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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*)

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Among 36 *Mycobacterium masilliense* and 22 *M. abscessus* isolates identified by *erm*(41) PCR and sequencing analysis of *rpoB* and 23S rRNA genes, the rate of accurate differentiation between these two subspecies was 100% by cluster analysis of spectra generated by Bruker Biotyper matrix-assisted laser desorption ionization–time of flight mass spectrometry.

Mycobacterium abscessus complex, a rapidly growing mycobacterium, is the cause of an increasing number of community- and health care-associated infections in humans (1). The isolation of *M. abscessus* complex from patients with various clinical infections has been reported in many countries, including Taiwan (2–7). The *M. abscessus* complex comprises three closely related subspecies, namely, *M. massiliense*, *M. bolletii*, and *M. abscessus* (*sensu stricto*) (4, 5, 7–9). Identification of *M. abscessus* complex members to the species level depends on sequencing analysis of several genes, including the *erm*(41) gene, the 23S rRNA gene, and several housekeeping genes (e.g., *rpoB* and *hsp65*) (7, 9–12). A previous report found that *erm*(41) PCR can differentiate *M. massiliense* from *M. abscessus* and *M. bolletii* but that sequencing analysis of *rpoB* and *hsp65* was less reliable at differentiating between the two (9). *M. abscessus* subsp. *bolletti* is now the recommended taxonomic name for *M. massiliense* (4, 12). Differences of *in vitro* susceptibilities to clarithromycin between *M. massiliense* (*M. abscessus* subsp. *Bolletti*) and *M. abscessus* (*sensu stricto*) isolates and of treatment response rates of lung diseases caused by these two species with clarithromycin-based antibiotic therapy have been reported (7–12). However, the use of molecular methods to differentiate among these subspecies is not possible in many routine microbiology laboratories.

The use of matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) is relatively new in the field of microbiology for species identification of yeasts and bacteria, including mycobacteria (13–17). Although MALDI-TOF MS has been shown to be a highly accurate method for identifying *M. abscessus* complex isolates to the species level, this method has not been shown to be able to differentiate between subspecies of the *M. abscessus* complex, i.e., *M. massiliense* (*M. abscessus* subsp. *bolletti*) and *M. abscessus* (*sensu stricto*) (15, 16).

In this study, we evaluated a total of 58 isolates of the *M. abscessus* complex obtained from various clinical specimens from patients treated during the period from January 2011 to December 2012 at the National Taiwan University Hospital, a 2,900-bed tertiary-care medical center in northern Taiwan. The isolates were presumptively identified as *M. abscessus* complex based on conventional biochemical methods as previously described (4–6). These isolates were further identified to the subspecies level by sequencing the *erm*(41) gene and by performing sequence analysis techniques targeting the *rpoB* and 23S rRNA genes. The following primer pairs were used: *erm*F (5′-GAC CGG GGC CTT CTT CGT GAT-3′) and *erm*R1 (5′-GAC TTC CCC GCA CCG ATT CC-3′) for the whole *erm*(41) gene; *rpoB* F (5′-GGCAAGGTCACCCCG AAGGG-3′) and *rpoB* R (5′-AGCGGCTGCTGGGTGATCATC-3′) for the *rpoB* gene; and 19 (5′-GTAGCGAAATTCCTTGTCG G-3′) and 21 (5′-TTCCCGCTTAGATGCTTTCAG-3′) for the 23S rRNA gene (4, 5). The sequences obtained were compared with published sequences in the GenBank database by using the BLASTN algorithm (http://www.ncbi.nlm.nih.gov/blast).

Among the 58 *M. abscessus* complex isolates, 22 were confirmed to be *M. abscessus* (*sensu stricto*) [smaller DNA of 673 bp amplified by *erm*(41) PCR; GenBank accession numbers JF346872.1 for the *rpoB* gene and EU590128.1 for 23S rRNA] and 36 were *M. massiliense* [smaller DNA of 397 bp amplified by *erm*(41) PCR; GenBank accession numbers CP003699.1 for the *rpoB* gene and GU143887.1 for 23S rRNA].

For MALDI-TOF MS analysis, a 10-μl inoculation loop was used to obtain *M. abscessus* complex colonies that had been grown in Middlebrook 7H11 agar plates (Becton, Dickinson-Diagnostic Systems, Sparks, MD) for 3 days. The MALDI-TOF MS protein extraction protocol for mycobacteria was performed as described previously, with modifications (16). A full loop of colonies was suspended in 500 μl of distilled water in a 1.5-ml screw-cap Eppendorf tube and was inactivated at 100°C for 30 min to kill the mycobacteria. The microtube was centrifuged at 13,000 rpm for 2 min, and the supernatant was discarded. The pellet was washed

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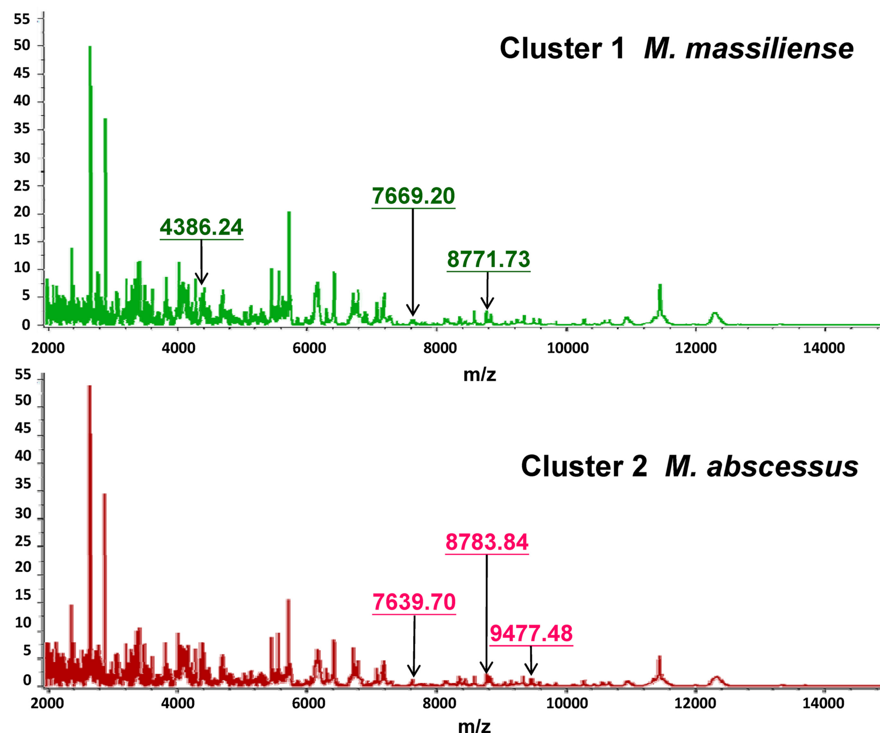

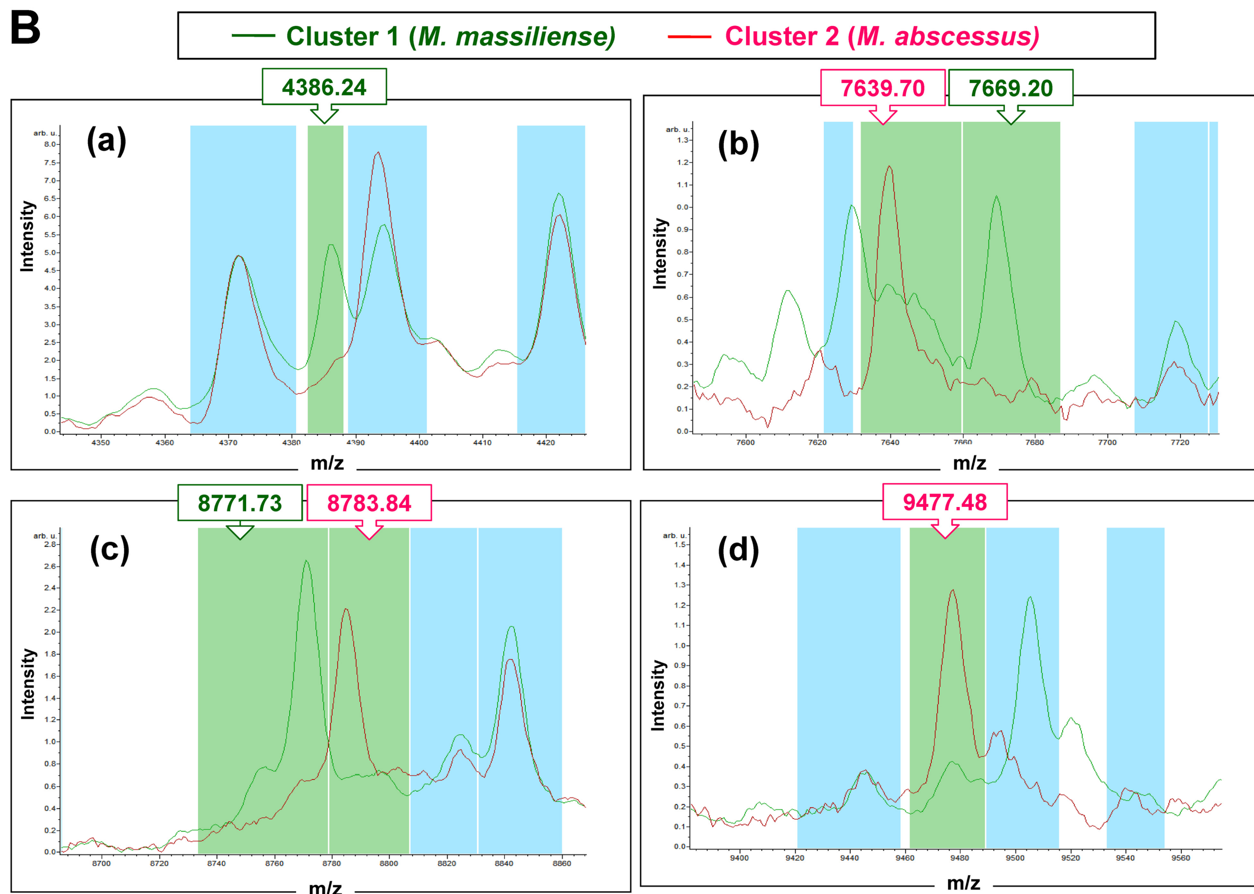

FIG 1 (A) Two clusters of *M. abscessus* complex spectra, i.e., cluster 1 (*M. massiliense*) and cluster 2 (*M. abscessus* [*sensu stricto*]), analyzed by clustering analysis of MALDI-TOF MS results. (B) The six peaks used to define cluster 1 (*M. massiliense*) and cluster 2 (*M. abscessus* [*sensu stricto*]), which were generated by ClinProTools with the genetic algorithm, are 4,386.24 *m/z*, 7,639.70 *m/z*, 7,669.20 *m/z*, 8,771.73 *m/z*, 8,783.84 *m/z*, and 9,477.48 *m/z*. The signals of 4,386.24 *m/z*, 7,669.2 *m/z*, and 8,771.73 *m/z* were observed in cluster 1 spectra but not in cluster 2 spectra, and those of 7,639.7 *m/z*, 8,783.84 *m/z*, and 9,477.48 *m/z* (class 2) were observed in cluster 2 spectra but not in cluster 1 spectra. The absolute intensities of the ions are shown on the *y* axis, and the masses (*m/z*) of the ions are shown on the *x* axis. The *m/z* values represent the mass-to-charge ratio.

TABLE 1 *In vitro* susceptibilities of *M. massiliense* and *M. abscessus* (*sensu stricto*) to 15 antimicrobial agents

Antimicrobial agent	MIC (μg/ml) for:					
	M. massiliense (*n* = 36)			*M. abscessus* (*sensu stricto*) (*n* = 22)		
	Range	50%	90%	Range	50%	90%
Amoxicillin-clavulanate	16/8–>64/32	>64/32	>64/32	64/32–>64/32	>64/32	>64/32
Cefoxitin	32–>128	64	128	32–>128	64	128
Ceftriaxone	64–>64	>64	>64	64–>64	>64	>64
Cefepime	>32	>32	>32	>32	>32	>32
Imipenem	8–>64	32	>64	16–>64	32	64
Clarithromycin (day 5)	0.06–>16	0.25	1	0.25–>16	4	16
Clarithromycin (day 14)	0.12–>16	1	>16	0.5–>16	>16	>16
Ciprofloxacin	>4	>4	>4	2–>4	>4	>4
Moxifloxacin	8–>8	>8	>8	1–>8	>8	>8
Tobramycin	8–>16	>16	>16	16–>16	16	>16
Amikacin	8–>64	16	>64	8–>64	16	64
Trimethoprim-sulfamethoxazole	1/19–>8/152	>8/152	>8/152	1/19–>8/152	8/152	>8/152
Doxycycline	16–>16	>16	>16	16–>16	>16	>16
Minocycline	8–>8	>8	>8	>8	>8	>8
Tigecycline	0.06–>4	2	>4	0.5–>4	2	4
Linezolid	16–>32	>32	>32	16–>32	>32	>32

with 70% ethanol, vortexed briefly, and centrifuged at 13,000 rpm for 2 min. The supernatant was decanted, the residual fluid was removed, and the pellet was dried at room temperature for 2 min. Twenty microliters of 0.5-mm-diameter silica beads and 20 μl of pure acetonitrile were added, and the mixture vortexed for 1 min. Twenty microliters of 70% formic acid was added, and the mixture vortexed for 10 s. One microliter of the supernatant was used for analysis by MALDI TOF MS.

MALDI-TOF MS measurement of the isolates was performed using a MALDI Biotyper system (Microflex LT; Bruker Daltonik GmbH, Bremen, Germany) with Compass Flex Series version 1.3 software and a 60-Hz nitrogen laser (337-nm wavelength). Spectra were collected in the linear positive mode in a mass range covering 1,960 to 20,132 *m/z*. ClinProTools (version 3.0.22; Bruker Daltoniks GmbH, Bremen, Germany) was used to generate classification models to differentiate among *M. massiliense* and *M. abscessus* (*sensu stricto*). All 58 isolates were initially identified by Bruker Biotyper MALDI-TOF MS as *M. abscessus* complex because of the lack of *M. massiliense* and *M. abscessus* (*sensu stricto*) in the database (MaldiBiotyperDB version 3.3.1.0 and Mycolib version 1.0.3). About 70 to 100 prominent ion peaks were noted in the *m/z* 2,000 to 12,000 range. Of these isolates, 50 (86.2%) had identification scores of >2.0 (range, 2.033 to 2.312) and 8 (13.8%) had identification scores of <2.0 (range, 1.74 to 1.911). Clustering analysis of the spectra from the 58 isolates of the *M. abscessus* complex, including 36 *M. massiliense* and 22 *M. abscessus* (*sensu stricto*) isolates, revealed two characteristic clusters (clusters 1 and 2) (Fig. 1A). Six peaks in the spectra of the isolates, i.e., 4,386.24 *m/z*, 7,639.70 *m/z*, 7,669.20 *m/z*, 8,771.73 *m/z*, 8,783.84 *m/z*, and 9,477.48 *m/z*, which were generated by ClinProTools with the genetic algorithm, were used to define clusters 1 and 2. The signals 4,386.24 *m/z*, 7,669.2 *m/z*, and 8,771.73 *m/z* were observed in the spectrum for cluster 1 (all were *M. massiliense*) but not in the spectrum for cluster 2 (all were *M. abscessus* [*sensu stricto*]), and those of 7,639.7 *m/z*, 8,783.84 *m/z*, and 9,477.48 *m/z* were observed in the cluster 2 spectrum but not in the cluster 1 spectrum (Fig. 1B).

The MICs of 15 antimicrobial agents against the 58 *M. abscessus* complex isolates were determined using the Sensititre RAPMYCO panel test (TREK Diagnostic Systems, West Sussex, United Kingdom) (5). The MICs of all agents tested were read on the fifth day (day 5) after incubation, and those of clarithromycin were also read on extended incubation (14 days). All the antimicrobial agents tested exhibited poor *in vitro* activities against both *M. abscessus* (*sensu stricto*) and *M. massiliense* isolates (Table 1). The MIC distribution of clarithromycin against *M. massiliense* and *M. abscessus* (*sensu stricto*) is shown in Fig. 2. The MIC_{90} of clarithromycin was 16 μg/ml for *M. abscessus* (*sensu stricto*) and 1 μg/ml for *M. massiliense* when the susceptibility assays were read on day 5 and >16 μg/ml when the susceptibility assays were read on day 14 for both species isolates (Table 1). Seven of the *M. abscessus* (*sensu stricto*) isolates had clarithromycin MICs of ≤2 μg/ml when the broth susceptibility test results were read on day 5. However, these seven *M. abscessus* (*sensu stricto*) isolates all showed

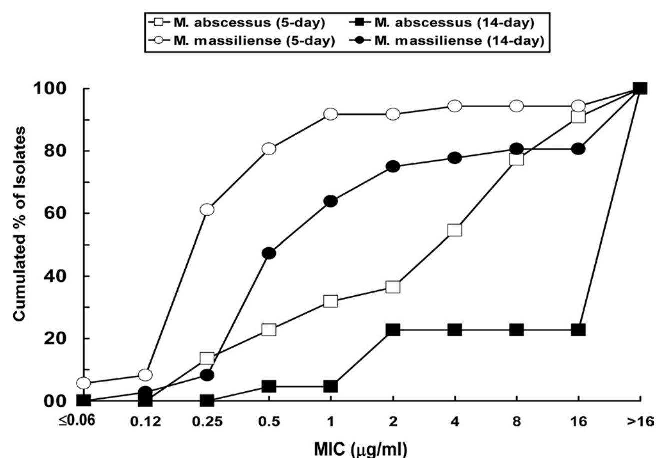

FIG 2 Distribution of MICs of clarithromycin against *M. massiliense* (*n* = 36) and *M. abscessus* (*sensu stricto*) (*n* = 22) determined by the Sensititre RAPMYCOI panel test (TREK Diagnostic Systems). Results were read on the 5th day of incubation and on day 14 (extended incubation).

clarithromycin MIC values of >16 (≥32) mg/ml on day 14, indicating that these isolates exhibited inducible resistance to clarithromycin (7). In contrast, the clarithromycin MIC values of 33 of 36 *M. massiliense* isolates were ≤2 μg/ml when the broth susceptibility test results were read on day 5; however, 11 (33.3%) of the 33 isolates exhibited clarithromycin MICs of >16 (≥32) μg/ml during the 14-day observation.

Previous studies have shown that rapidly growing mycobacteria, especially those of the *M. abscessus* complex, are the most common clinical isolates of mycobacteria and are a major cause of pulmonary disease due to nontuberculous mycobacteria (NTM) in Asia (2, 3, 18–20). Saleeb et al. reported that MALDI-TOF MS can be incorporated into the work flow of the microbiology laboratory for rapid and accurate identification of most species of mycobacteria (16). However, they also indicated that three pairs of closely related strains, namely, *M. abscessus* and *M. massiliense*, *M. mucogenicum* and *M. phocaicum*, and *M. chimaera* and *M. intracellulare*, could not be differentiated from each other by MALDI-TOF MS (16). Differentiation between two subspecies among *M. abscessus* complex isolates is important clinically because of the differences in their susceptibility profiles and clinical relevance (4, 5, 7, 9, 21). *M. massiliense* isolates tend to be more susceptible to clarithromycin than *M. abscessus* (*sensu stricto*) isolates (5, 7, 9, 21). In this study, we found that *M. abscessus* (*sensu stricto*) isolates were less susceptible to clarithromycin and had higher rates of resistance to that antibiotic than isolates of *M. massiliense*. Furthermore, a surprisingly high proportion of *M. massiliense* has been reported among *M. abscessus* complex central nervous system infections, and a higher proportion of *M. abscessus* (*sensu stricto*) among *M. abscessus* complex otologic infections (4, 5, 18).

In summary, our findings show that cluster analysis of spectra generated by MALDI-TOF MS can accurately differentiate between *M. massiliense* and *M. abscessus* (*sensu stricto*), although the number of isolates used in the study was small. This technique could be incorporated into the work flow of the microbiology laboratory for rapid and accurate differentiation between *M. massiliense* and *M. abscessus* (*sensu stricto*) among members of the *M. abscessus* complex.

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