Mycobacterial Inactivation Protein Extraction Protocol for Matrix-assisted Laser Desorption Ionization Time-of-Flight Characterization of Clinical Isolates

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Abstract

Background: Rapid identification of mycobacteria has been made possible with matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) in recent years. Working with high concentrations of mycobacteria in a PC-3 containment facility makes MALDI-TOF cumbersome and costly. Therefore removing the inactivated isolate’s protein extract from the PC-3 facility is needed for efficient identification in a routine PC-2 laboratory. Methods: This work describes a novel chemical and mechanical disruption protein extraction method, which provides reliable MALDI-TOF results from solid and liquid media, while ensuring laboratory safety. Results: When compared to sequencing results, 93.9% of the clinical isolates were identified in LJ media and 89% of the clinical isolates were identified in MGIT media. Conclusion: The MIPE protocol produces a high quality protein extract with improved isolate identification without compromising result turn-around-times or laboratory safety.

Keywords: Clinical isolates, MALDI-TOF, Matrix-assisted laser desorption ionization time-of-flight, mycobacterial identification

Introduction

The genus mycobacterium encompasses more than 150 aerobic species, some able to grow under reduced O₂ atmosphere. Mycobacterium as a genus can be broadly categorized into three groups: Mycobacterium tuberculosis complex (MTBC), Mycobacterium leprae, and other mycobacteria collectively known as nontuberculosis mycobacterium (NTM). They are nonspore forming (except Mycobacterium marinum, Mycobacterium avium under specific culture conditions), nonmotile, slightly curved, or straight rods with occasional branching. They contain a high concentration of mycolic acids (β-hydroxy-α-fatty acids) which explain their acid-fastness and microscopical morphology.

MTBC is the 9th leading cause of death from an infectious agent, with an estimated 33% disease prevalence worldwide. Members of the MTBC are strict intracellular pathogens of humans and animals causing tuberculosis. Its clinical presentation depends on the host’s immune system, presenting as a chronic respiratory illness with symptoms such as hemoptysis, fever, and nocturnal diaphoresis. NTM are not obligate pathogens and are found ubiquitously in the environment. In this group, members are opportunistic pathogens that can be associated with disease in immune-compromised hosts, cystic fibrosis patients, and chronic non-healing ulcers. M. leprae and Mycobacterium lepromatosis are the etiological agents for leprosy which cannot be routinely cultured in vitro; therefore, their inclusion is not relevant to this study.

Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF), fast-tracks identification results that conventionally would take days. Since the Bruker Biotyper MALDI-TOF was introduced in our laboratory, there has been substantial work in the optimization of the instrument to identify bacterial, mycobacterial, and fungal isolates. The manufacturer provided a working protocol for the identification of mycobacterial isolates (Bruker Daltonics). Furthermore,
several studies have strengthened the idea of mass spectrometry mycobacterial identification by slightly modifying this protocol to suit their respective workflows and practices. A group in Ireland modified that the original protocol and presented a two-step cell disruption protocol, which added 15 min of sonication to liberate mycobacterial proteins for further analysis. Another group from Spain added washing and ~20°C cold ethanol steps. Moreover, a washing step seems to be one of the most popular modifications to aid spectra production as shown by Kehrmann et al. It is clear that the manufacturer’s protocol has been exhaustively tested and modified. However, in previous library constructions (unpublished data), we found that heat inactivation causes changes in the innate protein composition with resulting low-quality spectra. This issue arises because of protein degradation or miss-folding while at high temperatures or natural methylation of the proteins which reacts negatively with the matrix solution used in the final step of the protocol. Heat inactivation of isolates may also activate protein clumping, thereby hindering mass spectrometry analysis. Buckwalter et al. suggested that 10 min in 70% ethanol was enough for bacterial inactivation, while a Brucella inactivation protocol describes how the matrix solvent can be used to inactivate bacteria. Although molecular-based techniques are more sensitive in identifying mycobacteria directly from clinical samples, routine culture is still needed for susceptibility testing. With this background, we opted to evaluate an isothermal, nonheating, inactivation, and protein extraction technique of cultured mycobacterial isolates that will maintain proteins in their native form for mass spectrometry analysis.

**Methods**

**Bacterial isolates**

Thirty-six mycobacterial clinical isolates collected and stored by SA Pathology (Adelaide, South Australia) including six control strains (Mycobacterium abscessus NCTC 13031, M. avium ATCC 700898, Mycobacterium chelonae ATCC 946, Mycobacterium fortuitum ATCC 6841, Mycobacterium kansasii ATCC 12478 and M. tuberculosis H37RV) were analyzed by MALDI-TOF and by 16S ribosomal RNA/rpoB sequencing. Isolates were retrieved from ~80°C storage and then cultured in Mycobacteria Growth Indicator Tube (MGIT, Becton Dickinson) and onto Lowenstein–Jensen (LJ) pyruvate media (Thermo Fisher Scientific, Adelaide, Australia), incubated at 30°C and 35°C, respectively, with ambient atmosphere for up to 12 weeks. Isolates were considered positive for culture once enough biomass was visible on the LJ media or once the BACTEC MGIT 950 TB system flagged positive. Both of these scenarios happened around 2–3 weeks of incubation.

**Inactivation protocol**

To select an inactivation protocol, a kill curve experiment was performed evaluating cellular killing by the Bruker ethanol inactivation method and the MALDI-TOF matrix solvent solution method described by Mesureur et al. respectively. As a modification to Mesureur’s work, this study used the matrix solvent solution provided by Bruker Daltonics (50% acetonitrile, 2.5% trifluoroacetic acid and 47.5% water). Three control strains were used for this initial work: M. tuberculosis H37RV, M. abscessus NCTC 13031, and M. avium ATCC 700898, respectively. Viable mycobacteria were exposed in duplicate to either killing agent for several contact time intervals (0, 10, 20, 30, and 60 min). Growth controls were similarly exposed to sterile water. After each exposure period lapsed, a 10 µl loop full of the exposed cells was cultured onto LJ pyruvate media, MGIT media, and horse blood agar, in duplicate and monitored over a period of 8 weeks. Media were inspected routinely for growth, daily for horse blood agar, weekly for LJ media, and BACTEC MGIT 950 TB system continuous monitoring for MGIT tubes. No viable growth at the end of the incubation period was determined as successful killing of mycobacterial cells.

**Mycobacterial inactivation and protein extraction protocol**

A 10 µl loop full of biomass from LJ pyruvate media or 3 ml of positive MGIT fluid (previously centrifugated at 14000 rpm for 5 min) were placed in a 1.5 ml Eppendorf tube (Eppendorf, South Pacific, NSW, Australia) with 200 µl of matrix solvent solution (50% acetonitrile, 2.5% trifluoroacetic acid, and 47.5% water) and homogenized with a micro-pestle as described elsewhere. After homogenization, the mixture was allowed to be in contact with the matrix solvent solution for 20 min in order to inactivate viable bacterial cells. The homogenized solution was centrifuged at 14,000 rpm (Eppendorf 5418/5418R, Eppendorf South Pacific, NSW, Australia) for 2 min. Once a pellet was formed, the supernatant was removed, and acetone (800 µl for LJ pyruvate media or 100 µl for MGIT fluid, respectively) was added to the 1.5 ml Eppendorf tube. The pellet and acetone were homogenized once again with the aid of a micropestle and centrifuged at 14,000 rpm for 2 min. The supernatant was removed, and the pellet was placed in molecular grade water (800 µl for LJ pyruvate or 100 µl for MGIT fluid). After centrifugation at 14000 rpm for 2 min, the pellet was left to air dry for 10 min. Subsequently, 70% formic acid and 100% acetonitrile (4 µl each for LJ pyruvate media or 1 µl each for MGIT media, respectively) were added to the dried pellet. Following repeat centrifugation at 14000 rpm for 2 min, 1 µl of the supernatant was spotted onto the MALDI-TOF target plate (MSP96 polished steel target, Bruker Daltonics) and was left to dry at room temperature. Finally, 1 µl of crystal matrix solution (α-cyano-4-hydroxycinnamic acid-saturated solution in 50% acetonitrile with 2.5% trifluoroacetic acid) (Bruker Daltonics) was added to each spot and left to crystallize at room temperature, before analyzed in the MALDI-TOF for data acquisition. Substitution of the acetone step with other commonly used compounds including absolute ethanol and cold ethanol, and Tween-20 was investigated and subsequently abandoned due to poor results.

**Mass spectrometry**

The laser on a Microflex MALDI-TOF instrument (Bruker Daltonics) was calibrated at 337 nm and ionized samples.
at a shot rate of 50 Hz for a total of 160 shots per target spot. Each spectrum had a mass to charge (m/z) range set of 1–21 kDa and was controlled by flexControl software (Bruker Daltonics). Calibration of the instrument was achieved running the Bruker Test Standard which provided mass accuracy (approximately ±2.5 Da per peak). Analysis of the spectra was achieved by comparing the spectra obtained to the Bruker database v. 6.0 (Bruker Daltonics) and the mycobacteria library v. 4.0 (Bruker Daltonics).

**Mycobacteria growth indicator tube media logarithmic score optimization**

Finally, optimization was performed to increase the yield of MALDI-TOF logarithmic scores obtained from isolates grown in MGIT media. The quality of spectra obtained by MALDI-TOF is proportional to the number of bacteria present during the analysis. Five isolates were selected for further analysis based on the difference between initial spectral scores obtained from both LJ and MGIT growth media, respectively. The criteria for selection relied on a relatively good (x > 1.9) score from LJ, but a low score (1.5 > x < 1.9) from MGIT media. This difference in scores can be attributed to the relative differences of growth density on both media, with fewer cells in positive MGIT compared to visible growth on LJ media. The isolates were tested at 0, 3, and 5 days after positivity (visible growth on LJ or positive signal from MGIT, respectively) while continuously incubating at 35°C. Identification at day 3 after MGIT positivity was proved to be the most reliable for identification, approximating results obtained from solid media.

**Molecular identification**

Mycobacterial isolates were harvested from LJ pyruvate media or 500 µl of MGIT fluid, respectively and inoculated (1 µl loop of culture from LJ pyruvate media or 500 µl of MGIT fluid) into 500 µl of molecular grade water (Thermo-Fisher, Australia). The homogenized mixture was subsequently boiled for 15 min to provide a sterile crude extract for 16S rDNA polymerase chain reaction (PCR) analysis. Amplification was performed on an Eppendorf® PCR instrument using the following conditions: 10 min at 94°C for polymerase activation and 40 cycles (94°C/30 sec, 55°C/1 min, and 72°C/2 min). Agarose gel electrophoresis was performed using 8 µl of the amplification product to confirm the purity and band intensity (1% agarose w/v, 3 v/cm stained with ethidium bromide). On confirmation, the remainder of the amplification product was cleaned using a column-based assay QIAquick™ (Qiagen Pty Ltd) and eluted in a final volume of 200 µl. One microliter of the cleaned PCR product was sequenced with a 3730/3730xl DNA Sequencer (Applied Biosystems, USA). Sequencing chromatograms were analyzed with Kodon 3.62 software (Applied Maths, USA).

**Results and Discussion**

Kill curve analyses were very informative and were the cornerstone of laboratory safety for this project. Despite the ability of the Bruker ethanol method to effectively kill mycobacteria (M. tuberculosis H37RV, M. avium, and M. abscessus) from MGIT media, it was found that this protocol was inadequate for the complete inactivation of mycobacteria from LJ media. Compared to a previously published method [17] modifications to the MALDI-TOF solvent inactivation method needed to be made to compensate for unknown variables such as contact time with the solvent for active mycobactericidal activity and differences in solvent composition. Bruker’s solvent solution used in this study consists of 50% acetonitrile, 2.5% trifluoroacetic acid, and 47.5% water, unlike the bioMérieux solvent solution utilized by Mesureur et al. (33% acetonitrile, 33% absolute ethanol, 3% trifluoroacetic acid, and 31% water). [17] Results showed that commercially available Bruker’s solvent is rapidly mycobactericidal for isolates obtained from both MGIT or LJ media [Table 1]. Based on this preliminary work, all further mycobacterial inactivation in this study was performed with Bruker’s matrix solvent solution.

Once the inactivation protocol was selected, the aim was to prove that while using the solvent inactivation method, reliable spectra can still be obtained to identify mycobacteria to the species level. When the mycobacterial inactivation and protein extraction (MIPE) protocol was used with acetone as an organic solvent for disrupting mycolic acid-containing cell walls, the best spectra were produced compared to other commonly used compounds including ethanol and Tween-20. To assess the effect of the solvent inactivation method on MALDI-TOF spectral quality, spectra of isolates with different exposure times were analyzed. Spectral analyses from isolates exposed for 20 min produced the best logarithmic scores approximating 1.98 [Figure 1]. Furthermore, it was also noticed that there is a window in which the spectra are highly accurate between 20 and 30 min exposure time to solvent killing. In this time window, there was the best peak sharpness and peak to noise ratio. After 30 min in contact with the solvent, the spectra started to deteriorate as shown by decreasing logarithmic scores [Figure 1].

It was noticed that the MIPE protocol was difficult to follow from MGIT culture media. MGIT medium produces less biomass density than LJ media, resulting in loss of the pellet once washed in deionized water. However, the wash step is needed to further clear any remains of acetone in the bacterial protein concentrate. For isolates obtained from MGIT media, reduction of the water volume added in the MIPE protocol to 100 µl, proved to be sufficient to clean the residual acetone from the bacterial biomass.

When identifying clinical isolates with the MIPE protocol, good results were obtained with isolates grown on LJ pyruvate media, 93.9% (31/33) isolates identified correctly compared with sequencing results. Using the MIPE protocol to identify the isolates from MGIT media produced acceptable results, accurately identifying 89% (26/29) of the mycobacterial isolates as compared to sequencing. Out of the 36 isolates included in
In this study, three cultures were contaminated and three isolates took longer than expected to grow. One *Mycobacterium interjectum* isolate did not yield recognizable peaks during MALDI-TOF analyses; two isolates were misidentified; one due to contamination of the subculture (*Mycobacterium kubicae*) and one due to a misidentification at the species level (*M. chelonae*). *Mycobacterium gordonea* cultured on LJ media were nonspecifically identified as *Mycobacterium* sp. nontuberculosis in contrast with 16S ribosomal RNA results. Furthermore, it was noted that MALDI-TOF spectrometry could not differentiate mycobacterial complexes such as *M. abscessus* complex and *Mycobacterium avium*/intracellulare complex.

### Table 1: Mycobactericidal activity of commercially available matrix solvent solution for control strains at various exposure intervals

| Control isolate and test conditions | Culture Inactivation time interval (min) |
|-----------------------------------|----------------------------------------|
|                                   | 0           | 10          | 20          | 30          | 60          |
| *M. abscessus* NCTC 13031 solvent solution | No growth   | No growth   | No growth   | No growth   | No growth   |
| *M. abscessus* NCTC 13031 Growth control (de-ionized water) | Growth 4 days* | Growth 4 days* | Growth 4 days* | Growth 4 days* | Growth 4 days* |
| *M. avium* ATCC 700898 solvent solution | No growth   | No growth   | No growth   | No growth   | No growth   |
| *M. avium* ATCC 700898 Growth control (de-ionized water) | Growth 28 days MGIT/14 days LJ | Growth 35 days MGIT/14 days LJ | Growth 7 days MGIT/14 days LJ | Growth 28 days MGIT/14 days LJ | Growth 28 days MGIT/14 days LJ |
| *M. tuberculosis* H37RV solvent solution | No growth   | No growth   | No growth   | No growth   | No growth   |
| *M. tuberculosis* H37RV Growth control (de-ionized water) | Growth 7 days MGIT/7 days LJ | Growth 7 days MGIT/7 days LJ | Growth 7 days MGIT/7 days LJ | Growth 7 days MGIT/7 days LJ | Growth 7 days MGIT/7 days LJ |

* Rapid-growing mycobacterial strain exhibiting growth on horse blood agar, LJ, and MGIT media. MGIT: Mycobacteria growth indicator tube, LJ: Lowenstein Jensen media, Solvent: Bruker Daltonics (50% acetonitrile, 2.5% trifluoroacetic acid, 47.5% water), *M. abscessus*: *Mycobacterium abscessus*, *M. avium*: *Mycobacterium avium*, *M. tuberculosis*: *Mycobacterium tuberculosis*

### Figure 1: Matrix-assisted laser desorption ionization time-of-flight mass spectromograms depicting results obtained with *Mycobacterium abscessus* at four different inactivation time intervals utilizing the mycobacterial inactivation protein extraction protocol. (a) 10 min, logarithmic score = 1.928; (b) 20 min, logarithmic score = 1.987; (c) 30 min, logarithmic score = 1.844; (d) 60 min, logarithmic score = 1.658
**Conclusion**

Despite demonstrating that commercial mass spectrometry can identify mycobacteria some improvements are needed to optimize the characterization of such isolates. The MIPE protocol produces a high-quality protein extract with improved isolate identification without compromising result turn-around-times or laboratory safety. Moreover, the addition of in-house libraries might help identification of local mycobacterial strains, decreasing nonspecific results and the need for further costly molecular identification.

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**Conflicts of interest**

There are no conflicts of interest.

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