PCR amplification of the *erm*(41) gene can be used to predict the sensitivity of *Mycobacterium abscessus* complex strains to clarithromycin

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Abstract:

A worldwide increase in the *Mycobacterium abscessus* (*M. abscessus*) complex has been reported. The purpose of this study was to investigate the diversity of the *rrl* and *erm*(41) genes, both of which are related to macrolide sensitivity in the *M. abscessus* complex. We also examined the efficacy of mass spectrometry as an alternative to molecular testing to classify subspecies of the *M. abscessus* complex. Fourteen strains of the *M. abscessus* complex were obtained, and based on conventional analyses using housekeeping genes, 57% were determined to be *M. abscessus* subsp. *abscessus*, 43% were *M. abscessus* subsp. *massiliense*, and none were identified as *M. abscessus* subsp. *bolletii*. However, depending on the strain, it was not always possible to distinguish between the subspecies by mass spectrometry. PCR products for the *rrl* and *erm*(41) genes were directly sequenced. Overall, 7.1% of the strains were found to have a *rrl* mutation, and 92.9% carried a T at position 28 of *erm*(41). Results presented here suggest that the main cause of treatment failure for *M. abscessus* complex infections is inducible macrolide resistance encoded by the erm(41) gene. From a strictly pragmatic standpoint, the phenotypic function of a putative *erm*(41) gene is the most important piece of information needed by clinicians to prescribe an effective treatment. Although PCR amplification of *erm*(41) is not sufficient to differentiate the *M. abscessus* complex subspecies, PCR can be easily and efficiently used to predict the sensitivity of members of the *M. abscessus* complex to clarithromycin.

1. Introduction

Recent epidemiology research has revealed a worldwide increase in non-tuberculous mycobacteria (NTM) infections. In Japan in particular, the *Mycobacterium abscessus* (*M. abscessus*) complex is the third most common pathogen in pulmonary diseases caused by NTM, after the *Mycobacterium avium* complex and *Mycobacterium kansasii* (1). The *M. abscessus* complex is categorized as rapidly growing mycobacteria, defined by visible growth

within seven days, and is one of the most difficult pathogens to treat. Over the past decade, the *M. abscessus* complex has been subclassified into three new subspecies: *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *massiliense* and *M. abscessus* subsp. *bolletii* (2). Macrolides are the key drugs used for treatment of *M. abscessus* complex infection; however, macrolides are not always effective or in some cases they lose effectiveness during the course of treatment. Acquired macrolide resistance is associated with point mutations in the *rrl* gene, which encodes 23S rRNA (3). An erythromycin ribosomal methylase, encoded by *erm*(41) in the *M. abscessus* complex, confers inducible resistance to macrolides (4). The functionality of the *erm*(41) gene differs depending on the subspecies. Most notably, *M. abscessus* subsp. *massiliense* has been proposed to have an incomplete *erm*(41) gene, which is associated with macrolide sensitivity. In addition, some *M. abscessus* subsp. *abscessus* strains have substitutions in the *erm*(41) gene that also lead to macrolide susceptibility. Thus it is important to distinguish the three kinds of subspecies and to analyze the sequences of the *rrl* and *erm*(41) genes. Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) has been used for microbial identification in recent years, and several researchers have attempted to apply this tool to differentiate the subspecies of the *M. abscessus* complex. However, different diagnostic criteria have been used at different institutions and the results of the method are inconsistent (5-10).

Few studies have investigated the ratio of subspecies of the *M. abscessus* complex in Japan, or examined their macrolide resistance genes. It is likely that regional differences in the ratios of the subspecies and the clinical features of such isolates may exist. The purpose of this study was to examine the *rrl* and *erm*(41) genes present in *M. abscessus* complex isolates obtained at our institutes located in the Tokyo-Yokohama area, and to compare these results to the sensitivity of the isolates to the macrolide antibiotic clarithromycin. We also compared the efficacy of using molecular testing and mass spectrometry to classify subspecies of the *M. abscessus* complex.

2. Methods

Samples and Data collection

Fourteen strains of the *M. abscessus* complex were obtained from each patient between July 2016 and April 2018 at Showa University Hospital (Tokyo) or at Showa University Fujigaoka Hospital (Yokohama). For reference, one strain of *Mycobacterium fortuitum* (*M. fortuitum*) was collected during the period. All strains were of sputum origin except for one *M. abscessus* complex isolate from a bronchoscopy. Clinical isolates were cultured in mycobacteria growth indicator tubes (MGIT) and in 2% Ogawa solid medium. *M. abscessus* complex and *M. fortuitum* were distinguished by DNA-DNA hybridization. All clinical data were collected from medical records. Official approval for the study was obtained in advance from the Ethics Committee for Research at Showa University (approved numbers 371 and 2016127). Informed consent was waived because of the retrospective nature of the study.

Molecular Testing

DNA was extracted from mycobacterial clinical isolates using InstaGene matrix (Bio-Rad Laboratories, Hercules, CA) and stored at -20 °C. The amount of DNA extracted ranged from 104 to 452 ng/μl. Primers for nucleic acid amplification were designed as indicated in Table I. PCRs were performed to amplify mutation hot spot regions in the housekeeping genes *hsp65*, *rpoB* and ITS to classify the strains into the three subspecies using a Mycycler ver.10.65 thermal cycler (Bio-Rad Laboratories, Hercules, CA). The *rrl* and *erm*(41) genes were also amplified in a similar manner. All PCR assays were carried out in 25-μl volumes containing 200 ng of template DNA, 0.1 units of Taq DNA polymerase (Roche Diagnostics, Mannheim, Germany), 25 pmol of each primer, and 10 nmol of dNTPs. Cycling parameters were 30 sec at 95 °C, 30 sec at 60 °C, and 60 sec at 72 °C for 30 cycles. PCR products were separated on a 5% polyacrylamide gel or 1.0% agarose gel. The gels were stained with ethidium bromide

and photographed under UV illumination. The PCR products were purified and were directly sequenced using a BigDye terminator kit and ABI Prism 3130 *xl* (Applied Biosystems, Foster, CA). When sequences could not be obtained by direct sequencing, the PCR products were ligated into a pGEM T easy vector (Promega, Madison, WI), which was then used to transform JM109 cells, as reported previously (11). Multiple clones were selected and plasmid DNA was purified from each and sequenced. The reference sequences for each gene were obtained from GenBank (accession numbers CU458896.1: *M. abscessus* subsp. *abscessus*, AP_014547.1: *M. abscessus* subsp. *massiliense*.)

Antibiotic susceptibility test

Minimum inhibitory concentrations (MICs) of amikacin and clarithromycin were determined by the broth microdilution method and were interpreted according to the Clinical and Laboratory Standards Institute document M24-A2 (12). Briefly, an appropriate volume of the culture was transferred into 3 ml of sterilized saline until the turbidity matched that of a 0.5 McFarland standard. A 10 μl aliquot of the suspension was used to inoculate 11 ml of cation-adjusted Mueller-Hinton medium and 100 μl was distributed into each of the 96 well panels. The panels were incubated for 72 h at 30 °C, and growth was determined. To test for inducible resistance to clarithromycin, the MICs for clarithromycin were also determined after 7 and 14 days of incubation.

Mass spectrometry

Colonies were transferred into microcentrifuge tubes containing 300 μl of sterile deionized water, and the tubes were incubated for 30 min at 95°C. Then samples were mixed with 900 μl of 70% ethanol by vortexing for 1 min. The suspensions were centrifuged at 13,000 rpm for 2 min, and the pellets were dried for 5 min at room temperature and resuspended in 20 μl of 100% acetonitrile with zirconia beads. The mixtures were vortexed for 1 min. The samples

were then suspended with 20 μl of 70% formic acid and centrifuged at 13,000 rpm for 2 min. Subsequently, 1 μl of the supernatant from each extract was spotted on a target plate. After drying, 1 μl of matrix solution (saturated α-cyano-4-hydroxycinnamic acid in 47.5% acetonitrile and 2.5% trifluoroacetic acid) was added onto each spot. Mass spectra were obtained on a MALDI Biotyper ver 4.0 configured with Micro flex LT/SH with Mycobacteria Library ver.5.0 (Bruker Daltonik, Bremen, Germany). Spectra were analyzed by Flex Analysis software 3.4 and MBT compass explore ver 4.1 (Bruker Daltonik, Bremen, Germany).

Statistical Analysis

The significance in each group was evaluated with Fisher's exact test or Pearson's Chi-square test, unpaired student's t-test, and the nonparametric Mann-Whitney test on ranks. $P < .05$ was considered significant. All analyses were performed using JMP 13.0 software (SAS Institute, Cary, NC).

3. Results

Determination of *Mycobacterium abscessus* complex subspecies by sequencing housekeeping genes

The results of sequence analyses of housekeeping genes are shown in Table II. To distinguish the three subspecies, *hsp65*, *rpoB* and ITS sequences were determined by direct sequencing and compared to reference sequences. The sequences of the *hsp65* genes from eight strains were consistent with the reference sequence from *M. abscessus* subsp. *abscessus*, while those from six strains were consistent with the *hsp65* reference sequence from *M. abscessus* subsp. *massiliense*, with the exception of one strain (No.9626), which had a change at position 280T>A. High heterogeneity of *rpoB* in the *M. abscessus* complex has been reported (13). The *rpoB* genes from eight strains were identical to the reference gene from *M. abscessus*

subsp. *abscessus*, while a 37C>T change was present in two strains (No.71740, No.9614), and two changes (52C>T and 391C>T) were found in another strain (No.8548). Six strains had *rpoB* sequences identical to the reference sequence from *M. abscessus* subsp. *massiliense*, with the exception of one substitution, 316T>C that was detected in four strains (No.74369, No.77944, No.9626, No.9388). No amino acid changes resulted from these nucleotide sequence differences. Together, eight strains were identified as *M. abscessus* subsp. *abscessus*, and six strains as *M. abscessus* subsp. *massiliense*, respectively. The results of sequence analyses of the ITS region were consistent with these findings. However, a novel insertion sequence (180_181GTTGT) was found in one strain of *M. abscessus* subsp. *abscessus* (No.71740)

Patients and characteristics

As mentioned above, results were obtained for all patients, 57% (8 of 14) of whom were infected with *M. abscessus* subsp. *abscessus*, and 43% (6 of 14) of whom were infected with *M. abscessus* subsp. *massiliense*, respectively. None were infected with *M. abscessus* subsp. *bolletii*. Table 3 shows the patient characteristics. There were seven males and seven females whose ages at diagnosis ranged from 30 to 83 years: thirteen were Japanese and one was Indian. According to the guidelines published by the American Thoracic Society/Infectious Diseases Society of America (14), all patients were newly diagnosed with *M. abscessus* complex pulmonary disease based on at least two positive culture results derived from pulmonary samples. As shown in Table III, there was no significant association of the subspecies with age, body-mass index, gender, smoking history, radiological findings, hemoptysis, sputum smear, or C-reactive protein.

Gene status of *rrl* and *erm*(41)

Sequence differences identified in the *rrl* and *erm*(41) genes are summarized in Table IV. In

the *rrl* gene, a A>G change was detected at position 2059 in one strain (No.8006), but no other alterations were found. As for the *erm*(41) gene, nucleotides at positions 64_65 and 159_432 were deleted in strains of *M. abscessus* subsp. *massiliense*, compared to the *M. abscessus* subsp. *abscessus* strains. Eight substitutions were found in the *M. abscessus* subsp. *abscessus* isolates, whereas no substitutions were found in the strains of *M. abscessus* subsp. *massiliense*. In those isolates of *M. abscessus* subsp. *abscessus*, 28T>C, 238A>G and 419C>T substitutions were responsible for the amino-acid changes W10R, I80V and P140L, respectively. As shown in Figure 1, the sizes of the PCR products amplified from the *erm*(41) genes were consistent with sequencing results (673 base pairs for *M. abscessus* subsp. *abscessus*, and 397 base pairs for *M. abscessus* subsp. *massiliense*), and identifications based on the size of the *erm*(41) gene were consistent with those based on *hsp65*, *rpoB* and ITS sequences.

Antimicrobial sensitivity

Table V shows the antibiotic susceptibility of the *M. abscessus* strains to amikacin and clarithromycin. The MICs of amikacin ranged from 2 to 16 μg/ml, which indicated that all strains were sensitive. There was no difference in the MICs between the two subspecies. *M. abscessus* subsp. *abscessus* isolates were sensitive to clarithromycin on day 3, but the MICs were significantly higher on day 14 with one exception (No.9944). In contrast, the strains of *M. abscessus* subsp. *massiliense* were susceptible to clarithromycin on days 3 through 14, except for one strain (No.8006), which showed resistance from the start. Strain No.9626 was sensitive early in the testing period, but the MIC was about 4-fold higher on day 14.

MALDI TOF-MS analysis

The details of mass spectra are shown in Figures 2 and 3. Of the 14 isolates, 11 were analyzed by MALDI TOF-MS. For reference, one clinical isolate of *M. fortuitum* was

simultaneously analyzed in a similar manner. The 11 strains were identified as *M. abscessus* complex (score range, 1.66 to 2.14) and were correctly differentiated from the strain of *M. fortuitum* (see supplemental figure). As shown in Figure 2, the representative spectra of *M. abscessus* complex subspecies were similar at a laser frequency of 50 Hz across 2,000 to 12,000 *m/z*. When magnifying the spectrum (Figure 3), distinctive peaks reported previously (8,10) were detected in some cases, but not in all samples. More specifically, peaks around 4390, 7639, 8781 and 9473 *m/z* for *M. abscessus* subsp. *abscessus* and peaks around 4385, 7669, and 8767 *m/z* for *M. abscessus* subsp. *massiliense* were found. However, each baseline was unstable and the peaks were wide and low for these strains. Moreover, discriminating peaks were mostly of low intensity or were overlapping. Thus, detection of clearly identifiable differences between strains of different subspecies was extremely difficult. Overall, it was possible to discriminate between *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *massiliense* by using mass spectra only in some cases.

4. Discussion

In the current study, *M. abscessus* complex isolates obtained from patients treated at our institutes located in the Tokyo-Yokohama area were analyzed. Overall, 57% (8 of 14) of the isolates were identified as *M. abscessus* subsp. *abscessu*s, 43% (6 of 14) as *M. abscessus* subsp. *massiliense*, and none as *M. abscessus* subsp. *bolletii*. It has been reported that *M. abscessus* subsp. *abscessus* is the most predominant subspecies of the complex followed by *M. abscessus* subsp. *massiliense*, and that *M. abscessus* subsp. *bolletii* is quite rare, ranging from 0 - 3% in Japan (15-19). There were no significant differences in clinical features between *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *massiliense*. Clinical characteristics of the *M. abscessus* complex did not help us to distinguish those subspecies, which is consistent with findings reported in the literature (15,20).

The proportion of the *M. abscessus* complex subspecies varies depending on the region

from which they are isolated. Compared to Western Europe, the prevalence of *M. abscessus* subsp. *bolletii* is lower in East Asian countries (21-24). Although, *M. abscessus* subsp. *abscessus* is the most predominant subspecies of the complex in most parts of the world, some studies have reported that *M. abscessus* subsp. *massiliense* is more abundant than *M. abscessus* subsp. *abscessus* in some parts of East Asia such as Taiwan and Korea (5,21,22,25) Actually whole-genome sequencing analyses revealed genetic distinctions between *M. abscessus* subsp. *abscessus* isolates in Asia and Western Europe (26), suggesting that the phylogenetic diversity correlates with the regional ratio of the subspecies.

The current commercial system for NTM differentiation in Japan consists of the DNA-DNA hybridization method, which is unable to differentiate subspecies of the *M. abscessus* complex. The three subspecies also cannot be distinguished by 16S rRNA gene sequencing, which is commonly used for bacterial taxonomy in academic research, because the 16S rRNA genes in the three subspecies are 100% identical (27,28). The differentiation requires sequencing of several housekeeping genes, which is not easy to accomplish in most mycobacteriology laboratories. Hence, these three subspecies have not been distinguished in hospital laboratories. Sequencing of a single target gene may lead to inaccurate identification of closely related subspecies; however, multilocus sequence analyses of the *M. abscessus* complex have been described using *hsp65, rpoB*, ITS, *gyrB, dnaA recA*, and *secA* (29). Although some other methods based on technology developed by multilocus sequence analyses have been designed, such as variable-number tandem repeat analysis (18,19,30,31) and multiplex PCR (17,32), those methods are complicated. In the present study, subspecies of the *M. abscessus* complex were differentiated based on partial sequences of the *hsp65* and *rpoB* genes, and results of ITS sequencing were also consistent in differentiating the two subspecies. In a subset of *M. abscessus* complex isolates, a hybrid genetic pattern for the *hsp65* and *rpoB* genes has been reported (33,34), presumably the result of horizontal gene transfer between the subspecies. In such cases ITS gene analysis was essential to identify the

subspecies. Sequencing of at least three housekeeping genes should therefore be carried out for subspecies identification.

Numerous institutions are seeking an alternative way to distinguish the *M. abscessus* subspecies in clinical practice. MALDI TOF-MS has been evaluated for the identification of microorganisms including mycobacteria. However in this study, it was not possible to distinguish between the *M. abscessus* subspecies of all isolates by MALDI TOF-MS. Although several institutes have reported the efficacy of MALDI TOF-MS in differentiating the three subspecies of the *M. abscessus* complex (5-10), methods for sample preparation and analysis, and diagnostic criteria have not been standardized. When defining the range from 2,000 to 20,000 *m/z*, ribosomal protein accounts for 50 to 70% of the peptide detected by MALDI TOF-MS. Thus, it is easy to distinguish between species that contain diverse ribosomal proteins. In fact, the current study revealed obvious differences between *M. fortuitum* and the *M. abscessus* complex by mass spectra (see supplemental figure). However, the mass spectrometry peaks may be affected by variations in culture media formulations, duration of growth, and other conditions. The utility of MGIT liquid medium (10), 5% sheep blood agar (6), Middlebrook 7H11 (7) and Lowenstein-Jensen agar (9) have been reported for MALDI TOF-MS. In our study, colonies derived from 2% Ogawa solid medium were prepared. Thus, the diagnostic criteria for MALDI-TOF analysis differ depending on the laboratory carrying out the analyses. Since there are few differences in the ribosomal proteins between the *M. abscessus* complex subspecies, and the sample preparation for mass spectra is even affected by climate, the procedure is often poorly reproducible. Considerable effort would be necessary to optimize and standardize a protocol to obtain reproducible mass spectrometry results for differentiating the *M. abscessus* complex subspecies.

rrl mutations confer acquired resistance and nucleotide T28 of *erm*(41) is associated with inducible resistance. Since most of the alterations identified in this study were in the *erm*(41) gene (92.9%; 13 of 14 isolates) rather than the *rrl* gene (7.1%;1 of 14), we concluded that the

main cause of treatment failure for *M. abscessus* complex infections was inducible resistance encoded by the *erm*(41) gene. One strain of *M. abscessus* subsp. *massiliense* (No.8006) harboring a point mutation 2059A>G in the *rrl* gene showed resistance to clarithromycin from day 3 through day 14 (MIC > 128 μg/ml). The presence of *rrl* mutations was reported in up to 30% of newly isolated strains in East Asia (16,35), whereas no *rrl* mutations were identified at the time of diagnosis in Spain (36). It has been reported that mutations in the *rrl* gene rapidly accumulate following clarithromycin use in monotherapy (3). Conversely, several studies have reported acquired resistance due to *rrl* mutations in the absence of any macrolide exposure (16,37). However, an in vitro study reported that *rrl* mutations at position 2058 or 2059 were observed during incubation with clarithromycin (38). In East Asia, low dose macrolides are often administered for prolonged periods to treat chronic respiratory disorders, such as diffuse panbronchiolitis, to stimulate an immunomodulatory effect (39). Hence, careful attention is needed when prescribing macrolides to patients initially diagnosed with pulmonary disease caused by *M. abscessus* complex. One strain of *M. abscessus* subsp. *abscessus* (No.9944) carried a substitution at position 28T>C in the *erm*(41) gene, which resulted in an amino acid change (W10R), and showed a low MIC value for clarithromycin. Other strains that harbored amino acid substitutions 238A>G (I80V) or 419C>T (P140L) showed inducible resistance. These data are in agreement with previous reports (4,37,40) suggesting that the 5' end of the *erm*(41) gene is a key region because this region is also predicted to carry a second open reading frame encoding a leader peptide that regulates expression of the *erm*(41) gene itself (4). In fact, two base deletions (61_62del) were commonly detected in *M. abscessus* subsp. *massiliense*, and in addition, strains harboring a C>T substitution at position 19 that led to a stop codon (R7stop) were identified in a case report of *M. abscessus* subsp. *abscessus* (40). With rare exceptions such as the presence of a full-length *erm*(41) gene (41,42), *M. abscessus* subsp. *massiliense* is generally not associated with inducible macrolide resistance because most strains harbor a truncated *erm*(41) gene

(4,21,43). Interestingly, in the present study, one strain (No.9626) showed a low initial MIC but the MIC increased 4-fold by 14 days. This observation suggests that mechanisms not involving *erm*(41) can cause inducible resistance. No strains that simultaneously harbored both a *rrl* mutation and nucleotide T28 in *erm*(41) were detected in this study; however, numerous studies have revealed that both resistance mechanisms can occur concurrently (36,38), and that a functional *erm*(41) gene does not exclude selection for *rrl* mutations (44).

As described above, the two subspecies of the *M. abscessus* complex were found, which is consistent with the results of other studies showing the predominance of *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *massiliense* in East Asia (15,16,21,24). Data from the current study indicate that the principal difference between the two subspecies was the size of the *erm*(41) gene. Several studies have revealed the presence of a complete *erm*(41) gene in *M. abscessus* subsp. *massiliense* strains (41,42) and a truncated *erm*(41) gene in *M. abscessus* subsp. *bolletii* (36), suggesting that *M. abscessus* subsp. *massiliense* acquired a full-length *erm*(41) gene by horizontal transfer from *M. abscessus* subsp. *abscessus* or *M. abscessus* subsp. *bolletii*, and that a truncated *erm*(41) gene was transferred from *M. abscessus* subsp. *massiliense* to *M. abscessus* subsp. *bolletii*. Additionally, a change at position 28T>C has been reported in *M. abscessus* subsp. *bolletii* (21). Therefore, although horizontal gene transfer between subspecies is probably quite rare, *erm*(41) PCR is not proposed as the best way to differentiate *M. abscessus* complex subspecies. However, *erm*(41) PCR can be easily and efficiently used for the prediction of sensitivity to clarithromycin in the *M. abscessus* complex. The phenotypic function of a putative *erm*(41) gene is important for the clinician from a strictly pragmatic standpoint. Likewise, *M. abscessus* complex subspecies should be categorized based on the presence of a functional *erm*(41) gene and macrolide sensitivity especially if horizontal gene transfer increases in the future.

In conclusion, the present study demonstrates the features of *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *massiliense*, isolated in the Tokyo-Yokohama area. No

strains of *M. abscessus* subsp. *bolletii* were detected. The two subspecies were quite similar to each other in clinical characteristics and on the basis of mass spectrometry analysis; however, it is possible to predict clarithromycin susceptibility in strains of the two species by PCR amplification of the *erm*(41) gene. This is a simple and useful method that can be carried out routinely in hospital laboratories, and is recommended to predict inducible resistance to macrolides before determining the MICs, which requires 14 days of incubation for *M. abscessus* complex subspecies.

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Availability of date and materials:

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Authors' Contributions:

A.M., F.Y., T.F., Y.Y., and Y.S. examined and cared for the current patients. All authors developed the concept, designed the experiments and analyzed the data. A.M. wrote the manuscript with the contributions from all authors, who commented on it at all stages.

Ethics approval and consent to participate:

Official approval for the study was obtained in advance from the Ethics Committee for Research at Showa University (approved numbers 371 and 2016127). Informed consent was waived because of the retrospective nature of the study.

Patient consent for publication:

Not applicable.

Competing interest:

The authors declare that they have no competing interests.

Reference

1. Namkoong H, Kurashima A, Morimoto K, Hoshino Y, Hasegawa N, Ato M and Mitarai S: Epidemiology of Pulmonary Nontuberculous Mycobacterial Disease, Japan. Emerg Infect Dis 22: 1116–1117, 2016.
2. Lee MR, Sheng WH, Hung CC, Yu CJ, Lee LN and Hsueh PR: Mycobacterium abscessus complex infections in humans. Emerg Infect Dis, 2015.
3. Wallace RJ, Meier A, Brown BA, Zhang Y, Sander P, Onyi GO and Böttger EC: Genetic basis for clarithromycin resistance among isolates of Mycobacterium chelonae and Mycobacterium abscessus. Antimicrob Agents Chemother 40: 1676–1681, 1996.
4. Nash KA, Brown-Elliott AB and Wallace 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 53: 1367–1376, 2009.
5. Teng SH, Chen CM, Lee MR, Lee TF, Chien KY, Teng LJ and Hsueh PR: Matrix-assisted laser desorption ionization-time of flight mass spectrometry can accurately differentiate between Mycobacterium masilliense (M. abscessus subspecies

bolletti) and M. abscessus (Sensu Stricto). J Clin Microbiol 51: 3113–3116, 2013.

6. Fangous M-S, Mougari F, Gouriou S, Calvez E, Raskine L, Cambau E, Payan C and Hery-Arnaud G: Classification Algorithm for Subspecies Identification within the Mycobacterium abscessus Species, Based on Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry. J Clin Microbiol 52: 3362–3369, 2014.
7. Panagea T, Pincus DH, Grogono D, Jones M, Bryant J, Parkhill J, Floto RA and Gilligan P: Mycobacterium abscessus Complex Identification with Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry. J Clin Microbiol 53: 2355–2358, 2015.
8. Suzuki H, Yoshida S, Yoshida A, Okuzumi K, Fukusima A and Hishinuma A: A novel cluster of Mycobacterium abscessus complex revealed by matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS). Diagn Microbiol Infect Dis 83: 365–370, 2015.
9. Buckwalter SP, Olson SL, Connelly BJ, Lucas BC, Rodning AA, Walchak RC, Deml SM, Wohifiel SL and Wengenack NL: Evaluation of Matrix-Assisted Laser Desorption Ionization− Time of Flight Mass Spectrometry for Identification of Mycobacterium species, Nocardia species, and Other Aerobic Actinomycetes. J Clin Microbiol 54: 376–384, 2016.
10. Kehrmann J, Wessel S, Murali R, Hampel A, Bange F-C, Buer J and Mosel F: Principal component analysis of MALDI TOF MS mass spectra separates M. abscessus (sensu stricto) from M. massiliense isolates. BMC Microbiol 16: 24, 2016.
11. Fukuchi K, Hagiwara T, Nakamura K, Ichimura S, Tatsumi K and Gomi K: Identification of the regulatory region required for ubiquitination of the cyclin kinase inhibitor, p21. Biochem Biophys Res Commun 293: 120–125, 2002.
12. Woods GL, Brown-Elliott BA, Conville PS, Desmond EP, Hall GS and Lin G: Susceptibility Testing of Mycobacteria, Nocardiae, and Other Aerobic Actinomycetes;

Approved Standard-Second Edition. Clin Lab Stand Inst 26: 1–61, 2011.

13. Macheras E, Roux AL, Bastian S, Leao SC, Palaci M, Tardy VS, Gutierrez C, Richter E, Gerdes SR, Pfyffer G, *et al.*: Multilocus sequence analysis and rpoB sequencing of Mycobacterium abscessus (sensu lato) strains. J Clin Microbiol 49: 491–499, 2011.
14. Griffith DE, Aksamit T, Brown-Elliott BA, Catanzaro A, Daley C, Gordin F, Holland SM, Horsburgh R, Huitt G, Iademarco MF, *et al.*: An official ATS/IDSA statement: Diagnosis, treatment, and prevention of nontuberculous mycobacterial diseases. Am J Respir Crit Care Med 175: 367–416, 2007.
15. Harada T, Akiyama Y, Kurashima A, Nagai H, Tsuyuguchi K, Fujii T, Yano S, Shigeto E, Kuraoka T, Kajiki A, *et al.*: Clinical and microbiological differences between Mycobacterium abscessus and Mycobacterium massiliense lung diseases. J Clin Microbiol 50: 3556–3561, 2012.
16. Yoshida S, Tsuyuguchi K, Suzuki K, Tomita M, Okada M, Hayashi S, Iwamoto T and Saito H: Further isolation of Mycobacterium abscessus subsp. abscessus and subsp. bolletii in different regions of Japan and susceptibility of these isolates to antimicrobial agents. Int J Antimicrob Agents 42: 226–231, 2013.
17. Nakanaga K, Sekizuka T, Fukano H, Sakakibara Y, Takeuchi F, Wada S, Ishii N, Makino M. Kuroda M and Hoshino Y: Discrimination of mycobacterium abscessus subsp. Massiliense from mycobacterium abscessus subsp. Abscessus in clinical isolates by multiplex PCR. J Clin Microbiol 52: 251–259, 2014.
18. Yoshida S, Arikawa K, Tsuyuguchi K, Kurashima A, Harada T, Nagai H, Suzuki K, Iwamoto T and Hayashi S: Investigation of the population structure of mycobacterium abscessus complex strains using 17-locus variable number tandem repeat typing and the further distinction of mycobacterium massiliense hsp65 genotypes. J Med Microbiol 64: 254–261, 2015.
19. Kusuki M, Osawa K, Arikawa K, Tamura M, Shigemura K, Shirakawa T, Nakamura T,

Nakamachi Y, Fujisawa M, Saegusa J, *et al.*: Determination of the antimicrobial susceptibility and molecular profile of clarithromycin resistance in the Mycobacterium abscessus complex in Japan by variable number tandem repeat analysis. Diagn Microbiol Infect Dis: 2016–2019, 2018.

20. Koh WJ, Jeon K, Lee NY, Kim BJ, Kook YH, Lee SH, Park YK, Kim CK, Shin SJ, Huitt GA, *et al.*: Clinical significance of differentiation of Mycobacterium massiliense from Mycobacterium abscessus. Am J Respir Crit Care Med 183: 405–410, 2011.
21. Kim HY, Kim BJ, Kook Y, Yun YJ, Shin JH, Kim BJ and Kook YH: Mycobacterium massiliense is differentiated from mycobacterium abscessus and mycobacterium bolletii by erythromycin ribosome methyltransferase gene (erm) and clarithromycin susceptibility patterns. Microbiol Immunol 54: 347–353, 2010.
22. Kim BJ, Yi SY, Shim TS, Do SY, Yu SK, Park YG, Kook YH and Kim BJ: Discovery of a novel hsp65 genotype within Mycobacterium massiliense associated with the rough colony morphology. PLoS One 7: 1–9, 2012.
23. Kim SY, Kang YA, Bae IK, Yim JJ, Park MS, Kim YS, Kim SK, Chang J and Jeong SH: Standardization of multilocus sequence typing scheme for Mycobacterium abscessus and Mycobacterium massiliense. Diagn Microbiol Infect Dis 77: 143–149, 2013.
24. Luo L, Li B, Chu H, Huang D, Zhang J, Gui T, Xu L, Zhao L, Sun X, *et al.*: Characterization of mycobacterium abscessus subtypes in Shanghai of China: Drug sensitivity and bacterial epidemicity as well as clinical manifestations. Med (United States) 95: 1–11, 2016.
25. Koh W-J, Jeong B-H, Kim S-Y, Jeon K, Park KU, Jhun BW, Lee H, Park HY, Kim DH, Huh HJ, *et al.*: Mycobacterial Characteristics and Treatment Outcomes in *Mycobacterium abscessus* Lung Disease. Clin Infect Dis 64: 309–316, 2017.
26. Davidson RM, Hasan NA, Reynolds PR, Totten S, Garcia B, Levin A, Ramamoorthy P,

Heifets L, Daley CL and Strong M: Genome sequencing of Mycobacterium Abscessus isolates from patients in the United States and comparisons to Globally Diverse clinical strains. J Clin Microbiol 52: 3573–3582, 2014.

27. Leao SC, Tortoli E, Paul Euzé J and Garcia MJ: Proposal that Mycobacterium massiliense and Mycobacterium bolletii be united and reclassified as Mycobacterium abscessus subsp. bolletii comb. nov., designation of Mycobacterium abscessus subsp. abscessus subsp. nov. and emended description of Mycobacteri. Int J Syst Evol Microbiol 61: 2311–2313, 2011.
28. Sassi M, Kahla I Ben and Drancourt M: Mycobacterium abscessus multispacer sequence Typing. BMC Microbiol 13, 2013.
29. National Committee for Clinical Laboratory Standards: Methods for dilution: antimicrobial susceptibility tests for bacteria that grow aerobically - Fifth Edition: Approved Standard M7-A5., 1997.
30. Kikuchi T, Watanabe A, Gomi K, Sakakibara T, Nishimori K, Daito H, Fujimura S, Tazawa R, Inoue A, Ebina M, *et al.*: Association between mycobacterial genotypes and disease progression in Mycobacterium avium pulmonary infection. Thorax 64: 901–907, 2009.
31. Wong YL, Ong CS and Ngeow YF: Molecular typing of Mycobacterium abscessus based on tandem-repeat polymorphism. J Clin Microbiol 50: 3084–3088, 2012.
32. Mougari F, Raskine L, Ferroni A, Marcon E, Sermet GI, Veziris N, Heym B, Gaillard JL, Nassif X and Cambau E: Clonal relationship and differentiation among mycobacterium abscessus isolates as determined using the semiautomated repetitive extragenic palindromic sequence PCR-based diversilab system. J Clin Microbiol 52: 1969–1977, 2014.
33. Kim HY, Kook Y, Yun YJ, Park CG, Lee NY, Shim TS, Kim BJ and Kook YH: Proportions of Mycobacterium massiliense and Mycobacterium bolletii strains among

Korean Mycobacterium chelonae-Mycobacterium abscessus group isolates. J Clin Microbiol 46: 3384–3390, 2008.

34. Kim B-J, Kim G-N, Kim B-R, Shim T-S, Kook Y-H and Kim B-J: Phylogenetic analysis of Mycobacterium massiliense strains having recombinant rpoB gene laterally transferred from Mycobacterium abscessus. PLoS One 12: e0179237, 2017.
35. Lee SH, Yoo HK, Kim SH, Koh WJ, Kim CK, Park YK and Kim HJ: Detection and assessment of clarithromycin inducible resistant strains among Korean mycobacterium abscessus clinical strains: PCR methods. J Clin Lab Anal 28: 409–414, 2014.
36. Rubio M, March F, Garrigó M, Moreno C, Español M and Coll P: Inducible and acquired clarithromycin resistance in the mycobacterium abscessus complex. PLoS One 10: 1–10, 2015.
37. Bastian S, Veziris N, Roux AL, Brossier F, Gaillard JL, Jarlier V and Cambau E: Assessment of clarithromycin susceptibility in strains belonging to the Mycobacterium abscessus group by erm(41) and rrl sequencing. Antimicrob Agents Chemother 55: 775–781, 2011.
38. Mougari F, Bouziane F, Crockett F, Nessar R, Chau F, Veziris N, Sapriel G, Raskine L and Cambau E: Selection of Resistance to Clarithromycin in Mycobacterium abscessus Subspecies. Antimicrob Agents Chemother 61, 2017.
39. Kudoh S and Keicho N: Diffuse panbronchiolitis. Clin Chest Med 33: 297–305, 2012.
40. Kim S-Y, Shin SJ, Jeong B-H and Koh W-J: Successful antibiotic treatment of pulmonary disease caused by Mycobacterium abscessus subsp. abscessus with C-to-T mutation at position 19 in erm(41) gene: case report. BMC Infect Dis 16: 207, 2016.
41. Shallom SJ, Gardina PJ, Myers TG, Sebastian Y, Conville P, Calhoun LB, Tettelin H, Olivier KN, Uzal G, Sampaio EP, *et al.*: New rapid scheme for distinguishing the subspecies of the Mycobacterium abscessus group and identifying Mycobacterium massiliense isolates with inducible clarithromycin resistance. J Clin Microbiol 51:

2943–2949, 2013.

42. Brown-Elliott BA, Vasireddy S, Vasireddy R, Iakhiaeva E, Howard ST, Nash K, Parodi N, Strong A, Gee M, Smith T, *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 53: 1211–1215, 2015.

43. Yoshida S, Tsuyuguchi K, Suzuki K, Tomita M, Okada M, Shimada R and Hayashi S: Rapid identification of strains belonging to the Mycobacterium abscessus group through erm(41) gene pyrosequencing. Diagn Microbiol Infect Dis 79: 331–336, 2014.

44. Maurer FP, Rüegger V, Ritter C, Bloemberg G V. and Böttger EC: Acquisition of clarithromycin resistance mutations in the 23S rRNA gene of mycobacterium abscessus in the presence of inducible Erm(41). J Antimicrob Chemother 67: 2606–2611, 2012.

Figure Legends:

Figure 1: Representative PCR products for the *erm*(41) gene. The amplified products from *Mycobacterium abscessus* subsp. *abscessus* strains (No.9016, No.8377, No.9944, No. 9854) were 673 bp in length, whereas those from *Mycobacterium abscessus* subsp. *massiliense* strains (No.9835, No.9626) were 397 bp in length. Far left lane, DNA size standard; Lane 1, No.8377; Lane 2, 9016; Lane 3, 9626; Lane 4, 9944; Lane 5, 9835; Lane 6, 9854.

Figure 2: Representative MALDI-TOF spectra of *Mycobacterium abscessus* subsp. *abscessus* and *Mycobacterium abscessus* subsp. *massiliense* (2,000~12,000 m/z).

Figure 3: Eight peaks differentiate the two *Mycobacterium abscessus* complex subspecies. Diagram shows the relative intensity (%) against the mass-to-charge ratio values of the

discriminating peak regions of MALDI-TOF averaged mass spectral profiles for each subspecies.

Figure S1: MALDI-TOF gel views for *Mycobacterium fortuitum*, *Mycobacterium abscessus* subsp. *abscessus* and *Mycobacterium abscessus* subsp. *massiliense* (4,000~12,000 m/z).

Table I Primer design.

Target	Primers	Sequence	bp
hsp65	hsp65F	5'-ACCAACGATGGTGTGTCCAT-3'	441
	hsp65R	5'-CTTGTCGAACCGCATACCCT-3'	
rpoB	rpoBF	5'-GAGGGTCAGACCACGATGAC-3'	408
	rpoBR	5'-AGCCGATCAGACCGATGTT-3'	
ITS	ITSF	5'-TTGTACACACCGCCCGTC-3'	490
	ITS336R	5'-CTTCTAGTGCCAAGGCATTCACC-3'	
rrl	rrl2145F	5'-GCGAAATTCCTTGTCGGGTAAGT-3'	283
	rrl2427R	5'-GGATATACGGTCCGAGGTTAG-3'	
erm(41)	erm-86F	5'-GACCGGGGCCTTCTTCGTGAT-3'	673
	erm64R	5'-GACTTCCCCGCACCGATTCC-3'	

bp: base pair.

Table II Sequence differences in clinical isolates of the *M. abscessus* complex.

Strain number	*hsp65*[a]						*rpoB*[b]															ITS[c]					
	115	118	127	190	280	340	10	31	37	52	88	124	127	136	202	277	316	343	376	379	391	25	60	98	180	276	insertion
CU458896 [d]	T	T	C	C	T	C	T	T	C	C	T	G	C	T	C	C	T	C	C	C	C	T	A	-	C	G	
9016	T	T	C	C	T	C	T	T	C	C	T	G	C	T	C	C	T	C	C	C	C	T	A	-	C	G	
8377	T	T	C	C	T	C	T	T	C	C	T	G	C	T	C	C	T	C	C	C	C	T	A	-	C	G	
9944	T	T	C	C	T	C	T	T	C	C	T	G	C	T	C	C	T	C	C	C	C	T	A	-	C	G	
71740	T	T	C	C	T	C	T	T	T	C	T	G	C	T	C	C	T	C	C	C	C	T	A	-	T	G	c.180_181insGTTGT
9614	T	T	C	C	T	C	T	T	T	C	T	G	C	T	C	C	T	C	C	C	C	T	A	-	C	G	
9854	T	T	C	C	T	C	T	T	C	C	T	G	C	T	C	C	T	C	C	C	C	T	A	-	C	G	
8548	T	T	C	C	T	C	T	T	C	T	T	G	C	T	C	C	T	C	C	C	T	T	A	-	C	G	
9419	T	T	C	C	T	C	T	T	C	C	T	G	C	T	C	C	T	C	C	C	C	T	A	-	C	G	
74369	**G**	**C**	**T**	**T**	T	**T**	**C**	**C**	C	C	**C**	**A**	**T**	**C**	**G**	**T**	C	**T**	**T**	**T**	C	C	**G**	**C**	T	**A**	
9835	**G**	**C**	**T**	**T**	T	**T**	**C**	**C**	C	C	**C**	**A**	**T**	**C**	**G**	**T**	T	**T**	**T**	**T**	C	T	**G**	**C**	C	**A**	
77944	**G**	**C**	**T**	**T**	T	**T**	**C**	**C**	C	C	**C**	**A**	**T**	**C**	**G**	**T**	C	**T**	**T**	**T**	C	C	**G**	**C**	T	**A**	
9626	**G**	**C**	**T**	**T**	A	**T**	**C**	**C**	C	C	**C**	**A**	**T**	**C**	**G**	**T**	C	**T**	**T**	**T**	C	T	**G**	**C**	C	**A**	
9388	**G**	**C**	**T**	**T**	T	**T**	**C**	**C**	C	C	**C**	**A**	**T**	**C**	**G**	**T**	C	**T**	**T**	**T**	C	C	**G**	**C**	T	**A**	
8006	**G**	**C**	**T**	**T**	T	**T**	**C**	**C**	C	C	**C**	**A**	**T**	**C**	**G**	**T**	T	**T**	**T**	**T**	C	T	**G**	**C**	C	**A**	
AP014547 [e]	**G**	**C**	**T**	**T**	T	**T**	**C**	**C**	C	C	**C**	**A**	**T**	**C**	**G**	**T**	T	**T**	**T**	**T**	**C**	T	**G**	**C**	C	**A**	

M. abscessus complex: Mycobacterium abscessus complex. Ins: insertion.

[a] Nucleotide positions are based on the *M. abscessus* subsp. *massiliense* sequence (accession no. AB548601).

[b] Nucleotide positions are based on the *M. abscessus* subsp. *massiliense* sequence (accession no. AB548600).

[c] Nucleotide positions are based on the *M. abscessus* subsp. *massiliense* sequence (accession no. AB548603).

[d] CU458896 (ATCC19977) is a reference sequence for *M. abscessus* subsp. *abscessus*.

[e] AP014547 (JCM15300) is a reference sequence for *M. abscessus* subsp. *massiliense*.

Table Ⅲ Characteristics of study participants .

	M. abscessus subsp. *abscessus* (n=8)	*M. abscessus* subsp. *massiliense* (n=6)	*P*-value
Age	69.1 ± 18.1	62.5 ± 9.7	0.435
BMI, kg/m^2	19.6 ± 3.4	18.6 ± 1.8	0.552
Gender			
Male	3 (21.4)	4 (28.5)	0.592
Female	5 (35.7)	2 (14.2)	
Smoking			
Never	6 (42.8)	2 (14.2)	0.277
Ever	2 (14.2)	4 (28.5)	
Radiological findings			
Cavity	1 (7.1)	2 (14.2)	0.538
Sympton of hemoptysis			
Yes	2 (14.2)	3 (21.4)	0.58
No	6 (42.8)	3 (21.4)	
Positive smear			
Yes	6 (42.8)	3 (21.4)	0.58
No	2 (14.2)	3 (21.4)	
Laboratory findings			
CRP, mg/dl	0.96 ± 1.22	1.68 ± 2.17	0.492

Date are expressed as numbers (%), values are means ± standard deviation. *M. abscessus* subsp. a*bscessus: Mycobacterium asbcessus* subspecies *abscessus. M. abscessus* subsp. *massiliense: Mycobacterium abscessus* subspecies *massiliense*, BMI: Body Mass Index, CRP: C-reactive protein.

Table Ⅳ Sequence differences in the *rrl* and *erm*(41) genes from clinical isolates of the *M. abscessus* complex.

	rrl[a]		*erm*(41)[b]																						
	2058	2059	-28	-4	28	41	46	64	65	85	90	109	120	123	159	238	255	279	330	336	419	438	466	amino acid change	
CU458896[c]	A	A	A	C	T	C	A	C	G	G	C	G	A	A	T	A	G	G	A	T	C	A	G		
9016	A	A	A	C	T	C	A	C	G	G	C	G	A	A	C	G**	G	G	C	T	C	A	G	I80V**	
8377	A	A	A	C	T	C	A	C	G	G	C	G	A	A	T	A	G	G	A	T	C	A	G		
9944	A	A	A	C	C*	C	A	C	G	G	C	G	A	A	C	G**	G	G	C	T	C	A	G	W10R*	I80V**
71740	A	A	A	C	T	C	A	C	G	G	C	G	A	A	C	G**	A	T	C	C	C	A	G		I80V**
9614	A	A	A	C	T	C	A	C	G	G	C	G	G	A	C	G**	A	T	C	C	C	A	G		I80V**
9854	A	A	A	C	T	C	A	C	G	G	C	G	A	A	T	A	G	G	A	T	C	A	G		
8548	A	A	A	C	T	C	A	C	G	G	C	G	A	A	C	G**	A	T	C	C	T***	A	G		I80V** P140L***
9419	A	A	A	C	T	C	A	G	G	G	C	G	A	A	C	G**	A	T	C	C	C	A	G		I80V**
74369	A	A	G	T	T	A	G	-	-	T	T	A	A	G	-	-	-	-	-	-	-	C	A		
9835	A	A	G	T	T	A	G	-	-	T	T	A	A	G	-	-	-	-	-	-	-	C	A		
77944	A	A	G	T	T	A	G	-	-	T	T	A	A	G	-	-	-	-	-	-	-	C	A		
9626	A	A	G	T	T	A	G	-	-	T	T	A	A	G	-	-	-	-	-	-	-	C	A		
9388	A	A	G	T	T	A	G	-	-	T	T	A	A	G	-	-	-	-	-	-	-	C	A		
8006	A	**G**	G	T	T	A	G	-	-	T	T	A	A	G	-	-	-	-	-	-	-	C	A		
AP014547[d]	A	A	G	T	T	A	G	-	-	T	T	A	A	G	-	-	-	-	-	-	-	C	A		

M. abscessus complex: *Mycobacterium abscessus* complex.

[a] Numbering system for the *rrl* gene from *Escherichia coli.*

[b] Numbering system for the *erm*(41) gene, with the GTG start codon as 1.

[c] CU458896 (ATCC19977) is a reference sequence for *M. abscessus* subsp. *abscessus*.

[d] AP014547 (JCM15300) is a reference sequence for *M. abscessus* subsp. *massiliense*.

[*] T to C transition at position 28 (28T>C) leading to a Trp>Arg amino acid change at codon 10.

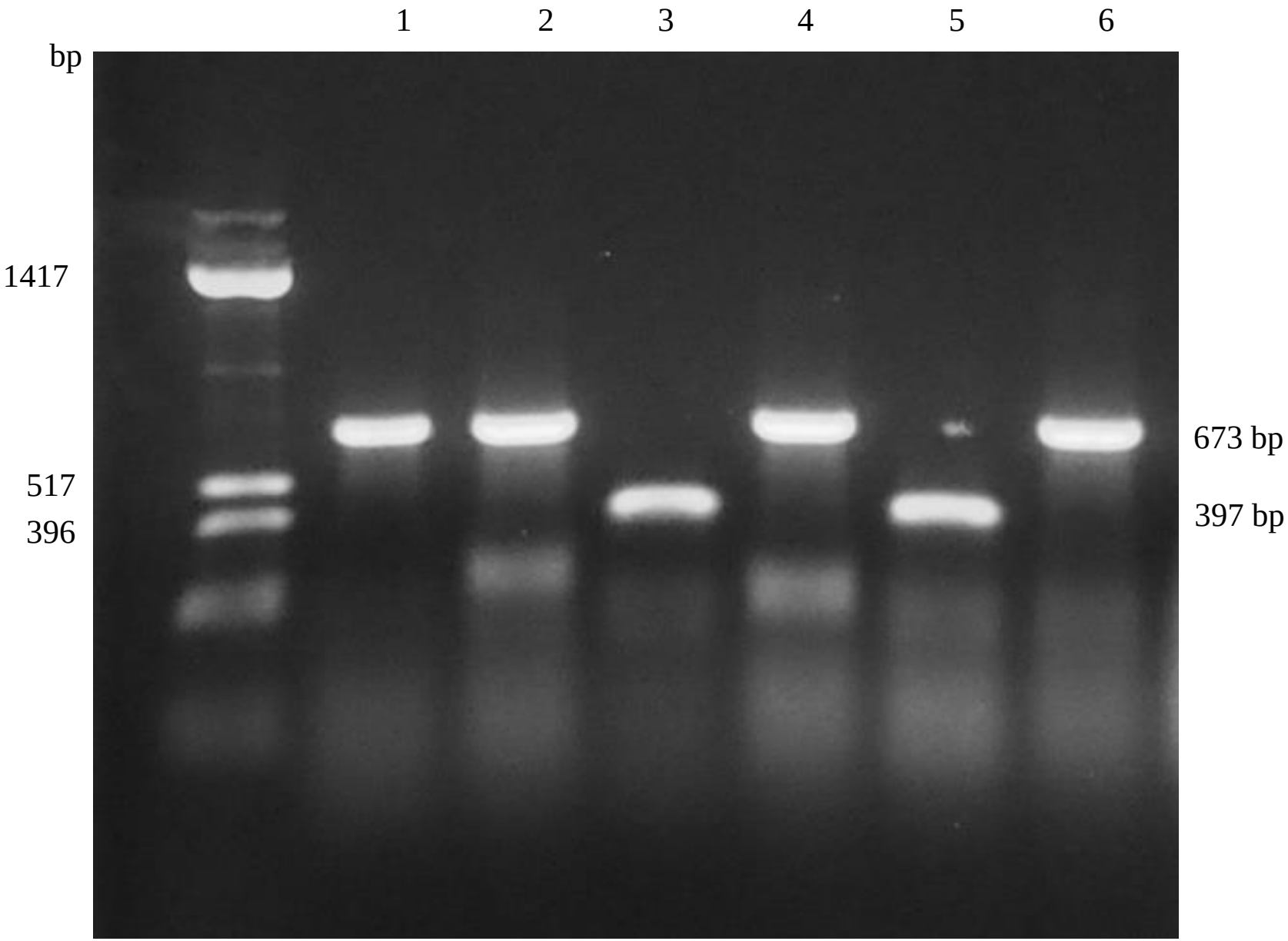
[**] A to G transition at position 238 (238A>G) leading to a Ile>Val amino acid change at codon 80.

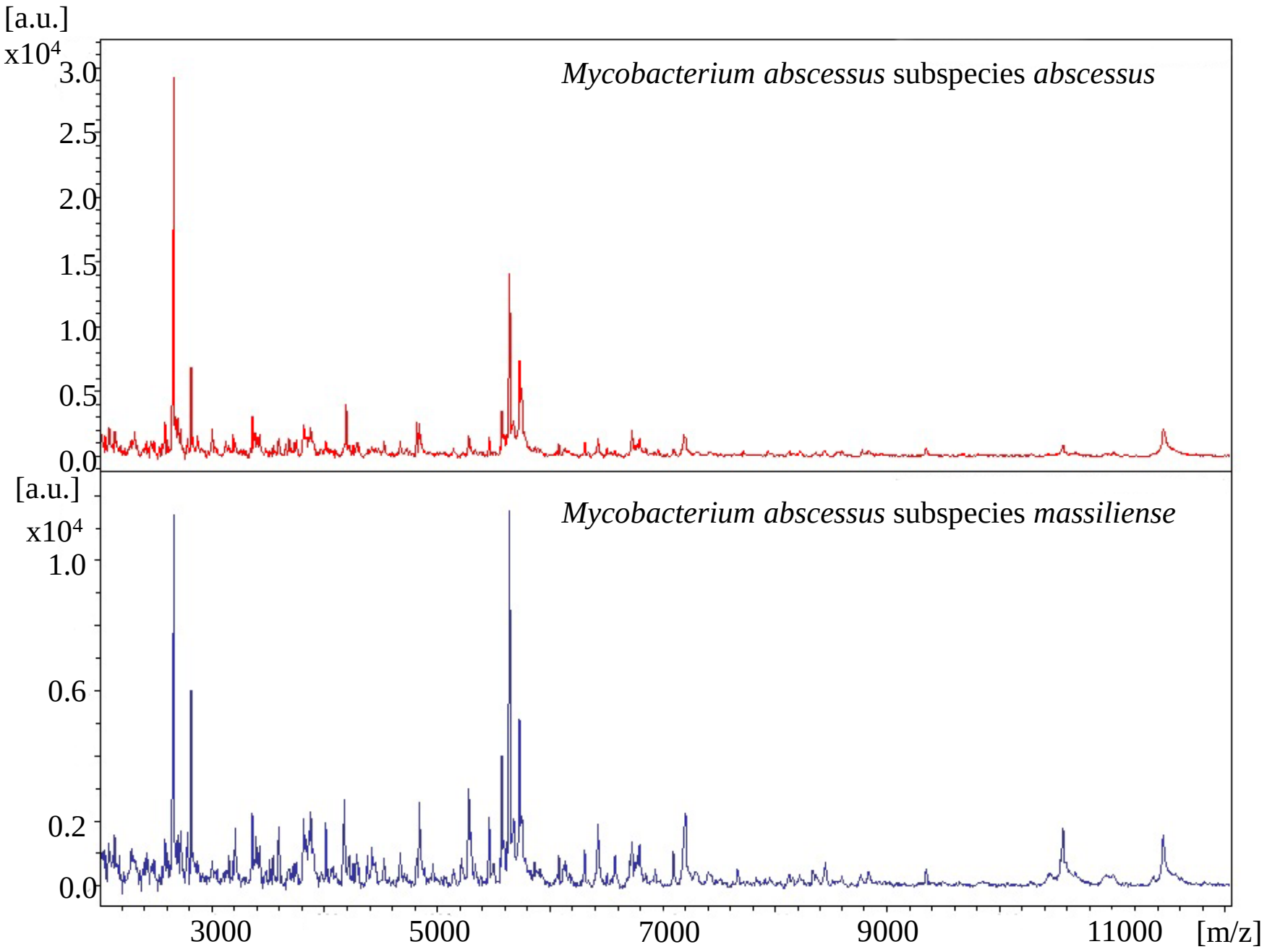
[***] C to T transition at position 419 (419C>T) leading to a Pro>Leu amino acid change at codon 140.

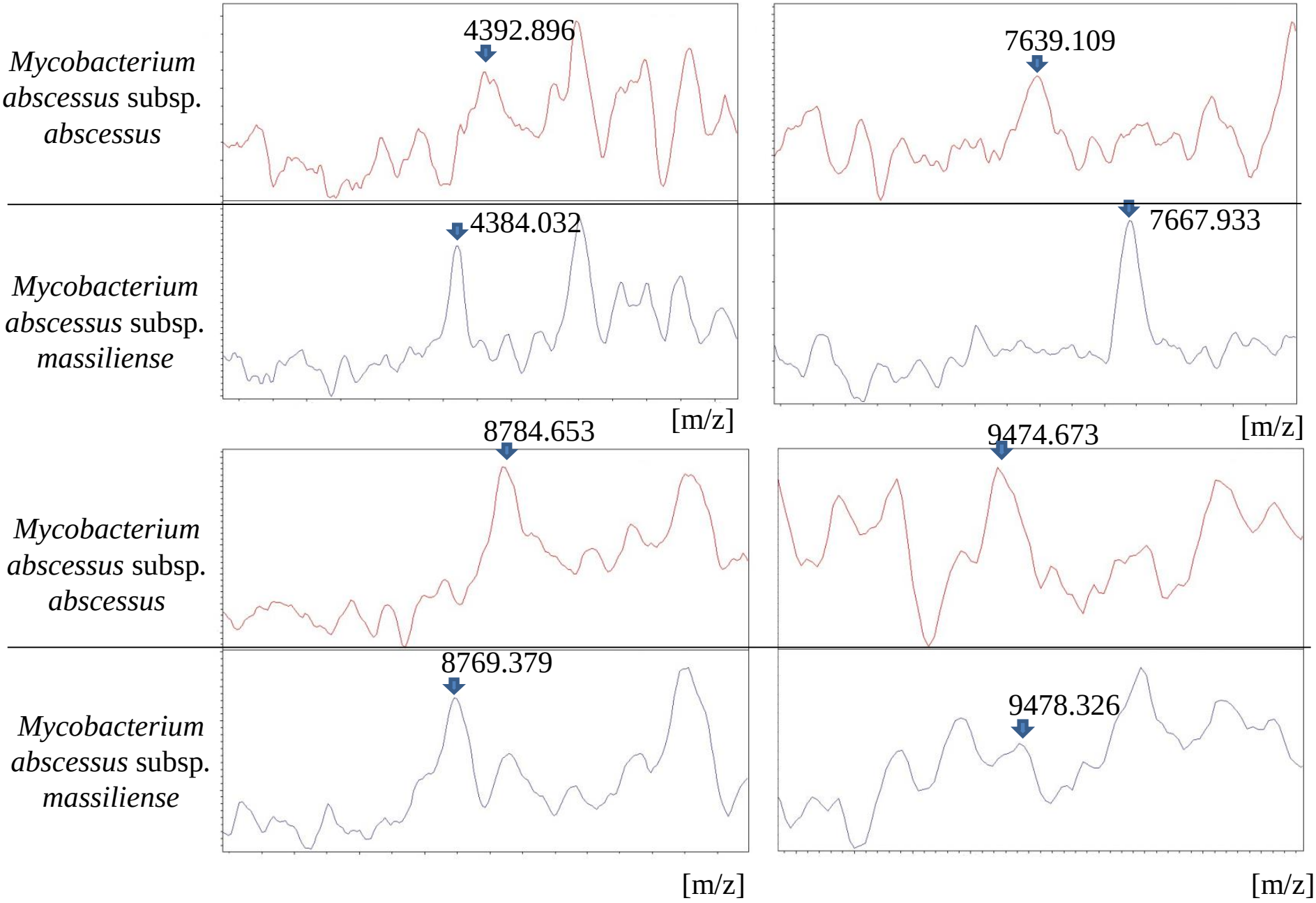
Table V Antibiotic susceptibilities of *M. abscessus* complex isolates based on MIC (μg/mL) values.

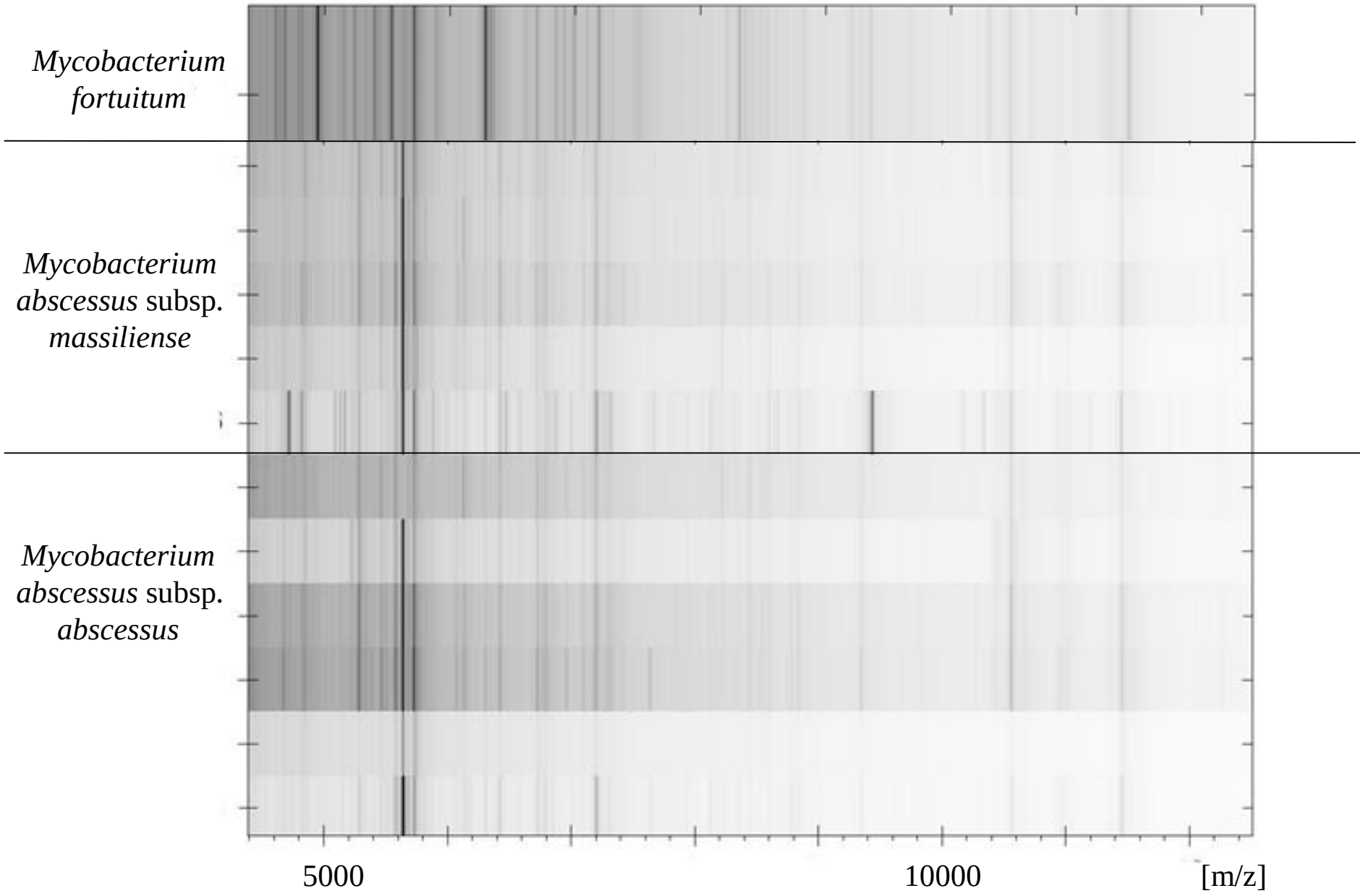
		Amikacin	Clarithromycin		
	strains no.	day 3	day 3	day 7	day 14
M. abscessus subsp. *abscessus*	9016	16	0.125	64	64
	8377	16	0.5	64	>128
	9944	8	≤0.06	≤0.06	≤0.06
	71740	8	0.25	32	32
	9614	8	0.25	64	64
	9854	8	0.125	>128	>128
	8548	8	≤0.06	64	>128
	9419	8	0.5	32	32
M. abscessus subsp. *massiliense*	74369	16	0.25	0.25	0.25
	9835	16	≤0.06	≤0.06	≤0.06
	77944	4	≤0.06	≤0.06	≤0.06
	9626	16	0.125	0.25	0.5
	9388	8	≤0.06	≤0.06	≤0.06
	8006	2	>128	>128	>128

MIC: minimum inhibitory concentration. *M. abscessus* subsp. a*bscessus: Mycobacterium abscessus* subspecies *abscessus. M. abscessus* subsp. *massiliense: Mycobacterium abscessus* subspecies *massiliense. M. abscessus* complex: *Mycobacterium abscessus* complex.


1
2
3
4
5
6
bp
1417
517
396
673 bp
397 bp


[a.u.]
x10^4
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Mycobacterium abscessus subspecies abscessus
[a.u.]
x10^4
1.0
0.6
0.2
0.0
Mycobacterium abscessus subspecies massiliense
3000
5000
7000
9000
11000
[m/z]


Mycobacterium abscessus subsp. *abscessus*
4392.896
7639.109
Mycobacterium abscessus subsp. *massiliense*
4384.032
7667.933
[m/z]
[m/z]
Mycobacterium abscessus subsp. *abscessus*
8784.653
9474.673
Mycobacterium abscessus subsp. *massiliense*
8769.379
9478.326
[m/z]
[m/z]


Mycobacterium fortuitum
Mycobacterium abscessus subsp. *massiliense*
Mycobacterium abscessus subsp. *abscessus*
5000
10000
[m/z]