | 1 | 1 to 16 | 4 | >16 | <0.12 to >16 | <0.12 | 0.5 | 0.25 to 2 | 0.25 | 2 |
| **Moxifloxacin** | <0.06 to 2 | 0.12 | 0.5 | 0.5 to >8 | 4 | >8 | <0.06 to 4 | 0.12 | 0.25 | 0.12 to 2 | 0.12 | 2 |
| **Cefoxitin** | 2 to >128 | 32 | 64 | 16 to >64 | >64 | 8 to 16 | 8 | 16 | 16 to 32 | 16 | 32 |
| **Ceftriaxone** | 2 to >64 | 32 | 64 | 16 to >64 | >64 | 8 to 16 | 8 | 16 | 16 to 32 | 16 | 32 |
| **Cefepime** | 4 to >128 | >64 | >64 | >64 | >64 | >64 | >64 | >64 | >64 | >64 |
| **Ethanbutol** | 8 to >128 | >32 | >32 | >32 | >32 | >32 | >32 | >32 | >32 | >32 |
| **Rifabutin** | 2 to >128 | 16 | 64 | 32 to >128 | >128 | >128 | 2 to >128 | 16 | 64 | 16 to 32 | 32 | 64 |
| **Minocycline** | <0.5 to >32 | 8 | >32 | <0.5 to >32 | 32 | >32 | <0.5 to 16 | 8 | >32 | 2 to 32 | 4 | 8 |
| **Tigecycline** | 0.12 to 4 | 0.5 | 1 | 0.12 to >4 | 1 | 2 | 0.12 to 4 | 0.5 | 1 | 0.12 to 1 | 0.25 | 1 |
| **AMPC/CVA** | 2/1 to >128/64 | 32/16 | 128/64 | 32/16 to >128/64 | >128/64 | >128/64 | 8/4 to >128/64 | 128/64 | >128/64 | 8/4 to 128/64 | 16/8 | 64/32 |
| **ST** | <0.25 to 4/8 | 1/19 | 4/76 | <0.5 to >8/152 | <4/76 | >152/8 | <0.25/4.8 to >1/19 | <0.25/4.8 | 1/19 | 1/19 to >8/152 | 4/76 | >8/152 |
Except in *M. fortuitum* and *M. wolinskyi* isolates, the MIC50 of arbekacin was the lowest among aminoglycoside antimicrobials (Tables 2, 3). The MIC50 of cefmetazole was equal to or lower than that of cefoxitin for all RGM species, although the values were similar (Tables 2, 3). The MIC50 of rifabutin was lower among MAB than among MMA. The proportion of isolates with MIC ≤ 2 mg/L for rifabutin was significantly higher than that of MMA isolates [MAB: 50/178 (28.1%) vs. MMA: 23/130 (17.7%); *p* = 0.041] (Table 2). Faropenem had a higher MIC50 than imipenem among all RGM isolates except *M. iranicum* (Tables 2, 3).

### Relationship between MAB *erm*(41) sequevar type and susceptibility to clarithromycin.

The sequence of *erm*(41) was obtained from 180 isolates, and the relationship between MAB *erm*(41) sequevar and clarithromycin MIC was determined (Table 5). For the remaining three isolates, we could not obtain any sequence data. None of the MAB isolates had a truncated *erm*(41) sequevar, whereas 2 of 133 MMA isolates had a functional *erm*(41) T28 sequevar. The clarithromycin (late-reading-time [LRT]) MICs for these two isolates were 0.5 mg/L and 8 mg/L, respectively. The *erm*(41) gene sequences of 131 MMA isolates were identical. In this survey, the proportion of the C28 sequevar in MAB was 12.2% (22/180), all of which were type 2. Several new sequevar types were identified in our isolates; the two most common of these new isolates were named jpn1 and jpn2. These new sequevars were similar to type 10, and all isolates were resistant to clarithromycin. The single nucleotide polymorphisms of *erm*(41) in each sequevar are shown in Table S3. Of the 158 isolates of the T28 sequevar, 7 showed clarithromycin MIC ≤ 4 mg/L, including isolates of types 1, 6, 7, 8, and 10.

### Relationship between *rrl* gene mutation of MAG and susceptibility to clarithromycin.

Among the 37 MAG isolates with acquired macrolide resistance, the proportions with *rrl* mutations were 2/24 for the MAB T28 sequevar, 0/2 for the MAB C28 sequevar, 1/2 for MAB unknown, 1/1 for MBO, and 3/8 for MMA (Table 6). However, the rate of *rrl* mutation among MMA isolates that acquired macrolide resistance was higher than that of the MAB T28 sequevar, although not significantly (MAB T28 sequevar: 2/24 [8.3%] vs. MMA: 3/8 [37.5%]; *p* = 0.085).

### Relationship between *rrs* gene mutation and susceptibility to amikacin.

*rrs* (A1408G) mutations were not found among the 73-amikacin non-susceptible (MIC ≥ 32 mg/L) isolates (MAG, *M. chelonae*, and *M. mageritense*).

### Quinolone resistance of *M. fortuitum* and its mechanism.

Of the three isolates of *M. fortuitum* that were resistant to ciprofloxacin, only one had a mutation in *gyrA*. In the mutant strain, the *gyrA* gene resulted in S83W amino acid substitution (TCG → TGG). None of the ciprofloxacin-susceptible isolates had mutations in *gyrA* and *gyrB*.

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**Table 4.** Characteristics of antimicrobial susceptibility of rapidly growing mycobacteria (RGM) species. ◎Susceptible isolates > 75%, ○ Susceptible isolates 51%–75%, △ Susceptible isolates 25%–50%, ▲ Susceptible isolates < 25% A) Trimethoprim/sulfamethoxazole.

|                      | Linezolid | Clarithromycin | Amikacin | Tobramycin | Imipenem | Cefoxitin | Moxifloxacin | Minocycline | ST(4) |
|----------------------|-----------|----------------|----------|------------|-----------|-----------|--------------|-------------|-------|
| MAB                  | △         | ◎              | △        | △          | △         | △         | △ △          | △ △ △ △    | △ △ |
| MMA                  | △         | ◎              | △        | △          | △         | △         | △ △          | △ △ △ △    | △ △ |
| MBO                  | △         | ◎              | △        | △          | △         | △         | △ △          | △ △ △ △    | △ △ |
| *M. fortuitum*       | ○         | △              | △        | △          | △         | △         | △ △ △        | △ △ △ △    | △ △ |
| *M. chelonae*        | △         | ◎              | △        | △          | △         | △         | △ △ △ △      | △ △ △ △    | △ △ |
| *M. peregrinum*      | ◎         | ○              | △        | △          | △         | △         | △ △ △ △      | △ △ △ △    | △ △ |
| *M. mageritense*     | ○         | △              | △        | △          | △         | △         | △ △ △ △ △    | △ △ △ △ △  | △ △ |
| *M. septicum*        | ○         | △              | △        | △          | △         | △         | △ △ △ △ △    | △ △ △ △ △  | △ △ |
| *M. musogenicum*     | ◎         | ○              | △        | △          | △         | △         | △ △ △ △ △    | △ △ △ △ △  | △ △ |
| *M. porcinum*        | ○         | △              | △        | △          | △         | △         | △ △ △ △ △    | △ △ △ △ △  | △ △ |
| *M. wolinskyi*       | ◎         | △              | △        | △          | △         | △         | △ △ △ △ △ △  | △ △ △ △ △ △ | △ △ |
| *M. senegalense*     | ○         | ○              | △        | △          | △         | △         | △ △ △ △ △ △  | △ △ △ △ △ △ | △ △ |
| *M. goodie*          | ○         | △              | △        | △          | △         | △         | △ △ △ △ △ △  | △ △ △ △ △ △ | △ △ |
| *M. iranicum*        | ◎         | ○              | △        | △          | △         | △         | △ △ △ △ △ △  | △ △ △ △ △ △ | △ △ |
| *M. canariense*      | ◎         | △              | △        | △          | △         | △         | △ △ △ △ △ △  | △ △ △ △ △ △ | △ △ |
| *M. immunogenum*     | ○         | △              | △        | △          | △         | △         | △ △ △ △ △ △  | △ △ △ △ △ △ | △ △ |
| *M. sphagni*         | ○         | △              | △        | △          | △         | △         | △ △ △ △ △ △  | △ △ △ △ △ △ | △ △ |
| *M. canariense*      | ◎         | ○              | △        | △          | △         | △         | △ △ △ △ △ △  | △ △ △ △ △ △ | △ △ |
| *M. immuno*          | ◎         | ○              | △        | △          | △         | △         | △ △ △ △ △ △  | △ △ △ △ △ △ | △ △ |
| *M. sphagni*         | ◎         | ○              | △        | △          | △         | △         | △ △ △ △ △ △  | △ △ △ △ △ △ | △ △ |
Discussion

In this study, we accurately identified 15 species of RGM from clinical isolates obtained from different locations around Japan. We characterized the susceptibility of these isolates to 24 antimicrobials, including tigecycline, sitafloxacin, rifabutin, and cefmetazole; none have defined MIC breakpoints in the CLSI, but they may have potential as therapeutic agents for RGM infections. We investigated not only MAG antimicrobial susceptibility, but also several gene mutations involved in antimicrobial resistance and prepared a summary of the susceptibility of the remaining 14 species of RGM.

The proportion of the C28 sequevar in MAB isolated from lower respiratory specimens (LRS) has been reported to be approximately 16–35%4, 10, 16, 17. However, in some previous Japanese reports, the ratio of the C28 sequevar among MAB from LRS was very low at 4.2% (2/48)18. In our survey, it was 12.2% (22/180), which is higher than that in the previous report18. In Japan, it is necessary to continue to evaluate whether the proportion of the C28 sequevar in MAB is lower than those in other countries.
A previous report from the USA indicated that sequevar types 4, 6, 7, 8, 9, and 10 (all T28 sequevars) may be associated with macrolide-induced resistance16. However, similar assessments outside of the USA have not been conducted so far. Among the 180 MABs in our study, only 4 isolates with erm(41) sequevar types 6, 7, 8, and 10 were susceptible to clarithromycin. Our data were generally consistent with the previous report19. Therefore, it was suggested that these sequevars are macrolide-resistant. So far, CLSI has recommended the determination of the erm(41) sequevar type for evaluation of induced macrolide resistance in MAB21, and our results support this recommendation. Further investigations on the relationship between sequevar types and macrolide resistance in other regions are required.

The rrl gene mutation is more likely to occur in the MAB C28 sequevar and MMA than in the MAB T28 sequevar among clarithromycin-acquired resistant strains in MAG2. In our survey, we found a similar trend but could not show a significant difference. Among MAG, more than half of the macrolide-acquired resistance occurred by mechanisms other than rrl gene mutation. The exact mechanism remains to be investigated.

Additionally, none of the amikacin non-susceptible isolates in our survey had the rrs gene mutation. A previous French study of antimicrobial susceptibility in 165 isolates of MAG showed that 7/8 strains with amikacin MIC > 64 mg/L had a rrs A1408G gene mutation16, which suggested that amikacin MIC > 64 mg/L is a criterion to suspect amikacin-acquired resistance16. In our survey, only one isolate of MAG showed MIC > 64 mg/L, and none of the isolates showed rrs mutation. MAG isolated in Japan may have fewer amikacin-acquired resistant isolates than those isolated in France.

As reported previously16,19, M. fortuitum was resistant to clarithromycin; however, it was susceptible to aminoglycosides, carbapenems, and fluoroquinolones in our study. Previous reports suggest that, in M. fortuitum, a serine residue at the 83rd position of gyrA constitutes QRDR and contributes to susceptibility to fluoroquinolones compared with other NTMs2. However, to date, only one report has shown quinolone resistance due to mutations in gyrA19. There has been no report of mutations in a serine residue at the 83rd position of gyrA. Fluoroquinolone resistance was found in 3 of 85 (3.5%) isolates in our study, and the S83W amino acid substitution was present in one of the three isolates. Our result also suggests that fluoroquinolone resistance can occur based on genetic changes other than QRDR mutations, and it is necessary to clarify the resistance mechanism in the future. In Japan, fluoroquinolones are being overused20, and there is a concern regarding the increase of fluoroquinolone-resistant isolates in M. fortuitum. Because M. fortuitum shows induced resistance to macrolides, fluoroquinolones play an important role in the treatment of M. fortuitum infections as an oral antibiotic. There is a great concern regarding treatment efficacy with the increase in resistant isolates.

Among M. chelonae isolates, resistance to clarithromycin was found in approximately 10% of isolates, consistent with previous reports18,21. Previous reports seem to indicate regional variability in tobramycin susceptibility, ranging from 54% in the UK21 to 83% and 17% in Japan16,22. In our study, approximately 40% of the strains were tobramycin-susceptible, an intermediate value between the values reported by the two previous reports from Japan. In addition, no rrs mutations were found in amikacin non-susceptible isolates. Arbekacin may be a potential therapeutic for isolates that are less susceptible to amikacin and tobramycin.

M. peregrinum was susceptible to most of the tested antimicrobials. M. mageritense isolates were resistant to clarithromycin, as has been previously reported21, and showed a low susceptibility to amikacin, although none had a rrs gene mutation (3 isolates showed an amikacin MIC > 64 mg/L). The mechanism of M. mageritense resistance to amikacin remains to be investigated. Conversely, it showed good susceptibility to quinolones, cefoxitin, and linezolid.

There are few reports on antimicrobial susceptibility for other rare RGM species using a sufficiently high number of clinical isolates. There is only one study involving M. mucogenicum and M. immunogenum reporting that most of the isolates were susceptible to linezolid, amikacin, and trimethoprim/sulfamethoxazole, while showing a poor susceptibility to clarithromycin21. In our study, although the number of isolates was small, we could show the tendency of antimicrobial susceptibility for rare RGM species. These rare RGM species tended to be susceptible to linezolid, quinolones, and trimethoprim/sulfamethoxazole.

Although there have been no reports regarding the MIC of arbekacin in RGM, this antimicrobial showed the lowest MIC among the aminoglycosides for almost all RGM species in this study (Tables 2, 3). The MIC50 value of sitafloxacin is reported to be lower than that of other fluoroquinolones in MAG, M. fortuitum, and M. chelonae18. However, in this study, we showed that the effect of sitafloxacin was similar on the 15 RGM species (Tables 2, 3). Cefmetazole and cefoxitin, cephahycin-based antimicrobials, had similar MICs, consistent with previous reports24,26 (Tables 2, 3). In countries such as Japan, when patients cannot be administered cefoxitin, cefmetazole may be an option for RGM treatment. In recent years, rifabutin has attracted attention as an oral treatment for MAG25,28, but, so far, there have been few reports of MICs measured by micro-dilution using cation-adjusted Mueller–Hinton broth medium26. Here, we have not only measured rifabutin MICs for many isolates using this standard method, but also showed that MICs were lower for MAB than for MMA (Table 2). MAB has a very high resistance rate not only to clarithromycin but also to fluoroquinolone; thus, finding an alternative orally administrated therapeutic option is essentially required in the future to determine whether rifabutin will be an effective orally administrated therapeutic option.

There are some limitations to our study. It was unclear whether there was prior administration of antibacterial drugs before susceptibility testing for all isolates. Some of the RGM species isolated in this study were rarely isolated to evaluate drug susceptibility. However, despite these limitations, our study reveals important epidemiological information about RGM in Japan and suggests several drugs that can be investigated as new treatment candidates. It is therefore necessary to accumulate and evaluate data from a larger set of samples and to verify the correlation between the actual therapeutic effect and the MIC values of these drugs in clinical trials.

We showed antimicrobial susceptibility profiles of 15 RGM species isolated in Japan. Amikacin and linezolid were the most effective against the 15 isolated RGM species. Arbekacin, sitafloxacin, and cefmetazole may be
possible therapeutic options for RGM infections. Based on the MIC values, rifabutin may be more potent in the treatment of MAB than MMA. Clinical trials are needed in the future to validate our findings.

Methods
Clinical isolates. From January 2012 to March 2019, 509 clinical specimens [409 LRS, 87 non-lower respiratory specimens, and 13 unknown] isolated from patients in Japan (one specimen per patient) were included in this study. From the specimens, 403 strains were isolated at BioMedical Laboratories (BML), Inc., a major clinical laboratory, and 106 were isolated at 45 hospitals in Japan.

PCR and sequence analysis. Bacterial genomic DNA was extracted using ISOPLANT II (NIPPON GENE CO., LTD, Japan). The three housekeeping genes, hsp65, rpoB, and sodA, of each isolate were sequenced for RGM species identification. For MAG, an additional PCR-based typing scheme was used for subspecies identification, and the erm(41) sequence type was determined. The sequence of rrl from MAG strains exhibiting acquired macrolide resistance was also determined. Similarly, the sequences of the rrs gene from MAG, M. chelonae, and M. mageritense, which are amikacin non-susceptible isolates, were analyzed. The sequences of the gyrA and gyrB genes encoding the QRDR were elucidated for all M. fortuitum isolates. All PCR procedures were performed as described previously, and the primers used are shown in Table S1.

Antimicrobial susceptibility test. All strains were subcultured on trypticase soy agar with 5% sheep blood (Becton, Dickinson and Company, New Jersey) at 35 °C for 3–5 days. Antimicrobial susceptibility testing was performed per the recommendations in the CLSI M24A-2 at 30 °C. A nephelometer (VITEK DENISCHEK, bioMerieux, France) was used to standardize the inoculum density (0.5 McFarland standard). The MICs of 24 antimicrobial agents (tigecycline, linezolid, clarithromycin, azithromycin, ampicillin, gentamicin, tobramycin, imipenem, doripenem, faropenem, levofloxacin, sitafloxacin, ciprofloxacin, moxifloxacin, cefmetazole, cefotaxime, cephalaxin, cefepime, ethambutol, rifabutin, minocycline, amoxicillin/clavulanic acid, and trimethoprim/sulfamethoxazole) were measured by the micro-dilution method using cation-adjusted Mueller–Hinton broth medium (Becton, Dickinson and Company). The MICs of clarithromycin and azithromycin were read two times to detect induced resistance. Positive growth of the control between days 3 and 5 was defined as early-reading-time. Inducible macrolide resistance was determined on day 14 and defined as LRT. Repetition of MIC measurement, as recommended by guidelines, was performed without exception.

Statistical analysis. Statistical analyses were performed using GraphPad Prism ver. 8.2.0 for Windows (GraphPad Software, San Diego, CA, USA). Data were compared using the Chi-square test for categorical variables, whereas Fisher’s exact test was used where the assumption of the Chi-square test was violated.

Data availability
The dataset generated and analyzed during the current study is available from the corresponding author on reasonable request.

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Author contributions
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Competing interests
The authors declare no competing interests.

Additional information
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