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Original Article

Moxifloxacin resistance and genotyping of *Mycobacterium avium* and *Mycobacterium intracellulare* isolates in Japan☆

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ABSTRACT

Background: Although fluoroquinolones are considered as alternative therapies of pulmonary *Mycobacterium avium* complex (MAC) disease, the association between fluoroquinolone resistance and MAC genotypes in clinical isolates from individuals not previously treated for MAC infection is not fully clear.
Methods: Totals of 154 *M. avium* isolates and 35 *Mycobacterium intracellulare* isolates were obtained from treatment-naïve patients with pulmonary MAC disease at the diagnosis of MAC infection at 8 hospitals in Japan. Their susceptibilities of moxifloxacin were determined by broth microdilution methods. Moxifloxacin-resistant isolates were examined for mutations of *gyrA* and *gyrB*. Variable numbers of tandem repeats (VNTR) assay was performed using 15 *M. avium* VNTR loci and 16 *M. intracellulare* VNTR loci.
Results: Moxifloxacin susceptibility was categorized as resistant and intermediate for 6.5% and 16.9%, respectively, of *M. avium* isolates and 8.6% and 17.1% of *M. intracellulare* isolates. Although the isolates of both species had amino acid substitutions of Thr 96 and Thr 522 at the sites corresponding to Ser 95 in the *M. tuberculosis* GyrA and Gly 520 in the *M. tuberculosis* GyrB, respectively, these substitutions were observed irrespective of susceptibility and did not confer resistance. The VNTR assays showed revealed three clusters among *M. avium* isolates and two clusters among *M. intracellulare* isolates. No significant differences in moxifloxacin resistance were observed among these clusters.
Conclusions: Although resistance or intermediate resistance to moxifloxacin was observed in approximately one-fourth of *M. avium* and *M. intracellulare* isolates, this resistance was not associated with mutations in *gyrA* and *gyrB* or with VNTR genotypes.

1. Introduction

The worldwide increase in the incidence of *Mycobacterium avium* complex (MAC) infections is a serious concern for physicians engaged in the clinical control of mycobacterial infections. Even after treatment with the standard regimen with clarithromycin,

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rifampin and ethambutol, sputum conversion is often not achieved [1,2]. In severe cases, fluoroquinolones can also be considered as alternative therapies [3]. Although the susceptibility of MAC isolates to various fluoroquinolones, including moxifloxacin, has been reported [3–11], it is unclear whether the clinical isolates used in the associated studies were provided from patients who had not received prior treatment.

Fluoroquinolone targets DNA gyrase subunits encoded by *gyrA* and *gyrB* and topoisomerase IV subunits encoded by *parC* and *parE*. Mutations in two short regions known as the quinolone resistance determining regions (QRDRs) have been identified as associated with fluoroquinolone resistance [12]. Because *Mycobacterium tuberculosis* lacks *parC* and *parE* homologs, the main targets of fluoroquinolones are the GyrA and GyrB proteins [13]. In *M. tuberculosis*, mutations in the QRDRs of GyrA that confer fluoroquinolone resistance occur at amino acid codons 88, 90, and 94; additional GyrA mutations occur at amino acid codons 74, 80, 89, 91 and 92 [14,15]. GyrB mutations conferring resistance to fluoroquinolone have been reported at amino acid codon 538, and other GyrB mutations have been found at codon 500 to codon 538 [14,15]. Although *M. avium* and *Mycobacterium intracellulare* also have *gyrA* and *gyrB* genes, mutations in the QRDRs of *gyrA* and *gyrB* in *M. avium* and *M. intracellulare* have been reported in only few studies [16,17].

Various genotyping methods, such as restriction fragment length polymorphism, pulsed field gel electrophoresis, and variable numbers of tandem repeats (VNTR)-based typing, have been conducted with *Mycobacteria* [18–21]. Correlation between VNTR clusters and susceptibility to levofloxacin in *M. avium* isolates were reported in Japan [22,23]. Two studies in China reported that moxifloxacin susceptibility did not differ between VNTR-clustered and VNTR-unclustered strains of *M. avium* [9] but was significantly more commonl in clustered strains than in unclustered strains of *M. intracellulare* [7].

We conducted a multicenter study to determine the moxifloxacin susceptibility of *M. avium* and *M. intracellulare* isolates from patients with pulmonary MAC disease who had not received prior treatment for MAC infection. We also examined mutations of *gyrA* and *gyrB* in moxifloxacin-resistant isolates and the association between moxifloxacin susceptibility and VNTR genotype.

2. Materials and methods

2.1. MAC strains

M. avium (154 isolates) and *M. intracellulare* (35 isolates) were collected from 8 Japanese hospitals between January 2013 and December 2014; Among these *M. avium* and *M. intracellulare* isolates, 71 and 12, respectively, were collected from the National Hospital Organization Kinki-chuo Chest Medical Center in Kinki District; 32 and 5, respectively, were collected from Tohoku University Hospital in Tohoku District; 23 and 3, respectively, were collected from Kyoto University Hospital in Kinki District, 7 and 10, respectively, were collected from the National Hospital Organization, Higashinagoya National Hospital in Tokai District; 7 and 2, respectively, were collected from the National Hospital Organization, Asahikawa Medical Center in Hokkaido District; 8 and zero, respectively, were collected from Keio University Hospital in Kanto District; 3 and 2, respectively, were collected from Fukujyuji Hospital in Kanto District; and 3 and 1, respectively, were collected from Nagoya City University Hospital in Tokai District. Each isolate was obtained from the sputum or bronchial lavage of pulmonary MAC patients at the diagnosis of MAC infection, which was based on the American Thoracic Society (ATS) guidelines for diagnosis of MAC infection [2]. None of the patients had received any antimicrobial treatments for MAC infection. Each strain was cultured on 7H11–C agar medium (Kyokutou Pharmaceutical Co., Ltd., Tokyo, Japan) and stored at −80 °C until examination of susceptibility, DNA sequencing and VNTR genotyping.

2.2. Drug susceptibility

Minimal inhibitory concentrations (MICs) of moxifloxacin were determined by cation-adjusted Muller-Hinton broth supplemented with 5% oleic acid, albumin, and dextrose. Microdilution trays were incubated at 37 °C in ambient air and examined after seven days. According to the protocol of the Clinical and Laboratory Standards Institute (CLSI), isolates with MICs of $\leq$1, 2, and $\geq$4 μg/ml were defined as susceptible, intermediate and resistant to moxifloxacin, respectively [24]. Drug concentrations that inhibited 50 and 90% of the tested isolates were designated MIC_{50} and MIC_{90}, respectively.

2.3. DNA extraction and sequencing of QRDRs

DNA was extracted from colonies using InstaGene Matrix (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. Primers were designed in house using Primer3 software (version 0.40) for *gyrA* and *gyrB* of *M. avium* TH135 (GenBank accession no. AP012555) and *M. intracellula*re ATCC13950 (GenBank accession no. NC_016946). The *gyrA* gene of *M. avium* was amplified using the primers *gyrA*Favi (5′-TGCTCTACGCCATGTACGAC-3′) and *gyrA*Ravi (5′-TCGACTGTCTCCTCGTCGAT-3′), and that of *M. intracellulare* was amplified using the primers *gyrA*Fint (5′-CGGGTGCTCTATGC-GATGTA-3′) and *gyrA*Rint (5′-TTTCAGGTCGGTCACGTTGT-3′). The *gyrB* gene of *M. avium* was amplified using the primers *gyrB*Favi (5′-GAATTGTATGTGGTCGAGGG-3′) and *gyrB*Ravi (5′-GCCATTT-CAACTTGTACAGC-3′), and that of *M. intracellulare* was amplified by using the primers *gyrB*Fint (5′-CGACTCGATGTTCCAGGCAA-3′) and *gyrB*Rint (5′- TTGATCTTCTTGCCGGCCTT-3′). Amplification of both *gyrA* and *gyrB* was performed using the following conditions: a denaturation step of 5 min at 95 °C; 30 cycles of 30 s at 94 °C, 30 s at 53 °C, and 1 min at 72 °C; and an extension step of 7 min at 72 °C. The sizes of the PCR-amplified fragments of the *M. avium* and *M. intracellulare gyrA* genes (at positions 167–455 and 163–498, respectively) were 289 and 336 bp, respectively.

The sizes of the PCR-amplified fragments for the *M. avium* and *M. intracellulare gyrB* genes (at positions 1366–1726 and 1419–1811, respectively) were 361 and 393 bp, respectively. The PCR products were purified and sequenced by Macrogen Japan Corp. (Kyoto, Japan). Sequencing reactions were performed in a BioRad DNA Engine Dyad PTC-220 Peltier Thermal Cycler using the ABI PRISM® BigDye® Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, MA USA) following the protocols supplied by the manufacturer. Single-pass sequencing was performed on each template using the corresponding primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Thermo Fisher Scientific).

2.4. VNTR analysis

For VNTR analysis, a single colony on an agar-solidified plate was used to extract DNA using InstaGene Matrix. Primer sets for 15 *M. avium* VNTR loci (MATR-VNTR) and 16 *M. intracellulare* VNTR loci (MITR-VNTR) were used in the VNTR analysis as described previously [18,19]. After the PCR products were electrophoresed on agarose gels and fragment sizes were estimated according to their

relationships to molecular size markers, the number of repeat units at each VNTR locus in each isolate was determined. Manhattan distance was determined based on the allele profile of the VNTR between each pair of isolates by Fitch-Margoliash analysis using PHYLIP software (version 3.68). Phylogenetic trees were constructed using FigTree software (version 1.3.1).

2.5. Statistical analysis

JMP version 9.0.0 (SAS Institute, NC, USA) was used for all statistical analyses. Group comparisons were made using the chi-square test and Fisher's exact test for categorical variables. The mean $\log_2$ values (±standard deviation) of the MICs were compared among the VNTR clusters using the Mann-Whitney *U* test, and P-values of less than 0.05 were considered significant.

2.6. Ethics

This study was approved by the Ethics Review Committee of Nagoya City University Graduate School of Medical Sciences and each of the hospitals involved.

3. Results

3.1. Moxifloxacin susceptibility of MAC isolates

Among the *M. avium* isolates, 76.6% (118/154) were susceptible to moxifloxacin, 16.9% (26/154) had intermediate susceptibility, and 6.5% (10/154) were resistant. Among the *M. intracellulare* isolates, 74.3% (26/35) were susceptible to moxifloxacin, 17.1% (6/35) had intermediate susceptibility, and 8.6% (3/35) were resistant. The MIC_{50} and MIC_{90} values of the isolates of both species were 1 and 2 μg/ml, respectively (Table 1). Eight patients had received fluoroquinolones (levofloxacin in 4 patients, garenoxacin in 3 patients and tosufloxacin in 1 patient) for pneumonia or bronchitis within 3 months before the diagnosis of MAC infection. Among the isolates from these patients, two *M. avium* isolates were susceptible to moxifloxacin, 2 showed intermediate susceptibility, and 2 were resistant, and two *M. intracellulare* isolates were susceptible to moxifloxacin.

There was no significant difference in susceptibility to moxifloxacin among the 8 hospitals (Supplementary Tables 1 and 2). The percentage of susceptible *M. avium* isolates was lower for Kinki District (central Japan) than for the other districts (northern and eastern parts of Japan) (70.2% *vs.* 86.7%, P = 0.02).

3.2. Sequencing of gyrA and gyrB

The *gyrA* and *gyrB* QRDRs of 3 susceptible, 26 intermediate and 10 resistant *M. avium* isolates and 3 susceptible, 6 intermediate and 3 resistant *M. intracellulare* isolates were sequenced. All of the *M. avium* and *M. intracellulare* isolates had an amino acid substitution of Thr 96 (ACC) at the site corresponding to Ser 95 (AGC) of the *M. tuberculosis* GyrA protein. The absence of differences in this amino acid among the susceptible, intermediate and resistant isolates indicated that the substitution of Thr 96 did not contribute to moxifloxacin resistance. Similarly, all of the *M. avium* and *M. intracellulare* isolates had an amino acid substitution of Thr 522 (ACC) at the site corresponding to Gly 520 (GGC) of the *M. tuberculosis* GyrB protein. The absence of differences in this amino acid among the susceptible, intermediate and resistant isolates of *M. avium* or *M. intracellulare* indicated that the Thr 522 substitution did not contribute to moxifloxacin resistance.

Table 1
Moxifloxacin susceptibility of 189 *Mycobacterium avium* complex isolates.

	susceptible	intermediate	resistant	MIC_{50}/MIC_{90}
M. avium (n = 154)	118 (76.6)	26 (16.9)	10 (6.5)	1/2
M. intracellulare (n = 35)	26 (74.3)	6 (17.1)	3 (8.6)	1/2

Data are presented as number (%) of isolates and μg/mL for MIC. MIC, minimum inhibitory concentration.

3.3. Genotyping and clustering of M. avium and M. intracellulare isolates

Among the 154 *M. avium* isolates, 133 MATR-VNTR patterns were identified. Thirteen pairs and four triplets of 38 *M. avium* isolates showed identical MATR-VNTR patterns. Of the 13 *M. avium* pairs sharing MATR-VNTR patterns, 11 comprised isolates obtained from patients living in the same prefecture; furthermore, among the 4 *M. avium* triplets sharing MATR-VNTR pattern, 2 isolates in each of two triplets and all three isolates in one triplet were obtained from patients from the same prefecture. Among the 35 *M. intracellulare* isolates, 33 MITR-VNTR patterns were identified. Two pairs of *M. intracellulare* isolates shared identical MITR-VNTR patterns; the isolates in each pair were obtained from patients living in different prefectures.

In the phylogenetic tree showing the relationships among the VNTR profiles, the *M. avium* isolates were separated into three major clusters. Cluster A, B and C consisted of 48, 63 and 43 isolates, respectively (Fig. 1). *M. intracellulare* isolates were separated into two major clusters, clusters A and B, which consisted of 16 and 18 isolates, respectively (Fig. 2). There was no significant difference in the VNTR clusters among the 8 hospitals (Supplementary Tables 1 and 2). The percentage of isolates belonging to *M. avium* cluster A was lower for Kinki District (central Japan) than for the other districts (northern and eastern parts of Japan) (24.4% *vs.* 41.7%, P = 0.03).

3.4. Association between VNTR genotype and moxifloxacin susceptibility

We determined the moxifloxacin susceptibility profiles of the *M. avium* and *M. intracellulare* isolates belonging to the three MATR-VNTR clusters and the two MITR-VNTR clusters, respectively (Tables 2 and 3). The proportion of *M. avium* isolates with moxifloxacin resistance was higher for MATR-VNTR cluster A than for clusters B and C, although the differences among the three clusters were not significant. The mean $\log_2$ MIC values in cluster A were significantly higher than those in cluster C (0.02 ± 1.18 vs. −0.53 ± 1.26, P = 0.048) but not cluster B (−0.40 ± 1.16, P = 0.09). No significant difference in moxifloxacin resistance or mean MIC $\log_2$ value was observed between the two MITR-VNTR clusters.

4. Discussion

In previous studies, moxifloxacin resistance was found in 10.8%, 14%, and 49.7% of *M. avium* strains [8,9,11]; in 1.6%, 34.6% and 64.0% of *M. intracellulare* strains [7,8,11]; and in 27.5% and 48.9% of MAC strains [3,10]. Furthermore, the MIC_{50}/MIC_{90} values reported in previous studies were 0.5–2/4–16 μg/ml for *M. avium* strains [4–6,8,9,11], 0.5–4/1–8 μg/ml for *M. intracellulare* strains [4,6–8,11] and 2/4–8 μg/ml for MAC strains [3,10]. In two studies, the strains were obtained from patients who were diagnosed according to the ATS guidelines [2]. Koh et al. reported a high rate of moxifloxacin resistance (27.5%) in MAC strains from patients with refractory pulmonary MAC disease [3]. Zhang et al. reported a low

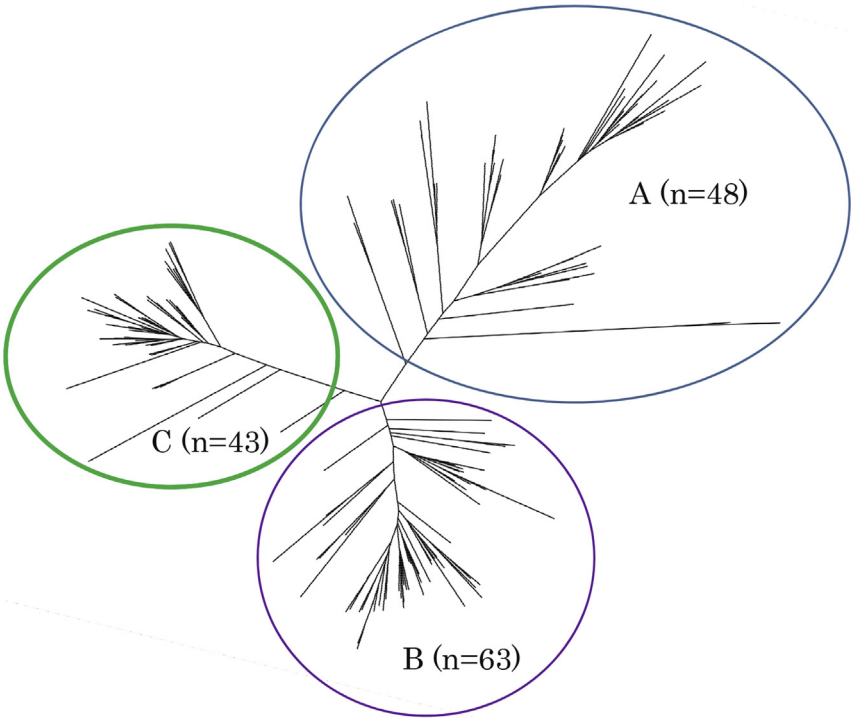

Fig. 1. Phylogenetic analysis of 154 *Mycobacterium avium* isolates from patients with pulmonary *M. avium* disease. The three major branches are designated clusters.

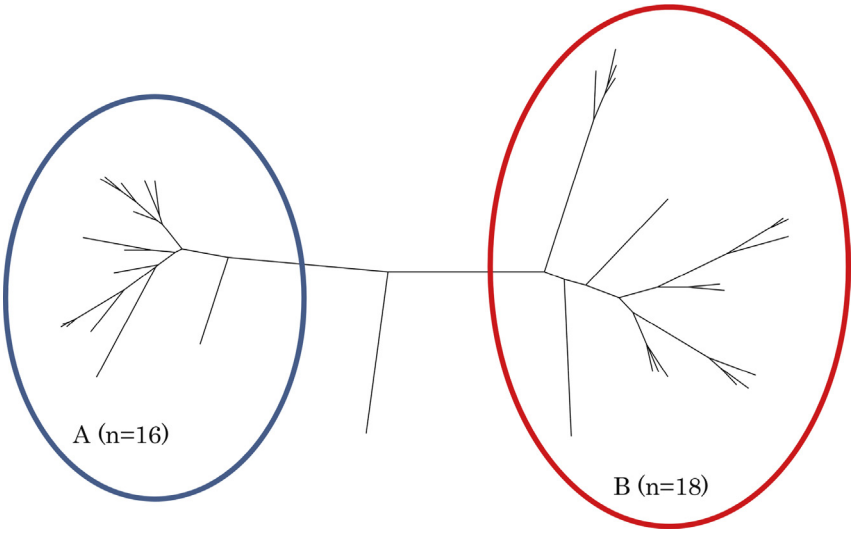

Fig. 2. Phylogenetic analysis of 35 *Mycobacterium intracellulare* isolates from patients with pulmonary *M. intracellulare* disease. The two major branches are designated clusters.

rate of moxifloxacin resistance (10.8% of *M. avium* strains and 1.6% of *M. intracellulare* strains) in patients whose previous treatments were unknown [8]. In this study, we found low rates of moxifloxacin resistance (6.5% of 154 *M. avium* isolates and 8.6% of 35 *M. intracellulare* isolates) and low MIC_{50}/MIC_{90} values (1/2 μg/ml for both the *M. avium* and *M. intracellulare* isolates). Each isolate was obtained at the diagnosis of MAC infection, and no patient had been treated with antimicrobials, including moxifloxacin. Although 8 patients had prior treatment with fluoroquinolones for pneumonia or bronchitis and 4 of the *M. avium* isolates from these patients were resistant or showed intermediate resistance to moxifloxacin, the remaining 181 patients had not received any fluoroquinolones before the diagnosis MAC infection. Therefore, the observed susceptibilities of the isolates in this study were likely to be greater than those observed in the previous studies. The findings that 23.4% of the *M. avium* isolates and 25.7% of the *M. intracellulare* isolates in the present study were resistant or showed intermediate resistance to moxifloxacin suggest that based on the current CLSI breakpoints [24], moxifloxacin does not have strong activity against *M. avium* and *M. intracellulare*. However, the CLSI breakpoints may need to be revised considering the emerging information on the distribution of MICs of presumably wild-type 'susceptible' isolates without prior treatment with fluoroquinolones.

We examined the amino acid sequence in the QRDRs of GyrA and GyrB in moxifloxacin-resistant *M. avium* and *M. intracellulare*

Table 2
Association between VNTR genotype and moxifloxacin susceptibility in 154 *Mycobacterium avium* isolates.

Susceptibility	Cluster A n = 48	Cluster B n = 63	Cluster C n = 43	P
Susceptible	33 (68.8)	49 (77.8)	36 (83.7)	0.23
Intermediate	9 (18.8)	12 (19.0)	5 (11.6)	
Resistant	6 (12.5)	2 (3.2)	2 (4.7)	

Data are presented as number (%) of isolates. VNTR, variable numbers of tandem repeats.

Table 3
Association between VNTR genotypes and moxifloxacin susceptibility in 34 *Mycobacterium intracellulare* isolates.

Susceptibility	Cluster A n = 16	Cluster B n = 18	P
Susceptible	12 (75.0)	13 (72.2)	0.88
Intermediate	3 (18.6)	3 (16.7)	
Resistant	1 (6.3)	2 (11.1)	

Data are presented as numbers (%) of isolates. VNTR, variable numbers of tandem repeats.

isolates. We observed an amino acid substitution of Thr 96 in GyrA at the site corresponding to Ser 95 of the *M. tuberculosis* GyrA protein and an amino acid substitution of Thr 522 of GyrB at the site corresponding to Gly 520 of the *M. tuberculosis* GyrB protein. However, the amino acid sequences in the QRDRs of GyrA and GyrB were identical between the susceptible and resistant isolates in this study. Therefore, we did not identify substitutions conferring moxifloxacin resistance in the QRDRs of *gyrA* and *gyrB* in our *M. avium* and *M. intracellulare* isolates. A natural polymorphism at Ser 95 of GyrA in *M. tuberculosis* was previously reported [25]. Recently, Kim et al. reported that moxifloxacin resistance was not associated with mutations in *gyrA* and *gyrB* in 30 *M. avium* isolates and 29 *M. intracellulare* isolates and that substitutions at amino acid codons 95 of GyrA and 520 of GyrB were present [17]. Although we analyzed the sequences outside the QRDRs according to reported mutations in *gyrA* and *gyrB* in *M. tuberculosis* [14,15], additional mutations were not found. Because the *M. avium* and *M. intracellulare* isolates in this study were obtained from patients without previous treatment, including fluoroquinolone treatment, mutations in *gyrA* and *gyrB* were unlikely to be present. Other mechanisms, such as efflux pumps, may be involved in moxifloxacin resistance in these species [26,27]. However, using reserpine and carbonyl cyanide *m*-chlorophenylhydrazone, which inhibit efflux pumps, we did not observe contributions of efflux pumps (data not shown).

Because we did not detect mutations in the QRDRs of *gyrA* and *gyrB*, we performed VNTR genotyping to reveal other genetic characteristics of the moxifloxacin-resistant isolates. Two previous studies reported that *M. avium* strains were separated into three major clusters of VNTR genotype, with the strains in one cluster showing lower MICs of levofloxacin than the strains in the other clusters [22,23]. Although the 154 *M. avium* isolates in the present study formed three major clusters and the 34 *M. intracellulare* isolates formed two major clusters, no association between moxifloxacin susceptibility profiles and cluster was observed for either the *M. avium* or *M. intracellulare* isolates (Tables 2 and 3). Our study included more *M. avium* isolates, 154, than used in previous studies, and all isolates were obtained from patients who had not received prior treatment. Similarly, Wei et al. reported no association of *M. avium* VNTR genotype with moxifloxacin resistance based on the MIC breakpoints of the CLSI criteria [9]. Therefore, the VNTR clusters are unlikely to predict resistance to moxifloxacin.

The susceptibility to moxifloxacin and the VNTR cluster distribution of *M. avium* isolates differed between Kinki District and the other districts in this study. In China, 22 of 23 clustered *M. avium* strains were isolated from Fujian Province, whereas only one was isolated from Beijing Province [9]. Considering that the epidemiology of *M. avium* and *M. intracellulare* infection are geographically diverse in Japan [28], it is possible that there are regional differences in susceptibility to moxifloxacin and VNTR clusters in Japan.

This study has several limitations. One limitation is that the number of isolates analyzed in this multicenter study varied among the hospitals. Furthermore, the sample size of 35 *M intracellulare* isolates was not sufficiently large for analysis. In addition, because the molecular mechanism of moxifloxacin resistance was not determined, we could not elucidate the association between moxifloxacin resistance and genotype.

In conclusion, in this study, moxifloxacin resistance and intermediate resistance was observed for 6.5 and 16.9% of *M. avium* isolates, respectively, and 8.6 and 17.1% of *M. intracellulare* isolates, respectively. All of the isolates were obtained from patients with pulmonary MAC disease who had not received prior treatment for MAC infection. Mutations in the QRDRs of *gyrA* and *gyrB* were not found among the moxifloxacin-resistant isolates. No significant association between VNTR cluster and moxifloxacin susceptibility profile was found, indicating that VNTR genotype does not predict moxifloxacin resistance. Further studies are needed to clarify the molecular mechanism of fluoroquinolone resistance in MAC strains.

Conflicts of interest

All authors report no potential conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jiac.2019.05.028.

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