A novel liquid media mycobacteria extraction method for MALDI-TOF MS identification using VITEK® MS

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ABSTRACT

A low-cost identification method that can be performed directly from a positive liquid medium culture is needed for the diagnosis of mycobacterial infections. Here, we describe a novel, cost-effective, and validated method that allows for direct and rapid identification of mycobacteria from a positive liquid culture using VITEK® MS with a total process duration under 45 min. From a liquid mycobacteria culture a 3.0 mL aliquot is removed 24–72 h post positivity and centrifuged to create a pellet. After decanting, the tube is blotted dry, the pellet is re-suspended in 0.5 mL of 70% ethanol and then transferred into a 2.0 mL tube containing glass beads. Mycobacteria are disrupted mechanically followed by a 10 min. incubation at room temperature to complete inactivation. Inactivated material is pelleted by centrifugation and then re-suspended in 10 μL of 70% formic acid and 10 μL of acetonitrile. After centrifugation, 1 μL of supernatant (protein extract) is deposited onto target slide, allowed to dry, and then 1 μL CHCA matrix is added.

A seeded study was conducted to demonstrate the reliability of the method, a total of 251 culture samples obtained from automated culture systems (BacT/ALERT® MP bottles, BACTEC MGIT™ 960 tubes, and VersaTREK® Myco bottles), were tested and resulted in 98.8% correct identification. Reproducibility was shown by testing three organisms across three reagent lots, between four laboratory technicians, over the course of five days for three liquid media systems resulting in a total of 180 deposits with an overall correct identification of 98.9% with the remaining results giving no identification. Additional studies were performed including comparison of different mechanical disruption techniques, stability of frozen extracts, and stability of slide deposits to allow for flexibility in a routine clinical workflow.

The described method proved to be safe while providing consistent and reproducible results for different species of mycobacteria and is compatible with the three most widely used liquid media medium detection systems.

1. Introduction

Mycobacteria infections are a significant threat to worldwide public health. In 2015, an estimated 1/3 of the world's population was estimated to be infected with *Mycobacterium tuberculosis*, and tuberculosis claimed the lives of an estimated 1.4 million people (World Health Organization, 2016). Non-tuberculous mycobacteria (NTMs) are increasingly recognized as mostly opportunistic pathogens of humans with the most frequent disease manifestation being chronic pulmonary infection (Buckwalter et al., 2015). The incidence of infection with NTM is rising due to an increased immunocompromised population (Marras et al., 2007). Alongside the rise in NTM infections, the number of mycobacteria species being recognized has increased over recent decades, which can largely be attributed to advances in genotypic analysis techniques (Tortoli, 2003).

Traditional identification of *Mycobacterium* spp. has been based on phenotypic traits and biochemical methods performed on solid media (Giger, 2011). These tests are simple but laborious, requiring long incubation periods, and are often not reliable (Buckwalter et al., 2015). Detection of *Mycobacterium* is accelerated by use of liquid medium detection systems which combine growth in broth with an automated instrument for detecting positive cultures (Kehrmann et al., 2015; Katila et al., 2000). The most widely used systems include BACTEC MGIT™ 960 tubes (MGIT) (Becton Dickenson Microbiology Systems, Sparks, MD), BacT/ALERT® MP bottles (MP) (bioMérieux, Durham, NC), and VersaTREK® Myco bottles (VersaTREK) (Thermo Fisher Scientific, Cleveland, OH).

The current gold standard for identifying mycobacteria to the species level is DNA gene sequencing targeting 16S rRNA, *rpoB*, *secA*, or *hsp65* (Hall et al., 2003). DNA sequencing improves the turnaround

time but is costly, labor intensive, requires specific equipment and a high level of expertise. Other molecular methods such as DNA probes provide fast and reproducible results for the more common mycobacteria species such as *M. tuberculosis* complex, *M. avium-M. intracellulare* complex (MAIC), *M. kansasii*, and *M. gordonae*. However, DNA probes are costly, lack specificity for less common *Mycobacterium* species probes, and often result in misidentifications (Tortoli et al., 2010; Wilson, 2013). An accurate, low-cost, and rapid identification method that can be performed directly from a positive liquid media culture is needed for the diagnosis of mycobacterial infections.

Matrix-assisted laser desorption/ionization time-of-flight mass-spectrometry (MALDI-TOF MS) is gaining wide acceptance as a rapid, accurate, and cost effective tool for identification of microorganisms in clinical laboratories (Ling et al., 2014; Patel, 2013; van Belkum et al., 2012). However, existing liquid extraction methods do not obtain a high rate of identification, are cumbersome, and require many processing steps, including heat inactivation (Buckwalter et al., 2015; Tortoli et al., 2010; Lotz et al., 2010; Tudó et al., 2015; Balada-Llasat et al., 2013; Mather et al., 2014; Wilen et al., 2015; El Khéchine et al., 2011). Moreover, identifying mycobacteria cultured from liquid media is difficult due to the low levels of biomass available from the positive sample as well as the potential interference from liquid media components (Lotz et al., 2010).

The VITEK® MS V3 with knowledgebase 3.0 is a database capable of identifying 49 *Mycobacterium* spp. or complexes encompassing over 2000 spectra from 494 strains (Girard et al., 2016). Here, we present a cost-effective validated method that allows for direct and rapid identification of mycobacteria from a positive liquid culture using VITEK® MS with total process duration under 45 min (Mediavilla-Gradolph et al., 2015; Tran et al., 2015). The method is designed to be safe while providing consistent and reproducible results for different species of mycobacteria and is compatible with the three most widely used liquid medium detection systems. Additionally there is a kit commercially available that includes the necessary components for performing the method (bioMérieux VITEK® MS *Mycobacterium/Nocardia* extraction kits) (*VITEK ® MS Mycobacterium/NocardiaVITEK (R) MS Mycobacterium/Nocardia reagent kit/ Vitek MS (r) liquid MYCO supplemental kit [Package Insert]*, 2015).

2. Materials and methods

2.1. Mycobacterium isolates

Clinically relevant mycobacteria species (rapid and slow growers) were selected for all studies (Clinical and Laboratory Standards Institute, 2008). Mycobacteria strains used in the studies are listed in Table 1. All strains were identified by 16S rDNA or *rpoB* prior to testing. Isolates were cultured from frozen stocks onto Lowenstein-Jenson slants or 7H11 plates prior to inoculation into MP, MGIT, and VersaTREK bottles containing the system-specific nutrient and antimicrobial supplements, at consistent inoculum of 5×10^5 CFU per bottle/tube.

2.2. Mycobacterium liquid media method

Automated liquid medium cultures grown in MP, MGIT, and or VersaTREK bottles were incubated until determined positive by the detection system. After 24–72 h post-positivity, a 3.0 mL aliquot was collected into a 5.0 mL frustoconical tube and centrifuged for 10 min at $3000 \times g$ to create a pellet. The supernatant was decanted and tube lip was blotted dry to remove residual medium. The pellet was re-suspended in 0.5 mL of 70% ethanol and transferred into a 2.0 mL tube containing 0.5 mm glass beads. Mechanical disruption was performed using either a BeadBeater or vortex with a horizontal tube adapter followed by a 10 min incubation in the same tube with ethanol at room temperature to complete sample inactivation. The supernatant was transferred to a 2.0 mL tube and centrifuged for 2 min at $14,000 \times g$.

Table 1
Test panel.

Microorganism	Strain #
M. abscessus	ATCC 19977, 03-337-31313[a], A035[a], A036[a]
M. africanum	NLA001300795, NLA001300953
M. bovis	C8131
M. chelonae	ATCC 35749
M. avium	ATCC 25291, C569, C1154, W111, W112
M. fortuitum	ATCC 6841, A038, A043
M. gordonae	ATCC 35756
M. intracellulare	ATCC 13209, ATCC 13950, C1152, C644, W106, W107, W110,
M. kansasii	ATCC12478, C557, W075, W076, W077, L078
M. lentiflavum	Mayo4[a], Mayo5[a], Mayo6[a], Mayo8[a], L112[a], L144[a]
M. marinum	L134[a]
M. malmoense	ATCC 29571
M. mucogenicum	04-295-31352[a], 04-304-30502[a], A073[a]
M. peregrinum	ATCC 14467, L040[a]
M. scrofulaceum	ATCC 19981, C2091, C1165, L105[a], MS12[a]
M. smegmatis	ATCC 19420 ATCC 19979, A064[a]
M. szulgai	ATCC 35799
M. tuberculosis	ATCC 25177, ATCC 35822, C2663, C2677, S02[a], S07[a], PT-15[a]
M. xenopi	ATCC 19250
M. immunogenum	W164[a]

[a] Clinical acquired strains.

Ethanol was removed and the pellet was re-suspended with 10 μL of 70% formic acid followed by 10 μL of acetonitrile for protein extraction. The 2.0 mL tube was centrifuged for 2 min at $14,000 \times g$ to pellet residual cell debris, 1 μL of supernatant (protein extract) was transferred onto target slide, allowed to dry, and then 1 μL CHCA matrix was added to complete the process (Fig. 1). Results were analyzed using VITEK® MS V3 with knowledgebase 3.0. Using this described protocol the following studies were conducted to challenge the method.

2.3. Protein extraction and identification from different media types

In order to evaluate the reliability of the method, a seeded study of 251 samples containing 33 strains representing 9 clinically relevant *Mycobacterium* species were conducted using MP bottles, MGIT tubes, and VersaTREK bottles. Positive samples were extracted and identified following the method described in Fig. 1.

2.4. Reproducibility

Reproducibility and robustness of the method were examined by seeding three rapid-growing mycobacteria into MP bottles, MGIT tubes, and VersaTREK bottles. Protein extracts were prepared by four technicians from two bottles/tubes of each organism over five separate days (total of 90 extractions and 180 deposits) using three different lots of bioMérieux VITEK® MS *Mycobacterium/Nocardia* extraction kits (*VITEK (R) MS Mycobacterium/Nocardia reagent kit/ Vitek MS (R) liquid MYCO supplemental kit [Package Insert]*, 2015).

2.5. Mechanical disruption evaluation

Once reliability and robustness of the method were established, flexibility of mechanical disruption was evaluated by comparing two options in parallel. The comparison examined 116 mycobacteria samples from 29 strains seeded into MP bottles and MGIT tubes inactivated using either a horizontal vortex (15 min) or a BeadBeater (5 min).

2.6. Extract and slide stability

Two studies were conducted to establish extract and deposit slide stability to ensure the method could support transport of extracts from satellite to reference laboratories. Based on biomass at positivity of the