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Substantial Improvement in Nontuberculous Mycobacterial Identification Using ASTA MicroIDSys Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry with an Upgraded Database

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Identifying *Mycobacterium* using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is challenging. We evaluated the performance of MALDI-TOF MS in identifying nontuberculous mycobacteria (NTM) using the ASTA MicroIDSys system (ASTA Inc., Suwon, Korea) with the MycoDB v1.95s and upgraded MycoDB v2.0-beta databases. We tested 124 NTM isolates collected from Ogawa medium at Severance Hospital, Seoul, Korea, between January and April 2019. MicroIDSys scores were categorized into three groups: ≥140, reliable identification; 130–139, ambiguous identification; and <130, invalid identification. To validate the results, we used the reverse blot hybridization assay (Molecutech REBA MycoID, YD Diagnostics Corp., Korea). Initial analysis using MycoDB v1.95s resulted in 26.6% (33/124) reliable, 43.5% (54/124) ambiguous, and 29.8% (37/124) invalid identifications. Re-analysis using the upgraded MycoDB v2.0-beta database resulted in 94.4% (117/124) reliable, 4.0% (5/124) ambiguous, and 1.6% invalid (2/124) identifications. The percentage of reliable identifications that matched with the reference increased from 26.6% (33/124) with MycoDB v1.95s to 93.5% (116/124) with MycoDB v2.0-beta. The upgraded databases enable substantially improved NTM identification through deep learning in the inference algorithm and by considering more axes in the correlation analysis. MALDI-TOF MS using the upgraded database unambiguously identified most NTM species. Our study lays a foundation for applying MALDI-TOF MS for the simple and rapid identification of NTM isolated from solid media.

Key Words: Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, Nontuberculous mycobacteria, Database upgrade, Identification, Performance evaluation

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More than one-third of nontuberculous mycobacteria (NTM) species are clinically relevant as they cause pneumonia, pediatric lymphadenitis, dermatitis, and systemic infections in patients with immunodeficiency [1, 2]. The treatment of NTM usually requires multidrug therapy, and the regimen is determined based on the NTM species identified [1, 3]. Rapid and accurate NTM

identification is required to avoid drug toxicity or the development of drug resistance resulting from unnecessary treatment. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been evaluated for NTM identification in clinical laboratories [4]. However, MALDI-TOF MS has limited applicability for *Mycobacterium* spp. detection owing to the thick lipid cell wall, making protein extraction challenging, and the limited availability of biomass from liquid cultures, which hampers accurate and timely species identification [5, 6]. MALDI-TOF MS is not suitable in clinical laboratories where high-throughput and highly accurate sample analysis is required. To overcome these limitations, the following strategies have been reported: protein extraction to obtain pure protein for analysis, database upgrades with reference spectra from additional NTM isolates and species, and creation of in-house libraries using spectra obtained from individual institutions [7-10].

We evaluated the NTM identification performance of the ASTA MicroIDSys system (ASTA Inc., Suwon, Korea) using the most up-to-date database for *Mycobacterium* spp. MycoDB v2.0-beta is a new version of the ASTA mycobacterial database with an advanced inference algorithm that gives more weight to specific peaks corresponding to specific NTM strains. Several studies have reported the competitive performance of MicroIDSys in diverse areas of clinical microbiology [11, 12]. However, NTM identification from clinical isolates has thus far only been reported using MicroIDSys with liquid culture specimens [13]. This study further demonstrates the performance of MicroIDSys and MycoDB v2.0-beta for NTM isolated from solid media.

We acquired 124 NTM clinical strains from clinical samples (sputum, bronchoalveolar lavage, and pus) between January and April 2019 from the Department of Laboratory Medicine and the Research Institute of Bacterial Resistance of Severance Hospital. The clinical samples were decontaminated using acetyl-L-cysteine-sodium hydroxide, neutralized with phosphate buffer, centrifuged, and inoculated into mycobacteria growth indicator tube (MGIT) liquid culture (Becton Dickenson, Sparks, MD, USA) and 3% Ogawa solid culture systems (BANDIO Bio Science, Pocheon, Korea). The cultures were incubated at 37°C for 6–8 weeks. For positive cultures, PCR was performed to differentiate NTM from *Mycobacterium tuberculosis* complex using AdvanSure™ TB/NTM real-time PCR kit (LG Life Sciences, Seoul, Korea) according to the manufacturer's instructions. Briefly, 5 µL of extracted nucleic acids was added to a PCR tube containing 5 µL of primer/probe mixture and 10 µL of PCR premix. The PCR cycling condition was as follows: one cycle of 50°C for 2 minutes and 95°C for 10 minutes; 35 cycles of 95°C for 10 seconds, 62°C for 40 seconds. The fluorescent signal was detected using SLAN real-time PCR detection system (LG Life Sciences). Isolates from Ogawa medium were collected for analysis, whereas Biomass grown in MGIT medium was not used. Protein was extracted from cultures grown in Ogawa medium according to the manufacturer's (ASTA) protocol. A single disposable loop was used to collect more than 10 µL of bacterial suspension, which was transferred into a tube containing 300 µL of distilled water and a silica bead, ensuring no contamination by media components. The bacteria in the tube were inactivated at 95°C for 10 minutes, washed with 700 µL of ethanol, and centrifuged at 13,000 rpm for 5 minutes. The supernatant was completely removed after the addition of 30 µL of 100% formic acid, the sample was subjected to 30 minutes of bead beating, followed by brief vortexing in the presence of 30 µL of 95% acetonitrile to break the cell walls and extract the proteins. After brief centrifugation, 1.5 µL of the supernatant was placed on a MALDI-TOF MS target plate (ASTA Inc.) and dried at 20–23°C. The spot was overlaid with 1.0 µL of α-cyano-4-hydroxycinnamic acid matrix.

The ASTA MicroIDSys MALDI-TOF MS system was used to acquire and analyze the spectra through laser exposure. The MycoDB v1.95s database was initially used to compare and analyze protein profiles, which were re-analyzed using the upgraded version MycoDB v2.0-beta. Both databases are based on the same dataset containing reference spectra from 71 mycobacterial species, including *M. fortuitum, M. abscessus, M. kansasii, M. avium, M. intracellulare, M. chelonae, M. gordonae*, and *M. szulgai*. However, the inference algorithm for drawing the most probable clinical species from the obtained spectral profile differs. MycoDB v1.95s simply determines the correlation between the prominent peaks in reference and obtained spectra, whereas MycoDB v2.0-beta calculates a weight for each peak and determines the correlation between spectral peaks considering the weight along with the m/z and intensity for each peak. MycoDB v2.0-beta uses deep learning to classify relatively strong signals into "likely true signals" and "likely noise signals" and reports the likelihood of a true signal as a weight.

Identification scores were categorized according to the manufacturer's recommendation: a score of <130 was defined as an invalid identification, a score of 130–139 as ambiguous identification, and a score of ≥ 140 as reliable identification. To validate the results, we used the reverse blot hybridization assay (Molecutech REBA MycoID, YD Diagnostics Corp., Yongin, Korea), which is a modification of the reverse hybridization assay widely used as a reference method for identifying NTM species in previous studies [14, 15].

Of the 124 NTM isolates, 58 and 66 isolates were categorized as rapidly growing and slowly growing mycobacteria, respectively (Table 1). The results of the initial analysis with MycoDB v1.95s were as follows: 26.6% (33/124) isolates with a score of ≥140, 43.5% (54/124) isolates with a score of 130–139, and 29.8% (37/124) isolates with a score of <130. The percentage of MALDI-TOF MS results with a score ≥140 that matched with the reference reverse blot hybridization assay was 100.0% (33/33). Re-analysis with the upgraded MycoDB v2.0-beta database yielded 94.4% (117/124) isolates with a score of ≥140, 4.0% (5/124) isolates with a score of 130–139, and 1.6% (2/124) isolates with a score of <130 (Table 2). The isolates that scored <130 were two strains of *M. avium*. Upon re-analysis, the percentage of MALDI-TOF MS results with scores ≥140 that matched with the reference was 99.1% (116/117) (Table 2). Using the upgraded database, the MALDI-TOF MS results matched with the reference results for 93.5% (116/124) of the isolates (Table 2). When only isolates with a score ≥140 were deemed correctly matched, the MALDI-TOF MS results matched with the references in 91.4% (32/35) of *M. avium* isolates, 94.3% (50/53) of *M. abscessus* isolates, 100% (5/5) of *M. fortuitum* isolates, 90.9% (20/22) of *M. intracellulare* isolates, and 100% (5/5) of *M. kansasii* isolates. Among the three misidentifications, two strains identified as *M. avium* by the reference method were identified as *M. intracellulare* using the upgraded database, and one strain identified as *M. intracellulare* by the reference method was identified as *M. gordonae* by MALDI-TOF MS.

Table 1. NTM isolates (N=124) collected, grouped according to the Runyon classification

Group	Species	N
Rapid growers	*Mycobacterium fortuitum* group	5
	M. abscessus	53
Slow-growing photochromogen	*M. kansasii*	5
Slow-growing scotochromogens	*M. gordonae*	3
	M. szulgai	1
Slow-growing nonchromogens	*M. avium*	35
	M. intracellulare	22

Abbreviation: NTM, nontuberculous mycobacteria.

Previous studies reported that database upgrades can significantly increase the ability to identify NTM species. Rodriguez-Sanchez, *et al.* [8] analyzed 109 NTM isolates from solid cultures using MALDI-TOF MS with Bruker Biotyper and Mycobac-

Table 2. Identification of NTM isolates using ASTA microIDSys MALDI-TOF MS with the MycoDB v1.95s and upgraded MycoDB v2.0-beta databases, along with percentages of species correctly identified using ASTA MALDI-TOF MS with MycoDB v2.0-beta

NTM species confirmed by reverse blot hybridization assay (reference method)	N	MALDI-TOF MS identification results							
		MycoDB v1.95s				MycoDB v2.0-beta			
		≥140 (N=33)	130–139 (N=54)	Total (N=124)	Correctly identified* isolates N (%)	≥140 (N=117)	130–139 (N=5)	Total (N=124)	Correctly identified* isolates N (%)
M. fortuitum group	5	1	1	2	1 (20.0)	5	0	5	5 (100.0)
M. abscessus	53	12	14	26	12 (22.6)	50	3	53	50 (94.3)
M. abscessus subsp. *abcessus*	39								
M. abscessus subsp. *massiliense*	14								
M. kansasii	5	1	4	5	1 (20.0)	5	0	5	5 (100.0)
M. avium	35	9	27	36	9 (25.7)	32	1	33†	32 (91.4)
M. intracellulare	22	10	7	17	10 (45.5)	20	1	21‡	20 (90.9)
M. chelonae	0	0	1	1§	0 (0.0)	0	0	0	
M. gordonae	3	0	0	0	0 (0.0)	4	0	4‡	3 (100.0)
M. szulgai	1	0	0	0	0 (0.0)	1	0	1	1 (100.0)
Invalid identification‖				37				2†	

Abbreviations: NTM, nontuberculous mycobacteria; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.
*Only isolates with an identification score ≥140 were deemed correctly identified; †Two *M. avium* isolates were unidentified by MALDI-TOF MS; ‡One *M. intracellulare* isolate was misclassified as *M. gordonae* by MALDI-TOF MS; §One *M. chelonae* isolate misidentified by MALDI-TOF MS was confirmed as *M. abscessus* subsp. *abscessus* by the reference method; ‖Isolates with an identification score <130 were labeled as invalid identifications.

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teria Library v2.0 and v3.0, and found improved confidence (identity scores >1.8 increased from 83.5% to 91.7%) following the library upgrade. Rodriguez-Temporal, *et al.* [9] analyzed 240 clinical NTM isolates from solid cultures using the same analytical instruments and found that the MALDI-TOF MS database upgrade from Mycobacteria libraries v2.0 to v3.0 improved mycobacterial identification (identity scores >1.7 increased from 73.8% to 80.0%). Together with these published results, our study demonstrates the utility of MALDI-TOF MS with the latest database in clinical laboratories for detailed and accurate NTM identification.

When applying MALDI-TOF MS in clinical microbiology, it is crucial to distinguish the unique signal peak for each microbial species from noise. This poses a significant obstacle, particularly in NTM identification due to artifact contamination from the culture medium during sample preparation and the limited amount of biomass that can be obtained. To overcome this obstacle, MycoDB v2.0-beta uses a strategy, in which additional weight is assigned to peaks that are likely to be true signals, while eliminating spectra in the database that include many peaks likely to represent noise from possible media contamination. Using MycoDB v2.0-beta, the 124 clinical NTM isolates were identified with high accuracy of 93.5%, as shown in Table 2. This is substantially better than most previously reported accuracies [6, 13, 16-18]. Notably, Yoo, *et al.* [13] reported slightly worse accuracy of 85.6% when using primary liquid culture and slightly better accuracy of 95.2% when using liquid media subcultures. Specifically, for *M. fortuitum, M. abscessus* subsp. *abscessus, M. kansasii, M. gordonae, M. szulgai,* and *M. abscessus* subsp. *massiliense*, all identification results were consistent with the reference data. The isolates with discordant results comprised two strains of *M. avium* and one strain of *M. intracellulare*. For the two *M. avium* strains, the identification scores were 122.2 and 125.1; thus, both strains were unidentifiable. As MycoDB v2.0-beta contains a reference for *M. avium*, the reason for the discordant results for these two strains is unknown and may be multifaceted. The *M. intracellulare* strain was identified by MALDI-TOF MS as *M. gordonae*, with a high identification score of 141.9. This discordance suggests the potential of infections from multiple species in the clinical sample or contamination in the pre-analytical stage, considering *M. gordonae* is often deemed a contaminant rather than a causative agent of human infection or colonization [19].

To our knowledge, only one study has used ASTA MicroIDSys MALDI-TOF MS for NTM identification [13]. Along with this previous report, our study provides valuable information about the utility of ASTA MicroIDSys MALDI-TOF MS. One of the main strengths of our study is that the sample size was relatively large compared with that of the previous study. Second, the accuracy of identification was substantially higher than that reported in other studies using solid cultures, suggesting that improvement in the analytical algorithms can greatly enhance the identification ability of MALDI-TOF MS. This study also had some limitations. First, we used only isolates from solid cultures, although the ability of ASTA MicroIDSys to identify mycobacteria directly from positive MGIT liquid cultures was evaluated recently [13]. Since mycobacterial growth would usually first be detected in liquid cultures, the clinical implications of this study are somewhat limited. Second, species that are rarely identified in clinical settings, including *M. xenopi, M. ulcerans*, and *M. marinum*, were not represented in our samples. Future studies targeting clinical isolates of rare species are required to evaluate the ability of MALDI-TOF MS to identify uncommon species.

In conclusion, our study demonstrated reliable NTM identification using ASTA MicroIDSys following MALDI-TOF MS, with substantially higher accuracy than previously reported. Our study lays a foundation for the future use of MALDI-TOF MS for the simple and rapid identification of NTM isolated from solid media.

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AUTHOR CONTRIBUTIONS

Conception and design of study: Yong D; Data acquisition: Song JH; Data analysis, and interpretation: Song JH, Yoon S, In Y; Drafting of manuscript: Song JH; Final approval of manuscript: Kim D, Lee H, Yong D, Lee K.

CONFLICTS OF INTEREST

None declared.

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