MALDI-TOF mass spectrometry and high-consequence bacteria: safety and stability of biothreat bacterial sample testing in clinical diagnostic laboratories

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Abstract

We considered the application of MALDI-TOF mass spectrometry for BSL-3 bacterial diagnostics, with a focus on the biosafety of live-culture direct-colony testing and the stability of stored extracts. Biosafety level 2 (BSL-2) bacterial species were used as surrogates for BSL-3 high-consequence pathogens in all live-culture MALDI-TOF experiments. Viable BSL-2 bacteria were isolated from MALDI-TOF mass spectrometry target plates after ‘direct-colony’ and ‘on-plate’ extraction testing, suggesting that the matrix chemicals alone cannot be considered sufficient to inactivate bacterial culture and spores in all samples. Sampling of the instrument interior after direct-colony analysis did not recover viable organisms, suggesting that any potential risks to the laboratory technician are associated with preparation of the MALDI-TOF target plate before or after testing. Secondly, a long-term stability study (3 years) of stored MALDI-TOF extracts showed that match scores can decrease below the threshold for reliable species identification (<1.7), which has implications for proficiency test panel item storage and distribution.

Clinical diagnostic laboratories have widely adopted matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS) systems for bacterial identification [1–3]. As part of a routine clinical laboratory workflow in hospitals, live bacterial culture is applied directly to the MALDI-TOF MS target plate as a thin smear and overlaid with a matrix solution [3]. This rapid, high-throughput ‘direct-colony’ (or ‘direct smear’) method is often performed on biosafety level 2 (BSL-2) laboratory benches and avoids the use of a more laborious full chemical extraction (with ethanol–formic acid) in a biological safety cabinet (BSC) [4, 5]. As MALDI-TOF MS systems are neither installed nor serviced in BSL-3 containment laboratories [6], exposures in BSL-2 laboratory areas are an unfortunate consequence following MALDI-TOF MS-based identification of rarely observed, yet highly pathogenic bacteria such as Francisella tularensis [7] and Burkholderia pseudomallei [8]. Recent issues surrounding inactivation of MALDI-TOF extractions of BSL-3 bacteria at a major US laboratory have been widely publicized, and this incident has drawn attention to biosafety concerns surrounding these samples [9]. Notwithstanding the potential risk of exposure from insufficient sample treatment, there also remains a risk of unknown exposure and delay of clinical diagnosis if a laboratory does not have inclusive MALDI-TOF mass spectral databases to indicate the potential presence of a biothreat bacterial agent after analysis [10–12], as was discovered at New York City laboratories with isolates of BSL-3 agent Brucella melitensis [13]. As our laboratory group focuses on BSL-3 high-consequence bacterial pathogens of increased biosafety and biosecurity risk, we routinely review aspects of testing that have a direct impact on safety, and we have performed several verification and validation studies in this area. In this short communication, we report the isolation of viable bacteria (using BSL-2 near-neighbour species as surrogates to BSL-3 ‘security-sensitive biological agents’, SSBA) after direct-colony application of live cultures to MALDI-TOF MS target plates, but we found no viable organisms upon sampling of the mass spectrometer instrument interior. Separately, we assessed the long-term stability of full chemical extractions for the MALDI-TOF MS Biotype identification of BSL-3 and near-neighbour bacterial species, which has implications for MALDI-TOF MS proficiency test panel designers and laboratory participants.

All chemicals and reagents were obtained from Sigma-Aldrich (Oakville, ON, Canada), unless stated otherwise.
All BSL-2 bacterial cultures used in the viability study (Bacillus cereus and Bacillus thuringiensis, n=5; Oligella urethralis, n=3; Yersinia pseudotuberculosis, n=5; Francisella philomiragia, n=5; Burkholderia thailandensis, n=1) were previously identified by genetic methods as part of an established workflow at the National Microbiology Laboratory (Winnipeg, Canada) and full chemical extractions with ethanol–formic acid–acetonitrile were performed as previously described [12]. Bacteria were cultured on 5% sheep blood agar (SBA) at 37°C in 5% CO₂ for 24 h. Spores were prepared from a 1 L Bacillus cereus ATCC 14579 liquid culture of 1/10 Columbia broth with 0.1 mM MnSO₄, incubated at 37°C for 96 h on an orbital shaker (150 r.p.m.). The spore culture was centrifuged, washed in sterile deionized water, re-suspended in 10 ml of deionized water and placed at 80°C for 10 min to kill vegetative

Fig. 1. Results from long-term stability testing of MALDI-TOF bacterial extracts on the Bruker Biotyper/Microflex system. Data from three sets of extract experiments are presented: BSL-2 and BSL-3 bacterial pathogen extracts (a) stored at −20°C and tested over 3 years, (b) stored at −20°C and tested over 6 months and (c) stored at −20 and −80°C, which were tested at shorter intervals over 3 months. All Biotyper match scores are the mean of four analysed sample spots for each individual bacterial extract. A Biotyper species-level identification is a match score of >2.0, and a genus-level identification is >1.7. Scores below 1.7 are unreliable for identification. For consistency, results were searched against mass spectral profile (MSP) entries for each species in the Bruker database library kept consistent throughout the study. †The Bruker libraries contain only B. melitensis profiles. An extract of B. cereus (1c) did match to a local MSP database (data not shown) with a species-level identification score (>2.0), but had only an initial genus-level against the designated Bruker database profile; a Bruker database was used throughout the study for consistency.
cells. Spores were enumerated by plate counts. As a test of the direct-colony method, we used the methodology of Cunningham and Patel [14] in applying bacterial culture directly to sterile steel discs (SPI Supplier, West Chester, PA, USA) as a routine thin film of culture, or as a larger excess amount of bacterial culture. Bacterial samples were covered in 1–1.5 µl α-cyano-4-hydroxycinnamic acid (CHCA) matrix solution (50% acetonitrile: 2.5% trifluoroacetic acid), dried, inoculated into a 5 ml brain heart infusion (BHI) broth tube for incubation at 37°C and checked for growth daily over 7 days. Any observed turbidity in BHI tubes was sub-cultured onto SBA on the same day, extracted, and analysed on the Bruker Microflex (Biotyper) as previously described [12, 15] to confirm the bacterial culture identity. We performed serial dilution plating to determine the bacterial sample application per direct colony spot: *Bacillus* spp. (1.7×10⁷/routine spot), *O. urethra- lis* (4.2×10⁶/routine spot), *Y. pseudotuberculosis* (3.5×10⁷/routine spot), *F. philomiragia* (3×10⁷/routine spot). ‘Excess culture’ spots were applied at approximately 4–6x of the routine spots. *B. cereus* spores were applied at 1×10⁷ or 5.4×10⁶ c.f.u. per spot. The positive control was a bacterial culture applied to a steel disc without matrix overlay, and negative controls were an uninoculated disc or a disc with matrix-only applied to the surface. We controlled for the possible inhibitory effect of chemicals by applying the MALDI-TOF reagents (formic acid, acetonitrile) directly to BHI broth tubes, along with the positive control bacterial culture on a steel disc.

To assess the viability of BSL-2 bacterial cultures and spores applied directly to target plates analysed in the MALDI-TOF MS instrument, as is routinely done in clinical diagnostic laboratories, live bacterial cultures were applied to the MSP-96 MALDI target plate (Bruker Daltonics, East Milton, ON, Canada) in a routine thin layer, overlaid with 1–1.5 µl of matrix solution and analysed on the Bruker Microflex (Biotyper). Estimated sample inoculum was as above for the routine direct colony application. Spores were applied at between 5.4×10⁶ and 2.2×10⁷ c.f.u. per spot, and overlaid with 1–1.5 µl matrix solution. The target plate was then removed from the MALDI-TOF MS instrument and placed in a BSC. Individually spotted samples were physically scraped off with a sterile loop or re-suspended in sterile deionized water (5 µl), and were then inoculated into BHI broth. We also tested ‘on-plate’ treatment of live BSL-2 bacterial culture, by applying 2 µl of 70% formic acid to culture on a MALDI-TOF target plate and inoculating into BHI broth for 7 days. To determine whether direct-colony analysis can contaminate the mass spectrometer instrument, we ran a MALDI-TOF target plate spotted with live culture direct-colony samples (*B. cereus*, *Y. pseudotuberculosis*, *B. cereus* spores) and subsequently sampled the instrument (during routine preventative maintenance service) with sterile swabs pre-wet with PBS, in the following locations: vacuum pump, target plate tray, exhaust fan, flight tube, electronics shelf and ion source. Swabs were used to inoculate 5 ml BHI broth and SBA agar, which were checked for growth daily over a 7-day incubation at 37°C.
Table 1. Viability (growth) of bacterial cultures applied to steel discs and covered with CHCA matrix solution, prepared as a mimic of live culture direct-colony testing on MALDI-TOF MS target plates

<table>
<thead>
<tr>
<th>Species</th>
<th>Surrogate for biothreat</th>
<th>Sample</th>
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<tr>
<td></td>
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<td>Routine direct-colony</td>
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<td></td>
<td></td>
<td>Culture-only (positive control)</td>
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<tr>
<td>Yersinia pseudotuberculosis</td>
<td>Yersinia pestis</td>
<td>5</td>
</tr>
<tr>
<td>Bacillus cereus complex spores</td>
<td>Bacillus anthracis</td>
<td>4</td>
</tr>
<tr>
<td>Francisella tularensis</td>
<td>Francisella philomiragia</td>
<td>5</td>
</tr>
<tr>
<td>Oligella urethralis</td>
<td>Brucella spp.</td>
<td>3</td>
</tr>
<tr>
<td>Burkholderia thailandensis</td>
<td>Burkholderia mallei</td>
<td>1</td>
</tr>
<tr>
<td>Burkholderia pseudomallei</td>
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*See text for number of spores applied per sample.
†See text for bacterial sample input amounts.

To determine the long-term stability of MALDI-TOF extracts, full chemical extractions (using ethanol–formic acid–acetonitrile) were performed on selected sets of BSL-2 and BSL-3 bacterial pathogens (listed in Fig. 1). All BSL-3 bacterial extractions were confirmed as non-viable before removal from the CL-3 containment laboratory, as per previously described methods [12]. Bacterial extracts were aliquoted, sealed with parafilm and stored in Sarstedt (Nürnberg, Germany) screw-cap tubes (with O-ring) at −20 °C, with a separate extract subset stored at −80 °C. At predetermined time points (15, 30, 60 and 90 days; 3 and 6 months; 2 and 3 years), single-use extract aliquots were removed from freezer storage and tested on the Bruker Microflex system with Biotyper software (ver 3.1, build 66). Each Biotyper run was calibrated with BTS (Bacterial test standard, Bruker). Biotyper match scores were recorded as the mean of four individual spots of the same extract, compared directly to a single mass spectral profile (MSP) species entry in the Bruker Biotyper libraries (Taxonomy, n=5989 MSPs; or Security-Restricted ‘SR’, n=123 MSPs), which was kept constant throughout the study.

Results showed that matrix solution inactivated bacterial culture applied as a routine thin layer on steel discs (Table 1), but that matrix was not sufficient to inactivate a larger amount of bacterial culture (Bacillus spp., Y. pseudotuberculosis and B. thailandensis) or spores. The latter mimics a situation when laboratory technicians apply an excess amount of live bacteria to a MALDI-TOF target plate and the matrix solution cannot entirely cover the bacterial culture. The physical removal of the applied direct-colony sample (by scraping or re-suspension of a thin-layer direct colony spot off a target plate into broth) found viable organisms with Y. pseudotuberculosis (sample re-suspension), Bacillus spp. (cells and spores, both scraped off and re-suspended) and B. thailandensis (scraped) from a plate run in the MALDI-TOF instrument. MALDI-TOF reagents (matrix, formic acid) did not have inhibitory effects on bacterial broth culture growth, as the liquid cultures grew within 1–2 days of inoculation. The ‘on-plate’ extraction of live bacterial cultures directly on a MALDI target plate with 70 % formic acid inactivated Gram-negative pathogens, but Bacillus spp. (n=4/5 cultures) remained viable. Based on these results, the matrix solution or on-plate extraction alone cannot be considered sufficient to inactivate bacterial culture and spores applied to MALDI-TOF target plates in all cases, as bacteria may remain viable before and after analysis.

After running a MALDI-TOF target plate of direct-colony applied cultures and spore samples, we sampled internal MS instrument surfaces to determine the risk of aerosolization of viable bacteria. To our knowledge, this is the first report of testing the internal surfaces of a MALDI-TOF MS instrument for viable bacteria. As no viable bacteria were isolated from the instrument itself, any potential risks to the laboratory technician would likely be associated with preparation of the MALDI-TOF target plate before and/or after testing.
in the MS instrument. Full chemical extraction has been suggested as the most safe and efficient method for MALDI-TOF MS analysis of highly virulent microorganisms, with filtration for spore-formers an important step for sample inactivation [6, 12, 16, 17]. However, this extraction method is not efficiently integrated into a hospital’s high-throughput workflow, as a BSL-3 agent would be rarely observed and MALDI-TOF plate preparation on a BSL-2 laboratory bench-top could potentially expose the laboratory technician to a high-consequence pathogen.

In addition to viability studies, we also routinely assess the long-term stability of reagents and samples in our lab for proficiency panels. By ensuring the stability of PT panel items, the provider can comply with accreditation standards such as those defined by ISO 17043 [18]. If PT panels contain BSL-3 pathogen extracts, they must be confirmed as being inactivated before distribution to BSL-2 public health laboratories, which requires advanced preparation time for viability testing and possibly necessitating the storage of extracts. Results from our MALDI-TOF extract stability study show a general reduction in Biotyper match scores below reliable species identification levels (<1.7) over longer-term storage at −20°C (Fig. 1a), although the decrease in match scores varied among the type of species and strain of bacteria (Fig. 1b). We observed lower-intensity MS peak profiles in the extracts stored for longer periods (6 months, 2–3 years), but our analysis did not assess the potential formation of satellite peaks from formylation of proteins, which may have an impact on the final Biotyper software identification accuracy [6]. Extracts of B. anthracis displayed the best long-term stability over a year, but dropped below reliable identification at 3 years in storage, as did all bacterial extracts tested at that time point (Fig. 1a). Over a 90-day period (Fig. 1c), storage of extracts at −80°C showed stable Biotyper match scores at above the species-level identification threshold (>2.0) versus storage at −20°C, which had reduced B. cereus match scores under the ‘not reliable’ identification level (<1.7). We attempted using alternate storage tubes (Protein ‘Lo-Bind’ tubes; Eppendorf, Hamburg, Germany), but observed that spectra from these extracts were unusable after long-term storage.

In summary, this study demonstrated the presence of viable bacteria on MALDI-TOF target plates after direct-colony application, but there was no evidence for dispersal of live bacteria from the target plate into the mass spectrometer instrument, or up through the flight tube and exhaust. This likely places the risk of exposure at the point of MALDI-TOF plate preparation and handling. These results may have biosafety implications for clinical diagnostic laboratories which employ the live culture/direct-colony method with MALDI-TOF MS for bacterial identification. Secondly, analysis of MALDI-TOF extract stability showed that Biotyper match scores can decrease below the threshold for reliable species identification (<1.7) after long-term storage. Laboratories involved in PT panel schemes, whether as the provider or the participant, may wish to review procedures related to storage and distribution of MALDI-TOF reagents and PT items in their quality systems. We will continue to work with our public health laboratory partners to improve MALDI-TOF MS-based diagnostics of high-consequence bacteria, specifically by addressing deficiencies in database quality and biosafety practices.

Funding information
Funding for this work is through the Government of Canada; no specific grant was received from any funding agency.

Acknowledgements
Thank you to the staff of the National Microbiology Laboratory, including Dr Andrea D. Tyler and the Bioforensics Assay Development and Diagnostics (BAADD) section, the NML Media core service and NML’s Mass Spectrometry & Proteomics core service.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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