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A comparison of the HAIN Genotype CM reverse hybridisation assay with the Bruker MicroFlex LT MALDI-TOF mass spectrometer for identification of clinically relevant mycobacterial species

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The *Mycobacterium* genus encompasses more than 160 species [1] and is frequently divided into two groups: *Mycobacterium tuberculosis* complex (MTC) and Nontuberculous Mycobacteria (NTM). The pathogenicity of MTC is well documented and the complex is comprised of *M. tuberculosis, M. bovis, M. africanum, M. microti* and *M. canetti* species. Many NTM are considered environmental contaminants and their role in human disease is not well understood [2]. There are exceptions, most notably, *Mycobacterium avium* complex isolates, *Mycobacterium fortuitum* and *Mycobacterium abscessus* [3,4]. These mycobacteria are frequently isolated from immunocompromised patients and are increasingly being isolated from immunocompetent patients. This accentuates the need for systems that allow timely, reliable identification of NTM and MTC isolates. Rapid identification of mycobacteria facilitates early and appropriate therapeutic and infection control measures. For many years, the mainstay of mycobacterial identification rested with phenotypic, biochemical and molecular methods [5]. The Genotype CM (HAIN, system, can identify MTC, and some NTM species: *M. avium, M. abscessus, M. chelonae, M. fortuitum, M. gordonae, M. intracellulare, M. interjectum, M. kansasii, M. malmoense, M. marinum/M. ulcerans, M. peregrium, M. xenopi* and a less specific identification listed as *Mycobacterium* species or high GC Gram-positive bacterium. These species are considered the most commonly encountered clinically relevant NTM [6], but for more infrequently encountered mycobacteria, an additional assay for alternative species would need to be used. A difficulty when using this technology is in the interpretation of the staining pattern, as many of the species listed may only have one unique band. The approach when using MALDI-tof MS is that an identification is provided with a log score that is considered to give an indication of confidence in the strain identification. The use of the HAIN Genotype CM assay became the established method for primary identification in CUH following an extensive comparison with the previous assay [7] used in the laboratory and has proven to be a reliable method of identification, as supported by external quality assurance scheme results concordance with expected results.

In recent years, traditional methods of identification have been replaced by sequencing and proteomic methods; namely Matrix-Assisted Laser-Desorption/Ionisation time-of-flight (MALDI-tof) mass spectrometry (MS). MALDI-tof MS may offer an appropriate replacement for traditional methods in the routine diagnostic mycobacteriology laboratory. MALDI-tof MS has shown efficacy as a mycobacterial identification method [8–10]. Its use has, however, been accompanied by reports of ineffective extraction methods and laboriousness [11,12]. We hypothesised that MALDI-tof MS could be a potential replacement for the HAIN assay for MTC and NTM.

The mycobacteria tested in the current study were from patient samples (N = 99) and from the United Kingdom National External Quality Assessment Scheme (UKNEQAS) distributions (N = 18), respectively. All GenoType identifications of UKNEQAS isolates were consistent with expected results. All MTC isolates were referred to the Irish Mycobacterial Reference Laboratory (St James' Hospital, Dublin, Ireland) for confirmation of Cork University Hospital laboratory identification. Following storage, and prior to testing with the MALDI-tof, all isolates were Gram-stained to ensure the absence of contaminating bacteria, and fluorescent-stained (Auramine-O) to confirm acid fastness. The identification of mycobacteria using the GenoType CM assay was conducted in accordance with the manufacturer's instruction [7].

For the identification by MALDI-tof MS, all mycobacteria were recovered from frozen storage into MP BacT/Alert (Middlebrook 7H10) mycobacteria bottles and

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This work represents an advance in biomedical science because it demonstrates the efficacy of MALDI-TOF MS for the presence of mycobacteria.

incubated in the BacT/Alert system. When the bottles flagged positive, the mycobacterial biomass underwent an extraction protocol as described previously within a biosafety level three laboratory [13]. Briefly, 1.2 mL of biomass was centrifuged at 13,160 g for 5 min and the supernatant was decanted, 300 µL of deionised water was added to the tube and the culture was inactivated in a calibrated dry water bath at 95°C for 30 min. A total of 900 µL of ethyl alcohol (100%) was added and the tube was incubated at room temperature for 3–5 min. The samples were then vortexed at maximum for 15 s and centrifuged at 13,160 g for 15 min. The supernatant was discarded, 2 × 10 µL loopfuls of 0.5 mm zirconia/silica beads were added to each tube, which was followed by the addition of 10–50 µL (depending on biomass size) of pure acetonitrile (ACN). This was followed by vortexing at maximum speed for 15–20 s. The samples underwent sonication for 15 min. An equal volume of 70% formic acid (as ACN) was added to each sample followed by another brief vortexing step. The samples were then centrifuged at 13,160 g for 2 min. A 1 µL aliquot of supernatant was added to a MSP 96 target polished steel target plate (Bruker Daltronics, Bremen, Germany) and each sample was tested five-fold (technical replicates). Following drying, each extract was immediately overlaid with 1µl of α-Cyano-4-hydroxycinnamic acid matrix. Once the rough source vacuum was below 3.7 mbar, the spectra were acquired in a linear positive ion mode at a laser frequency of 60 hz across a mass/charge ratio within the range of 2–20 kDa using the MicroFlex LT mass spectrometer (Bruker Daltronics). For each spectrum, 240 laser shots in 40 shot steps from different areas of the spot were automatically accumulated and analysed. Where peaks were seen and not accumulated, these spots were analysed with manually acquired spectra (a minimum of 240). The spectra were analysed using the Mycobacterial Library (Bead Method) version 1µl (Bruker Daltronics). The best log-score of the five MALDI-tof replicate results for each isolate is recorded in Table 1. The result of MALDI-tof identification of the collection of isolates showed 97% accordance with those attained with the Genotype CM. There were no incorrect identifications by MALDI-tof and there were three isolates that failed to identify (through generating a log score of less than 1.7 when using version 1 of Bruker's Mycobacterial Library). The results are shown in Table 1.

Bruker has assigned levels of confidence for results depending on the log-score obtained for an isolate. For mycobacteria a log-score of 1.7 or greater is considered to be an acceptable identification and that a value of 2.0 or more is considered a high confidence identification [14]. Bruker has also recently reduced the log-scores assigned as high and low confidence levels for mycobacteria for the most recent versions of the mycobacterial library [15]. Only four of the mycobacteria isolates tested in the present study scored between 1.7 and 1.8 (one each of MTC, *M. xenopi, M. scrofulaceum* and *M. lentiflavum*). Overall, 66% of all MTC, 45% of NTM and 58% of all isolates tested had a log score of greater than 2.0. The log-score intervals for each species are shown in Table 2.

Table 1. Comparison of Genotype CM identification and MALDI-TOF MS identification results for *Mycobacterium tuberculosis* complex and nontuberculous *Mycobacterium* spp. isolates.

Genotype CM identification	Number of isolates	Number of concordant MALDI-TOF MS identifications i.e. a log score of >1.7 (%)
* *Mycobacterium tuberculosis* complex	73	72 (99)
Nontuberculosis *Mycobacterium* spp.	44	42 (95)
M. abscessus	8	8
M. avium	19	18
M. fortuitum	3	3
M. gordonae	2	2
M. intracellularae	4	4
M. malmoense	2	2
M. scrofulaceum	2	2
M. xenopi	3	2
^ *Mycobacterium* spp. *(M. lentiflavum)*	1	1
Total	117	114 (97)

*The MTC isolates were comprised of 66 *M. tuberculosis* isolates, 5 *M. bovis* BCG isolates, 1 *M. bovis* isolate and 1 *M. africanum* isolate.
^The *M. lentiflavum* was identified by the GenoType CM as *Mycobacterium* species (not provided as an option on CM strips). Identification of this isolate was provided by the Irish Mycobacterial Reference Laboratory, Dublin, Ireland.

There are many factors to consider when replacing a mycobacterial identification method in the laboratory with a new one, including capital costs, day-to-day running costs and staff training requirements. These factors are detailed in Table 3. Both Genotype CM and MALDI-tof MS require capital investment if the clinical laboratory is not already in possession of a PCR platform or MALDI-tof MS.

The timely identification of NTM bacteria is very important for choosing a suitable antimycobacterial therapy. The American Thoracic Society (ATS) has recognised the developing clinical importance of NTM infection and has described criteria to facilitate the diagnosis and treatment of pulmonary NTM disease [16]. It is important to note that some NTM are more likely to be resistant to the more common antimycobacterial agents. For instance, when choosing an empiric therapy for *M. abscessus* and *M. chelonae* infections, knowing the species is critical [17].

When MALDI-tof MS was compared directly to the HAIN Genotype CM assay, there were three isolates that failed to identify, thereby showing 97% concordance between the two systems. All three isolates had log scores of less than 1.7 and the result for one isolate was listed as having no peaks. This latter isolate, which had been identified as an MTC isolate by the HAIN system, was a particularly slow-growing isolate, whose failure to identify by MALDI-TOF may be attributable to a poor biomass. This problem has been alluded to

Table 2. Log-scores of MALDI-TOF MS identification results for *Mycobacterium tuberculosis* complex and nontuberculosis *Mycobacterium* spp. isolates.

MALDI-TOF MS identification	Number of isolates	Log-score >2.00 (%)	Log-score 1.7–1.99 (%)	No Identification: Log-score <1.7 (%)
Mycobacterium tuberculosis complex	73	48 (66)	24 (33)	1 (1)
Nontuberculosis *Mycobacterium* spp.	44	20 (45)	22 (50)	2 (5)
M. abscessus	8	6	2	0
M. avium	19	5	13	1
M. fortuitum	3	2	1	0
M. gordonae	2	1	1	0
M. intracellularae	4	3	1	0
M. malmoense	2	2	0	0
M. scrofulaceum	2	1	1	0
M. xenopi	3	0	2	1
M. lentiflavum	1	0	1	0
Total (%)	117	68 (58)	46 (39)	3 (3)

Table 3. A comparison of parameters affecting the clinical laboratory when using the HAIN Genotype CM and the Bruker MALDI-TOF MS as mycobacterial identification tools.

Parameter	Genotype CM	MALDI-TOF MS
Purity	Does not require mycobacteria to be pure	Requires pure growth of mycobacteria
Requirement for batching	Yes: Samples will be batched due to high running cost, unlikely to be run for a single sample	No: Single sample can be identified, low running cost
Capital cost	Moderate: May require acquisition of PCR and hybridisation instrumentation	High: However, many laboratories will have a MALDI-TOF in-situ and will only require access to the *Mycobacterium* database
Running cost	High, due to expense associated with amplification and commercial kit usage	Low, negligible day to day running costs (up to 25x less than the Genotype CM [8,20]
Staff training	Will require considerable staff training, particularly for new staff and those unfamiliar with PCR methodologies	Will require some staff training. The procedure is relatively straightforward
Hands-on time of staff	Requires substantial hands-on time of staff, particularly the preparation of master mix and addition of hybridisation reagents	Requires substantial hands-on time, however the time of preparation is considerably less (minimum of 1 h less hands-on time than Genotype assays)
Space requirements	May require four pieces of instrumentation. Instrument sizes vary depending on specification and through-put. The method may require three rooms, one for extraction, a clean room and another room post amplification	The Bruker Biotyper MALDI-TOF MS is a bench top instrument with an associated PC that occupies limited space.
Identification turn-around time	Long if batching is in place, i.e. in low prevalence regions	Can be substantially reduced as batching need not apply and negates need for referral of unusual NTMs for identification and associated delays associated with subculture
Culture independent identification	No	No
Number of mycobacteria that can be identified using this system	14 mycobacterial species and will indicate 'mycobacterial species' that will require Genotype AS testing or referral	149 mycobacterial species in Mycobacteria library v3.0 [15]
Can system differentiate between members of *Mycobacterium tuberculosis* complex?	No, but the Genotype MTBC assay can be used to speciate.	Not currently elucidated

previously [18]. In an attempt to overcome this, the mycobacterial biomass was allowed to settle at the top of the bottle prior to drawing, as previously described [19]. After centrifugation, the biomass pellet was observed and compared with a 50 µL volume of liquid. Where the biomass was visually less than this, another 1.2 mL aliquot of the settled mycobacterial biomass was drawn and centrifuged. The amount of acetonitrile and formic acid was also adjusted to match the volume of the mycobacterial biomass. This resulted in a low 'No Identification' or 'No Reliable Identification' rate of 3%. It has been shown previously that MALDI-tof MS was inferior for the identification of mycobacteria from primary liquid culture compared to a solid medium subculture [18]. The present study identified mycobacteria that were recovered from frozen storage in a liquid medium, this may have resulted in an increased biomass and a reduced time to positivity using a continuously monitored instrument compared to a primary mucous digested, decontaminated respiratory specimen for example. However, in the routine diagnostic mycobacteriology laboratory issues surrounding low biomass could be overcome through the steps outlined above and, if necessary, by extending incubation time of the failed identifications, particularly for very slow-growing mycobacteria. The identification of the three isolates that failed to identify by MALDI-tof MS was confirmed by a reference laboratory.

In an examination of other parameters relevant to the use of HAIN and MALDI-tof systems in the clinical laboratory as shown in Table 3, MALDI-tof offers advantages over Genotype CM, provided that the initial capital cost of the

MALDI-tof instrument is offset by high-throughput use. The main limitation in the use of MALDI-tof for mycobacterial identification currently lies in its inability to differentiate between members of the MTC and the requirement for isolate purity. Our study demonstrates the clinical utility of MALDI-tof MS for the identification of mycobacteria using a two-step cell disruption protocol. We suggest this can be used to facilitate the introduction, verification and implementation of MALDI-tof MS for the reliable identification of mycobacteria. Furthermore, we speculate that the implementation of MALDI-tof MS using these methods will help future-proof clinical laboratories against a background of increasing rates and varieties of NTM.

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