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

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Abstract

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*, *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 antibiotics. The MIC of rifabutin for *M. abscessus* subsp. *abscessus* (MAB) was lower than that for *M. abscessus* subsp. *massiliense* (MMA).

The number 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.

Introduction

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 [1]. The prevalence of infections caused by the *Mycobacteroides abscessus* group (MAG), a major group of RGM, has increased in Japan [2]. Several mycobacterial species causing RGM infections have a natural resistance to several antibiotics, rendering standard treatment regimens inefficient [3]. 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 [4]; *rrl*, which is associated with acquired resistance to macrolides [4]; *rrs*, which affects aminoglycoside resistance [5]; and *gyrA* and *gyrB*, which encode a quinolone resistance determining region (QRDR) related to emerging quinolone resistance [6].

Susceptibility of RGM to antibiotics 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 antibiotic susceptibility tests have not always identified RGM species with sufficient accuracy [7]. The minimum inhibitory concentration (MIC) breakpoints of only 11 antibiotics are described in the Clinical and Laboratory Standards Institute (CLSI) M24A-2 [8], and the MIC values of other antibiotics that have been measured according to the method by CLSI are not sufficiently evaluated. Second, recent reports demonstrate that susceptibility to macrolides correlates with the response rate [9,10], and that the use of azithromycin, imipenem, and amikacin is associated with good therapeutic results [11] for pulmonary infections caused by MAG. However, the correlation between breakpoints proposed by CLSI and treatment outcomes remains unclear for most antibiotics in many settings of RGM infection. Gathering information regarding the MIC values of antibiotics 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 [5 *M. abscessus* subsp. *abscessus* (MAB), 3 *M. abscessus* subsp. *massiliense* (MMA), 2 *M. chelonae*, and 1 *M. senegalense*] 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, 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 remarkable resistance to clarithromycin and amikacin, but were susceptible to fluoroquinolones, imipenem, and cefoxitin (Table 3). The results of the antimicrobial susceptibility test and 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. MIC50 of sitafloxacin was the lowest among all the fluoroquinolones for all RGM species. Except in *M. fortuitum* and *M. wolinskyi* isolates, the MIC50 of arbekacin was the lowest among aminoglycoside antibiotics. The MIC50 of cefmetazole was less than that of cefoxitin for all RGM species, although the values were almost similar. The MIC50 of rifabutin was lower among MAB than among MMA. The proportion of isolates with MIC $\leq$ 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]. Faropenem had higher MIC50 than imipenem among all RGM isolates except *M. iranicum*.

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 C28 sequevar in MAB was 12.2% (22/180), all of which were type 2. Several new sequevar types were identified in our isolates; 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 (SNPs) 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 MAB T28 sequevar, 0/2 for 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 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 C268G (numbering system used for *Escherichia coli*) mutation in the *gyrA* gene resulted in S83W amino acid substitution. None of the ciprofloxacin susceptible isolates had mutations in *gyrA* and *gyrB*.

Discussion

In this study, we accurately identified 15 species of RGM from clinical isolates obtained from different locations around Japan and characterized the susceptibility of these isolates to 24 antibiotics, including tigecycline, sitafloxacin, rifabutin, and cefmetazole, none of which has defined MIC breakpoints in the CLSI but 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 C28 sequevar in MAB isolated from lower respiratory specimens (LRS) has been reported to be approximately 16–35% [4,12–14]. However, in some previous Japanese reports, the ratio of C28 sequevar among MAB from LRS was very low at 4.2% (2/48) [15]. In our survey, it was 12.2% (22/180), which is higher than that in previous report [15]. In Japan, it is necessary to continue to evaluate whether the proportion of C28 sequevar in MAB is lower than those in other countries.

The relationship between specific *erm*(41) sequevar types and clarithromycin susceptibility in 349 MAB strains without *rrl* gene mutations was reported in the USA [12]. Only 7 of the 85 isolates with clarithromycin (LRT) MIC≤ 8 mg/L had any of the *erm* (41) sequevar types 4, 6, 7, 8, 9, and 10. Therefore, it was suggested that these sequevars are involved in macrolide resistance [12]. However, similar assessments outside of the USA have not been conducted so far. Among the 180 MABs in our study, only 4 of the 26 isolates with clarithromycin (LRT) MIC ≤ 8 mg/L had any of the *erm*(41) sequevar types 4, 6, 7, 8, 9, and 10. Our data were generally consistent with the previous report [12]. 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 MAB [16], 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 MAB C28 sequevar and MMA than in MAB T28 sequevar among clarithromycin-acquired resistant strains in MAG [4]. 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 mutation [13], which suggested that amikacin MIC > 64 mg/L is a criterion to suspect amikacin-acquired resistance [13]. 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 previously [15,17], *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 to other NTMs [6]. However, to date, only one report has shown quinolone resistance due to mutations in *gyrA* [17]. 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 overused [24], and there is a concern regarding increase of fluoroquinolone-resistant isolates in *M. fortuitum*. Since *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% isolates, consistent with previous reports [15,19]. Previous reports seem to indicate regional variability in

tobramycin susceptibility, ranging from 54% in the UK [25] to 83% and 17% in Japan [15, 20]. 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 antibiotics. *M. mageritense* isolates were resistant to clarithromycin, as has been previously reported [21] 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 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 clarithromycin [22]. 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 MIC of arbekacin in RGM, this antibiotic showed the lowest MIC among the aminoglycosides for almost all RGM species in this study. The MIC50 value of sitafloxacin is reported to be lower than that of other fluoroquinolones in MAG, *M. fortuitum*, and *M. chelonae* [15]. However, in this study, we showed that the effect of sitafloxacin was similar on the 15 RGM species. Cefmetazole and cefoxitin, cephamycin-based antibiotics, had similar MICs, consistent with previous reports [23,24]. 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 MAG [25,26], but, so far, there have been few reports of MICs measured by micro-dilution using cation-adjusted Mueller-Hinton broth medium [26]. 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. MAB has a very high resistance rate not only to clarithromycin but also to fluoroquinolone; thus, finding an alternative orally administrated therapeutic option is essential. A detailed evaluation is required in the future to determine whether rifabutin will be an effective orally administered 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 of 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.

In conclusion, 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 and sitafloxacin may be possible therapeutic options for RGM infections. Because cefmetazole and cefoxitin had similar MICs, cefmetazole may be a substitute for cefoxitin. The MIC of rifabutin for MAB was lower than that for MMA.

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., one of the major clinical laboratories, 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* [27], *rpoB* [28], and *sodA* [29], of each isolate were sequenced for RGM species identification. For MAG, additional PCR-based typing scheme [30] was used for subspecies identification, and *erm*(41) sequence type was determined [12, 31]. The sequence of *rrl* from MAG strains exhibiting acquired macrolide resistance was also determined [32]. Similarly, the sequences of the *rrs* gene from MAG, *M. chelonae*, and *M. mageritense,* which are amikacin non-susceptible isolates, were analyzed [5]. 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 [5,27–32], and the primers used are shown in Table S1.

Antibiotics 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. Antibiotic susceptibility testing was performed per the recommendations in the CLSI M24A-2 at 30 °C [8]. A nephelometer (VITEK DENSICHEK, bioMerieux, France) was used to standardize the inoculum density (0.5 McFarland standard). The MICs of 24 antimicrobial agents (tigecycline, linezolid, clarithromycin, azithromycin, arbekacin, amikacin, gentamycin, tobramycin, imipenem, doripenem, faropenem, levofloxacin, sitafloxacin, ciprofloxacin, moxifloxacin, cefmetazole, cefoxitin, ceftriaxone, 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) [8]. 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 Fisher's exact test for categorical variables. $p < 0.05$ was considered statistically significant.

Declarations

Ethical approval

Not required

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Competing interests

The authors declare no competing interests.

Data availability

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

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Author contributions

KK: Investigation, Data curation, Visualization, Writing- Original draft preparation. AY: Conceptualization, Investigation, Writing- Reviewing and Editing, Funding acquisition. SI: Data curation. MS: Resources. YA: Investigation. YU: Investigation. SK: Supervision, Writing- Reviewing and Editing. K Kikuchi: Conceptualization, Methodology, Data curation, Writing- Reviewing and Editing.

Competing interests

None declared.

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Tables

Tables 1-6 are available in the Supplementary Files.