MALDI-TOF mass spectrometry: An emerging tool for unequivocal identification of non-fermenting Gram-negative bacilli

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Background & objectives: Matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) has been instrumental in revolutionizing microbiological identification, especially in high-throughput laboratories. It has enabled the identification of organisms like non-fermenting Gram-negative bacilli (NFGNB), which has been a challenging task using conventional methods alone. In this study an attempt was made to validate MALDI-TOF MS for the identification of clinical isolates of each of the three most common NFGNB, other than Pseudomonas spp., taking molecular methods as the gold standard.

Methods: One hundred and fifty clinical isolates of NFGNB, confirmed by molecular methods such as Acinetobacter baumannii [oxa-51 polymerase chain reaction (PCR)], Burkholderia cepacia complex (expanded multilocus sequence typing) and Stenotrophomonas maltophilia (species-specific PCR), were taken. Isolated colonies from fresh cultures of all 150 isolates were smeared onto ground steel plate, with and without formic acid extraction step. The identification was carried out using MALDI-TOF MS Biotyper database.

Results: A concordance of 100 and 73.33 per cent was found between the molecular techniques and MALDI-TOF MS system in the identification of these isolates up to genus and species levels, respectively. Using a cut-off of 1.9 for reliable identification, rate of species identification rose to 82.66 per cent. Principal component analysis dendrogram and cluster analysis further increased discrimination of isolates.

Interpretation & conclusions: Our findings showed MALDI-TOF MS-based identification of NFGNB as a good, robust method for high-throughput laboratories.

Key words Acinetobacter baumannii - Burkholderia cepacia complex - matrix-assisted laser desorption ionization time-of-flight-mass spectrometry - MLST - non-fermenting Gram-negative bacilli - Stenotrophomonas maltophilia

Non-fermenting Gram-negative bacilli (NFGNB) cause a multitude of severe and disabling infections. Though many varied pathogenic organisms come under the term NFGNB, the majority of serious infections are caused by Pseudomonas aeruginosa, Acinetobacter baumannii, Stenotrophomonas maltophilia and Burkholderia cepacia complex (Bcc). Unlike P. aeruginosa, the rest of the NFGNBs lack easily discernable phenotypic characteristics. Their contrasting susceptibilities, high intrinsic resistance and remarkable ability to develop resistance to essentially all commonly used antibiotics including
the anti-pseudomonal drugs limit the therapeutic options, causing a delay in patient management. Bec is intrinsically resistant to aminoglycosides and polymyxins while S. maltophilia is intrinsically resistant to aminoglycosides and commonly used carbapenems. A. baumannii is increasingly becoming resistant to the carbapenems, leaving polymyxins as the only option. Consequently, their correct identification is essential as no single drug is effective against all, which hinders the initiation of appropriate empirical treatment resulting in increased morbidity and mortality.

It takes several days for a routine laboratory to identify these organisms due to their inert biochemical activities and difficulty in interpretation of phenotypic characteristics. These analyses require expertise and are time-consuming and often give unreliable and overlapping results. The automated systems are not much discriminatory, and molecular methods are cost-and labour-intensive. Thus, a simple, rapid and reliable technique is the need of the hour. Matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-TOF MS) is emerging as an efficient, accurate and cost-effective alternative tool in microbial identification.

In this study the identification accuracy of MALDI-TOF MS was compared with standard molecular methods for identifying clinical isolates of NFGNB other than P. aeruginosa.

**Material & Methods**

The study was conducted in the department of Medical Microbiology, Post Graduate Institute of Medical Education and Research, Chandigarh, India, between August 2013 and October 2014. The isolates were obtained from clinical samples over the years (2005-2013) and were well preserved by stocking up in 20 per cent glycerol vials and reviving from time to time. These were isolated from various sources such as blood, endotracheal aspirates, cerebrospinal fluid, wounds and drain fluids.

It was noted that in literature most studies though have quoted 100 per cent genus identification for NFGNBs, but correct species identification was as low as 72 per cent also. Thus, we took the best possible result of 100 and 72 per cent given by the available data for species concordance, giving an average of 86 per cent with a margin of error of 14 per cent. Using the standard formula \((Z\text{-score})^2 \times SD \times (1-SD)/(\text{margin of error})^2\), the sample size was calculated to be 49 taking a confidence level of 95 per cent (corresponding to Z-score of 1.96), standard deviation of 0.5 and margin of error of 14 per cent. Hence, 150 clinical isolates of NFGNB (50 isolates each of A. baumannii, S. maltophilia and Bec) were included in the study.

**Criteria of selection of isolates:** All isolates that could be revived from stock cultures that gave pure colony growth and fulfilled the molecular identification for their respective organisms were included. Those isolates which did not revive in pure form, showed any contamination or were misidentified by molecular gold standards were excluded.

**Identification of isolates:** These organisms were presumptively identified by conventional techniques such as colony morphology and a set of standardized biochemical reactions. The identity was further confirmed using standard molecular techniques for each as, oxa-51 polymerase chain reaction (PCR) for confirming A. baumannii, species-specific PCR for S. maltophilia and expanded multilocus sequence typing (E-MLST) for Bec. In Bec isolates, identification was also carried out by subjecting the amplicon of recA-PCR to sequencing using Big Dye Terminator Cycle Sequencing Kit, version 3.1 (Applied Biosystems, USA). The nucleotide sequences were analyzed on ABI 3130 Genetic Analyzer (Applied Biosystems) and compared with sequences available on the internet (http://www.ncbi.nlm.nih.gov/BLAST/).

**MALDI-TOF MS:** The Microflex LT MALDI-TOF MS (Bruker Daltonics, Germany) with a 60-Hz nitrogen laser was used to analyze spectra over a mass range of 2000-20000 Da. All specimens were processed as per the manufacturer’s instructions. Pre-analytic preparation of samples was performed by using a sterile wooden tip to pick an isolated bacterial colony freshly grown on defined agar medium and then smearing a thin film in duplicate onto a ground steel MALDI biotarget 96 plate (Direct Transfer procedure). One of the microbial films was overlaid directly with 1.0 µl α-cyano-4-hydroxycinnamic acid (MALDI TOF HCCA) matrix solution while the other pair was first treated with 1 µl 100 per cent formic acid (FA) for on-plate extraction. The sample-matrix mixture was dried at room temperature and subsequently inserted into the system for data acquisition. A sum spectrum was acquired by summing the laser shots. Quality controls were internally calibrated using Escherichia coli DH5α supplied by Bruker Daltonics, following the same procedure. The data were processed automatically by the instrument software and the spectra were compared with reference libraries for bacterial identification matching.
Spectra were analyzed using MALDI Biotyper database version 3 (Bruker Daltonics, Germany). Manufacturer-recommended score cut-offs were used to determine genus level (1.7000 to 1.999) or species level (≥2.000) of the organism. A score of <1.7 was considered unreliable for genus identification. For this study, a Biotyper score cut-off of ≥2.0 was considered for reliable species identification when analyzing under automatic mode. Any isolate getting lower score was reanalyzed before labelling an isolate as ‘Not reliable identification’. The NFGNB were also analyzed taking a cut-off score of ≥1.9 for species-level identification, instead of ≥2.0 as also suggested by Ford and Burnham13. An attempt to harness the potential of MALDI-TOF MS as a typing technique was also carried out by creating main spectra for all the isolates and studying them by principal component analysis (PCA) dendrogram. The correlation distance from peaks measured by the software was used to construct dendrogram on the basis of ribonucleic protein profile of the isolates. In cases of misidentification, cluster analysis using Biotyper version 3 was also carried out.

Statistical analysis: Statistical analysis was performed using the Mann–Whitney Wilcoxon test with GraphPad software (GraphPad software Inc., California, USA).

Results

Acinetobacter baumannii: Oxa-51 PCR confirmed all 50 isolates presumptively identified as A. baumannii. Using manufacturer’s cut-offs, the concordance rate of MALDI-TOF MS with oxa-51 PCR for correct identification up to genus level was obtained in 100 per cent isolates, while higher discriminatory resolution up to species level was noted in 88 per cent (44/50). A four per cent increase in the rate of species identification was obtained when the cut-off of ≥1.9 was taken instead of ≥2. Direct transfer procedure could identify all the isolates, whereas two per cent remained unidentified with FA on-plate extraction method. There were no misidentifications (Table I). On PCA dendrogram, all 50 isolates fell into two distinct groups without any outliers (Fig. 1).

Stenotrophomonas maltophilia: All 50 isolates presumptively identified on the basis of biochemical reactions were confirmed to be S. maltophilia by species-specific PCR. The concordance rate of MALDI-TOF MS was 100 per cent for the genus and 50 per cent (25/50) for the correct species identification. An increase in species identification to 70 per cent (35/50) was noted when altered cut-off of ≥1.9 was considered. Four per cent of isolates remained unidentified with FA on-plate extraction method and four per cent remained unidentified with no direct transfer. No isolate was misidentified by MALDI-TOF (Table I). PCA dendrogram revealed two distinct groups with four clusters representing all the 50 isolates of S. maltophilia (Fig. 2).

| NFGNB               | Level of discrimination | Number of isolates (cut-off) |
|---------------------|-------------------------|-----------------------------|
|                     |                         | DT                          | DT + on-plate FA extraction | DT                          | DT + on-plate FA extraction |
| Acinetobacter baumannii (n=50) | To genus level | 50                          | 49                          | 50                          | 49                          |
|                     | To species level       | 44                          | 41                          | 46                          | 43                          |
|                     | Unidentified           | 0                           | 1                           | 0                           | 1                           |
|                     | Misidentified          | 0                           | 0                           | 0                           | 0                           |
| Stenotrophomonas maltophilia (n=50) | To genus level | 50                          | 48                          | 50                          | 50                          |
|                     | To species level       | 25                          | 24                          | 35                          | 31                          |
|                     | Unidentified           | 0                           | 2                           | 0                           | 0                           |
|                     | Misidentified          | 0                           | 0                           | 0                           | 0                           |
| Bcc (n=50)          | To genus level         | 50                          | 49                          | 50                          | 48                          |
|                     | To species level       | 41                          | 40                          | 43                          | 43                          |
|                     | Unidentified           | 0                           | 1                           | 0                           | 2                           |
|                     | Misidentified*         | 9                           | 9                           | 9                           | 9                           |

*Missidentified at the species level. DT, direct transfer; FA, formic acid; Bcc, Burkholderia cepacia complex.
Burkholderia cepacia complex (Bcc): Of the 50 isolates labelled as Bcc by conventional methods, E-MLST identified 37 isolates as B. cenocepacia (35-B. cenocepacia IIIA, 2-B. cenocepacia IIIB) and 13 as B. cepacia. MALDI-TOF MS gave a concordance rate of 100 per cent for genus identification and 82 per cent (41/50) for correct species identification when a cut-off of \( \geq 2 \) was considered. An increase of four per cent was noted with \( \geq 1.9 \) cut-off. Direct transfer method could identify all the 50 isolates, while two and four per cent remained unidentified by FA on-plate extraction method using cut-off \( \geq 2 \) and \( \geq 1.9 \), respectively (Table I). There were no misidentifications with respect to 37 B. cenocepacia isolates; however, they were not resolved to lineage level of IIIA and IIIB. Of the 13 B. cepacia isolates, only four were correctly identified while the rest were misidentified as B. cenocepacia by MALDI-TOF MS. The overall concordance rate of MALDI-TOF MS for correct identification up to species level was 100 per cent for B. cenocepacia and 30.77 per cent for B. cepacia (Table II). The \( \text{recA} \) sequencing correctly identified 29 of 37 B. cenocepacia and 12 of 13 B. cepacia, giving a concordance of 78.37 and 92.30 per cent, respectively. PCA dendrogram constructed by MALDI-TOF MS placed all B. cenocepacia and 12 of 13 B. cepacia isolates into two distinct groups (Fig. 3). Cluster analysis also identified all B. cenocepacia and 12 of 13 B. cepacia correctly, 11 of which were closely placed (Fig. 4). Only one B. cepacia isolate remained ‘misidentified’ by both the methods.

**Discussion**

In spite of continuous advancements in the field of diagnostics, the identification of NFGNB by conventional methods alone is neither satisfactory nor reliable. Keeping in view the inter-species differences in these NFGNB, the need for reliable identification beyond genus level cannot be understated. The importance of speciation of A. calcoaceticus- A. baumannii complex is well known. Not only is A. calcoaceticus an environmental non-pathogenic species, but the other species of the complex

![Fig. 1. MALDI-TOF MS based principal component analysis dendrogram for 50 isolates of Acinetobacter baumannii. The isolates fell into two distinct groups based on their main spectra. Distance level represents diversity in percentage. Numbers indicate isolates in study.](image-url)
also vary in their clinical outcomes and show contrasting susceptibilities to antimicrobial agents\(^\text{14,15}\). Toh \textit{et al}\(^\text{16}\) have highlighted the role of MALDI-TOF MS in \textit{Acinetobacter} speciation. Within the genus \textit{Stenotrophomonas}, much of the clinical significance is attached to \textit{S. maltophilia} in being both environmental as well as opportunist pathogen\(^\text{17}\). The different species within Bcc not only differ in their geographical distribution, but also in their pathogenic potential, ranging from environmental contaminants to those posing an epidemic threat necessitating infection control measures\(^\text{2}\).

MALDI-TOF MS has not only reduced the average turnaround time but also the cost per isolate, with no loss in accuracy\(^\text{18,19}\). MALDI-TOF MS is a promising tool, but it needs optimization. This study was aimed to validate MALDI-TOF MS identification of NFGNB by comparing with the gold standard molecular

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**Table II.** Comparison of MALDI-TOF MS and \textit{recA} sequencing in identification of 50 \textit{Burkholderia cepacia} complex isolates and their concordance with multilocus sequence typing (MLST)

| Number of isolates | Identification by MLST | Correct ID by MALDI TOF-MS | Correct ID by \textit{recA} sequencing |
|--------------------|------------------------|-----------------------------|-------------------------------|
|                    |                        | Genus level | Species level | Mis-identified | Concordance with MLST (%) | Genus level | Species level | Mis-identified | Concordance with MLST (%) |
| 37                 | \textit{Burkholderia cenocepacia} | 37      | 37\(^*\)      | 0               | 100                      | 37      | 29\(^*\)      | 8               | 78.37                 |
|                    | \textit{B. cenocepacia} IIIA (35) |         |                |                |                          |         |                |                |                      |
|                    | \textit{B. cenocepacia} IIIB (2) |         |                |                |                          |         |                |                |                      |
| 13                 | \textit{B. cepacia} | 13      | 4              | 9               | 30.77                    | 13      | 12              | 1               | 92.30                 |

\(^*\)Both MALDI-TOF and \textit{recA} sequencing did not identify species of \textit{B. cenocepacia} upto IIIA and IIIB lineage level
techniques. An overall concordance of 100 per cent was obtained in correctly identifying the genus of NFGNB using MALDI-TOF MS, which corroborated well with earlier studies\textsuperscript{7,8}. For the identification of isolates up to species level, a concordance of 73.33 per cent with the molecular methods was obtained. However, when the cut-off was optimized to $\geq 1.9$, the identification rate increased to 82.6 per cent without any compromise in accuracy. Earlier studies have shown rates in the range of 72-77 per cent\textsuperscript{8,13,20} for species-level identification of NFGNB. It was worth noting that although the score was less for a few isolates of \textit{A. baumannii} and \textit{S. maltophilia}, yet there were no misidentifications. This was in contrast to the work of Espinal \textit{et al}\textsuperscript{21} in which a few species of genus \textit{Acinetobacter} were misidentified. One possible explanation could be the upgradation of the database in the Biotyper version 3 used in our study. Further, the isolates of \textit{A. baumannii} and \textit{S. maltophilia} which were not resolved up to species level due to scores $< 1.9$ could also be correctly characterized by their close grouping in the PCA dendrogram. Spinali \textit{et al}\textsuperscript{22} have also shown an upcoming role of MALDI-TOF MS in microbial typing. Though the concordance in species identification for \textit{B. cepacia} isolates was only 30.77 per cent, similar to that reported by Fehlberg \textit{et al}\textsuperscript{23}, when they were subjected to PCA dendrogram, all but one isolate were placed correctly in their respective \textit{B. cenocepacia} and \textit{B. cepacia} groups. The cluster analysis method was also able to correctly group together 11 out of 13 \textit{B. cepacia} isolates. In the current study, \textit{recA} sequencing misidentified several isolates giving a concordance of 78.37 per cent for \textit{B. cenocepacia} and 92.30 per cent for \textit{B. cepacia}. Correct species identification in as low as 65.4 per cent of Bcc isolates using single gene target, i.e. \textit{recA} sequencing has been noted earlier also\textsuperscript{24}. A probable explanation could be that classification on the basis of a single gene will have lower discriminatory power than

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Fig. 3. MALDI-TOF MS based principal component analysis dendrogram for 50 isolates of \textit{Burkholderia cepacia} complex. All 37 \textit{B. cenocepacia} isolates were grouped together; 12 of 13 \textit{B. cepacia} isolates formed a distinct group. The 13\textsuperscript{th} isolate (45), misidentified by MALDI-TOF MS as \textit{B. cenocepacia}, is encircled.
that based on a group of seven putative housekeeping genes, as done in E-MLST.

*B. cenocepacia* IIA was found to be the most prevalent species in our setup\(^2\), and MALDI-TOF MS was able to identify all these isolates, although not to the lineage level. MALDI-TOF MS has shown promising results in correct identification of commonly occurring *Bcc* isolates elsewhere in the world as well\(^2\). Thus, harnessing the various applications of MALDI-TOF MS, beyond mere matching with the latest database, may not only improve the sensitivity of microbial identification but also give an insight into the predominant strains circulating in the patient population on the basis of different groups formed by PCA dendrogram.

The FA extraction step has aided in improving identification rates in case of Gram-positive cocci\(^3\), but such correlation has not been found in case of NFGNBs\(^4\). This pre-analytic step of extraction has not shown any significant improvement in our study also. Our findings support a cut-off of ≥1.9 for reliable species identification of these NFGNBs.

In conclusion, leaving aside the high setup cost, the prompt identification with a high discriminatory power at low running cost per isolate makes MALDI-TOF MS a suitable tool for the characterization of difficult to identify microorganisms in high-throughput laboratories.

**Conflicts of Interest:** None.

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