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The impact of protein extraction protocols on the performance of currently available MALDI-TOF mass spectrometry for identification of mycobacterial clinical isolates cultured in liquid media

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ABSTRACT

Background: Protein extraction step is particularly important for identification of mycobacterial isolates by MALDI-TOF mass spectrometry (MS) because of its thick and solid cell wall. This study compared the performance of MALDI-TOF MS for identification of mycobacterial clinical isolates cultured in liquid media between heating-based protocol and non-heating protocol.

Methods: Clinical mycobacterial isolates cultured in liquid media were prospectively analyzed. Reference identification was real-time PCR and restriction fragment length polymorphism. The specimens prepared by heating protocol and non-heating protocol were tested using MALDI Biotyper (Bruker Daltonics, Bremen, Germany) and Vitek MS (bioMérieux, Marcy l'Etoile, France), respectively.

Results: Among the 206 clinical specimens prepared by heating method, identification rates were 90.3% and 60.7% in MALDI Biotyper and Vitek MS, respectively. Identification accuracy of MALDI Biotyper and Vitek MS was 100% for the isolates of *Mycobacterium tuberculosis* complex (MTBC), *Mycobacterium avium*, *Mycobacterium intracellulare*, *Mycobacterium abscessus* and *Mycobacterium fortuitum*. Among the 121 clinical specimens prepared by non-heating method, identification rate for MALDI Biotyper and Vitek MS were 61.2% and 69.4%, respectively. Identification accuracy of MALDI Biotyper/Vitek MS were 92.9%/94.1% for MTBC, 92.9%/100% for *M. avium*, 90%/100% for *M. intracellulare*, 100%/100% for *M. abscessus* and 100%/100% for *M. fortuitum*.

Conclusions: The performance of MALDI-TOF MS for identification of mycobacterial clinical isolates is affected by protein extraction protocol. For best performance, protein extraction protocol should be chosen considering the MALDI-TOF MS system. In the present study, heating protocol with MALDI Biotyper system showed reliable identification results for mycobacterial clinical isolates.

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1. Introduction

Tuberculosis (TB) remains one of the world's deadliest communicable diseases. In 2013, an estimated 9.0 million people developed TB, and 1.5 million died from the disease [1]. The *Mycobacterium* genus consists of over 100 species of rapid-growing and slow-growing acid-fast bacilli (AFB). When mycobacterial infection is suspected, rapid and accurate diagnosis of it is important for patient care and public health. Inappropriate identification of the *Mycobacterium tuberculosis* complex (MTBC) can lead to unnecessary isolation of patients and implementation of toxic treatment regimens [2]. Rapid identification of mycobacteria has proven difficult due in part to their fastidious growth requirements and slow growth rate [3,4]. Molecular probe, hybridization-based techniques or polymerase chain reaction (PCR) are relatively fast and simple, but are expensive, can require specific equipment and expertise, and often have a limited availability to clinically common species [3,5–8]. Recently the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) technique provides a rapid alternative for the identification of microorganisms including *Mycobacterium* spp. based on differences in their protein profile [9–11].

For more rapid identification, MALDI-TOF MS is increasingly being used by clinical laboratories for identifying isolates obtained from solid medium as well as directly from liquid samples such as blood culture and urine [12–14]. These studies demonstrated advantages including rapid identification, high accuracy, and low reagent cost associated with the use of MALDI-TOF MS in clinical laboratories. For the isolates obtained from solid medium, MALDI-TOF MS showed 95% to 99% identification accuracy [3,5,6,15]. However, not many studies have been

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conducted about the application of MALDI-TOF MS to the identification of mycobacterial clinical isolates obtained directly from liquid media. In addition, to minimize the potential exposure of laboratory personnel to MTBC and to maximize the quality of the spectra obtained, inactivation of the mycobacterial cells and extraction of mycobacterial proteins are imperative prior to the performance of MALDI-TOF MS [6,8]. Importantly, few studies have examined the impact of protein extraction protocol on mycobacterial identification using MALDI-TOF MS.

The present study evaluated the impact of protein extraction protocols (heating-based method *vs.* non-heating simplified method) on the identification rate and accuracy of the two MALDI-TOF MS systems, MALDI Biotyper (Bruker Daltonics, Bremen, Germany) and Vitek MS (bioMérieux, Marcy l'Etoile, France). In addition, the performance of MALDI-TOF MS for the routine identification of mycobacterial clinical isolates obtained from liquid culture was analyzed for each species.

2. Method and materials

2.1. Clinical isolates and identification

This study was composed of two parts. In Part A, positive specimens were randomly selected from mycobacterial broth culture. They were prepared with heating-based method (Method 1: see the next section). Prepared samples were simultaneously tested with MALDI Biotyper (Bruker) and Vitek MS (bioMérieux). In Part B, randomly selected broth culture specimens were prepared with non-heating simplified method (Method 2: see the next section). We used two automated culture systems: MGIT 960 (Becton Dickinson, Sparks, MD, USA) or VersaTREK (Thermo Scientific, Oakwood village, OH, USA). Reference identification was conducted using a previously published method [16]. Briefly, MTBC and nontuberculous mycobacteria (NTM) were simultaneously detected and identified by laboratory-developed real-time PCR, multiplex real-time PCR/melting curve analysis and *rpoB* PCR restriction fragment length polymorphisms. This study was approved by the Institutional Review Board of the Seoul National University Bundang Hospital (B-1103/124-105).

2.2. Preparation of isolates for MALDI-TOF MS analysis

2.2.1. Method 1

After positive signal, culture broths were removed from culture system, and tested after one to two weeks of room-temperature storage. The test was intentionally delayed for obtaining sufficient biomass. A 2.0-mL aliquot was removed from positive broth cultures and centrifuged for 2 min at 14,000 rpm. The supernatant was then removed from the pellet; 500 μL of distilled water was added to the pellet and centrifuged for 2 min at 14,000 rpm. The supernatant was then removed and the pellet was suspended in 300 μL of distilled water followed by 900 μL of 100% ethanol was added (final concentration 75% ethanol). The ethanol suspension was vortexed and centrifuged for 2 min at 14,000 rpm. The supernatant was then removed and the pellet was suspended in 500 μL of distilled water, vortexed, and centrifuged for 2 min at 14,000 rpm. The supernatant was removed and the pellet was suspended in 50 μL of distilled water by vortex, and then put in a 95 °C ± 5 °C heat block for 30 min to inactivate the organism. After heat inactivation, the suspension was allowed to cool to room temperature followed by adding 1200 μL of chilled 100% ethanol. This suspension was then vortexed to homogenize and centrifuged for 2 min at 14,000 rpm. The supernatant was removed and the pellet was dried completely. Once the pellet was completely dry, 25 to 50 μL (depending on size of the pellet) of 100% acetonitrile and a spatula of zirconia/silica beads (diameter 0.5 mm; Biospec, Bartlesville, OK, USA) was added to the tube. The tube was vortexed for 1 min to disrupt any aggregates, and then an equal amount of 70% formic acid was added to the tube. This acetonitrile/formic acid suspension was again vortexed and then centrifuged for 2 min at 1400 rpm. A 1-μL portion of supernatant was applied to MALDI-TOF target plates for each of the mass spectrometry systems. When dry, 1 μL of matrix (alpha-cyano-4-hydroxy cinnamic acid) was used to overlay the analyte and allowed to dry followed by analyzed using MALDI-TOF MS. Each extract was analyzed in duplicate and the higher score was used for data analysis.

2.2.2. Method 2

Like Method 1, the test was conducted after one to two weeks from positive signal. A 1.8-mL aliquot was removed from positive broth cultures and centrifuged for 10 min at 8000 rpm. The supernatant was then removed and the pellet was suspended in 500 μL of 70% ethanol. The ethanol suspension was moved into the 2 mL tube containing 200 μL of glass beads (diameter 0.5 mm; Bertin Technologies, Montigny le Bretonneux, France) and vortexed for 15 min and centrifuged for 2 min at 14,000 rpm. The supernatant was removed and the pellet was dried completely. Once the pellet was completely dry, 25 to 50 μL (depending on size of the pellet) of 100% acetonitrile was added to the tube. The tube was vortexed for 1 min, and then an equal amount of 70% formic acid was added to the tube. This acetonitrile/formic acid suspension was again vortexed and then centrifuged for 2 min at 14,000 rpm. A 1-μL portion of supernatant was applied to MALDI-TOF target plates for each of the mass spectrometry systems. When dry, 1 μL of matrix (alpha-cyano-4-hydroxy cinnamic acid) was used to overlay the analyte and allowed to dry before analyzed using MALDI-TOF MS. Each extract was analyzed in duplicate and the higher score was used for data analysis.

2.3. MALDI-TOF analysis

2.3.1. MALDI Biotyper

A target plate prepared with protein extracts, as described in previous section, was inserted into the Bruker Microflex LT MALDI-TOF MS (Bruker) for analysis. A composite profile of proteins with a mass-to-charge ratio (m/z) of 2000 to 20,000 was generated based on a minimum of 240 measurements (laser shots) for each analyte. The composite profile was analyzed using either MALDI Biotyper Mycobacteria Library v1.0. Mycobacteria Library v1.0 is a mycobacterium-specific library that contains 173 unique mycobacterial spectra representing 94 species. Query of either reference library returned the top 10 identification matches along with confidence scores ranging from 0.0 to 3.0.

2.3.2. Vitek MS

The spectra were acquired in linear positive ion mode at a laser frequency of 50 Hz across m/z 2000 to 20,000 Da using the Vitek MS system (bioMérieux). For each target slide, the *Escherichia coli* reference strain ATCC 8739 was used for instrument calibration according to the manufacturer's specifications. For each test, up to 100 mass profiles were produced from 500 laser shots at different areas of the target that were then summed into a single raw mass spectrum. Each spectrum was then processed by baseline correction, denoising, and peak detection to identify well-defined peaks. After spectrum acquisition, the data were transferred from the Vitek MS acquisition station to the SARAMIS analysis server, where the data were reported as the number of peaks. All spectra generated on the Vitek MS were compared to both SuperSpectra and ReferenceSpectra in the SARAMIS 4.13 database. ReferenceSpectra are the spectral fingerprints generated from a single isolate. In contrast, SuperSpectra are computed from multiple (15 +) reference spectra by extracting conserved mass signals and weighting those signals according to their specificities for different taxonomic levels of identification (*i.e.*, family, genus, species, and subspecies).

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