MALDI-TOF: A useful tool for laboratory identification of uncommon glucose non-fermenting Gram-negative bacteria associated with cystic fibrosis

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The predisposition of patients with cystic fibrosis (CF) for recurrent pulmonary infections can result in poor prognosis of the disease. Although the clinical significance in CF of microorganisms, such as Staphylococcus aureus, Haemophilus influenzae and Pseudomonas aeruginosa, is well established, the implication of uncommon glucose non-fermenting Gram-negative bacilli (UGNF-GNB) in respiratory samples from CF patients is still unclear. Because of limitations of traditional methods used in most clinical laboratories, the accurate identification of these microbes is a challenge. Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) is an alternative tool for efficient identification of bacteria. This was a retrospective study to evaluate different identification methods in a collection of UGNF-GNB isolated from children with CF during a period of three years. The performance of MALDI-TOF was compared to that of 16S rDNA gene sequencing and to a conventional and automated phenotypic identification. The discriminatory power of MALDI-TOF (75.0% agreement) was superior to automated techniques (67.1% agreement) and to conventional phenotypical identification (50.0% agreement). MALDI-TOF also demonstrated high accuracy in identifying Stenotrophomonas maltophilia, Achromobacter xylosoxidans and Chryseobacterium indologenes, but had limited utility in identifying Pandoraea spp. and some species of Acinetobacter and Chryseobacterium (other than C. indologenes). Although MALDI-TOF identified only 75% of the isolates in comparison with 16S rDNA gene sequencing, the prompt identification and high discriminatory power exhibited by MALDI-TOF make it a useful tool for the characterization of microorganisms that are difficult to identify using routine methods.

INTRODUCTION

The airways of cystic fibrosis (CF) patients show increased adhesiveness and viscoelasticity of surface liquids. A microbial niche may develop in the respiratory tract of these patients, which increases their likelihood of developing chronic respiratory infections (Paschoal & Pereira, 2010).

Abbreviations: APID, automated phenotypic identification; BCC, Burkholderia cepacia complex; CF, cystic fibrosis; CPID, conventional phenotypic identification; GNF-GNB, glucose non-fermenting Gram-negative bacilli; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; UGNF-GNB, uncommon glucose non-fermenting Gram-negative bacilli.
The prognosis of these infections associated with microorganisms such as *Staphylococcus aureus*, *Haemophilus influenzae*, *Pseudomonas aeruginosa* and *Burkholderia cepacia* complex (BCC) are well established among the medical scientific community (Coenye et al., 2002; Marko et al., 2012). However, the clinical value of finding uncommon glucose non-fermenting Gram-negative bacilli (UGNF-GNB) is still unknown for CF patients.

Identification of glucose non-fermenting Gram-negative bacilli (GNF-GNB) in the laboratory is hampered by their metabolic and physiological properties (Desai et al., 2012). In addition, automated techniques exhibit limitations when the micro-organism is a UGNF-GNB (Bosshard et al., 2006).

In this scenario, the use of matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) may be a potential tool for faster, more accurate and less-expensive identification of micro-organisms than conventional techniques (Desai et al., 2012; Marko et al., 2012).

MALDI-TOF is based on the spectral analysis of bacterial proteins, mainly ribosomal proteins, ionized by laser irradiation of the bacterial cell. The identification is performed by comparison of the spectrum of the investigated microorganism with spectra in a reference database (Desai et al., 2012; Fernández-Olmos et al., 2012b; Lay, 2002; Marko et al., 2012), which allows rapid identification of bacteria, mycobacteria and yeasts (Desai et al., 2012).

The aim of this study was to evaluate the performance of MALDI-TOF in the identification of UGNF-GNB isolated from the cultures of oropharyngeal swabs obtained from young children with CF. The results were compared with those obtained with 16S rDNA gene sequencing and conventional techniques used predominantly for clinical microbiology (i.e. manual and automated phenotypic identification).

**RESULTS AND DISCUSSION**

The 76 samples analysed by 16S rDNA gene sequencing represented 24 species and/or bacterial groups. Eight species included multiple strains and accounted for 78.9 % of the studied samples. The remaining UGNF-GNB groups comprised 16 genera/species that included 21.1 % of the studied group (Table 1).

The overall concordances of identification between the different methods tested and 16S rDNA gene sequencing were the following: MALDI-TOF 75 % (57/76), the automated system 67.1 % (51/76) and the conventional phenotypic method 50 % (38/76) (Table 1).

Genera and species identified with 100 % agreement between MALDI-TOF and 16S rDNA gene sequencing were *Achromobacter* sp. (1/1), *Achromobacter xylosoxidans* (6/6), *Acinetobacter calcoaceticus* (1/1), *Bordetella bronchiseptica* (1/1), *Myroides* sp. (1/1), *Pseudomonas fluorescens* (1/1), *Rhizobium radiobacter* (1/1) and *Stenotrophomonas maltophilia* (33/33) (Table 1). Six isolates produced spectra inconsistent with any micro-organism database of VitekMS and could not be identified by MALDI-TOF, including one isolate of *Chryseobacterium bovis*, two isolates of *Pandorea sp.utorum/oxalativorans* and one isolate of *Acinetobacter guillouiae*, because of the absence of identification spectra in the equipment’s database. One isolate of *Acinetobacter*

**METHODS**

**Study design.** This was a retrospective study that compared different methods for the identification of micro-organisms. The commonly isolated GNF-GNB from CF patients, such as *P. aeruginosa* and BCC, were excluded. The criteria used for comparing the results among techniques were agreement between genus and species, or at least between genera in cases where sequencing identified only the genus of the micro-organism.

**Bacterial isolates.** In total, 76 bacterial isolates from oropharyngeal swab cultures obtained from 44 children, between 2 and 90 months of age, were studied. These children all had CF and were attending the outpatient paediatric pneumology clinic at the Clinics’ Hospital of Federal University of Paraná in the period from August 2003 to June 2006.

**Processing of samples.** After having been routinely identified, bacterial isolates were kept frozen at −80 °C in tryptic soy broth containing 15 % glycerol and reactivated by growth on blood agar and incubation for 24–48 h at 35 °C for the execution of this study.

**Conventional phenotypic identification.** Conventional phenotypic identification (CPID) was performed according to the methods described by Schreckenberger et al. (2003).

**Automated phenotypic identification.** The Vitek2 Compact System with a GN card (bioMérieux) was used to perform automated phenotypic identification (APID). The results were considered acceptable when the confidence score was higher than 90 %.

**MALDI-TOF.** Identifications by MALDI-TOF were performed in duplicate by using a VitekMS Spectrometer (bioMérieux) as previously described (Marko et al., 2012; Richter et al., 2013). The result was considered acceptable when the confidence score was greater than 90 % for the first spot and the second spot result agreed with the first. *Escherichia coli* ATCC 8739 was used for every acquisition group on the target slide to calibrate the mass spectrometer. Matrix solution (z-cyano-4-hydroxycinamnic acid) without micro-organisms was used as the negative control.

**16S rDNA gene sequencing.** DNA was extracted using the Accuprep Genomic DNA Extraction kit (Bioneer). The 16S rDNA gene sequencing was performed with the MicroSeq 500 16S rDNA Bacterial Sequencing Kit (Applied Biosystems). The obtained amplicon was purified using the ExoSAP-IT reagent (Affymetrix). Sequencing was performed using an Applied Biosystems 3130 Genetic Analyzer. All steps were performed according to the manufacturers’ instructions. The sequences obtained were analysed and edited when necessary using BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html), and the consensus sequence obtained was evaluated by two genomic databases, leBibi’s paralogy database (http://www.ncbi.nlm.nih.gov/BLAST), and the consensus sequence obtained was evaluated by two genomic databases, leBibi’s paralogy database (http://www.ncbi.nlm.nih.gov/BLAST), and the consensus sequence obtained was evaluated by two genomic databases, leBibi’s paralogy database (http://www.ncbi.nlm.nih.gov/BLAST), and the consensus sequence obtained was evaluated by two genomic databases, leBibi’s paralogy database (http://www.ncbi.nlm.nih.gov/BLAST), and the consensus sequence obtained was evaluated by two genomic databases.
and one of Acinetobacter baumannii were not identified even after repeated analysis.

The identification by the APID method fully agreed with the results obtained with the 16S rDNA-sequence-identification method for the following micro-organisms: Acinetobacter ursingii (4/4), B. bronchiseptica (1/1), Myroides sp. (1/1), P. fluorescens (1/1) R. radiobacter (1/1) and S. maltophilia (33/33). The identification of the remaining strains agreed partially in comparison with the reference technique: A. xylosoxidans (1/6), A.baumannii (4/6) and Chryseobacterium indologenes (4/5).

CPID presented complete concordance with 16S rDNA gene sequencing in the identification of Acinetobacter sp. (1/1) and Myroides sp. (1/1). A. xylosoxidans (2/6), A. baumannii (4/6) and C. indologenes (1/5) showed some discrepancies between CPID and the identification by 16S rDNA gene sequencing.

The high agreement of the APID results (67.1 %) with 16S rDNA gene sequencing compared with CPID (50 %) can be explained by the greater set of biochemical tests provided by the APID identification cards than those provided by the CPID. The greater availability of biochemical tests in APID provides more accurate differentiation of the species involved, because it covers wider metabolic properties of UGNF-GNB (Desai et al., 2012). Despite the better performance of APID than CPID identification, for several reasons, all phenotypic identification systems for GNF-GNB have inherent limitations. First, not all of the strains of the same species have the same particular characteristics; second, the same strain may produce different results in repeated tests; and, third, the databases associated with phenotypic tests are limited (Bosshard et al., 2006). Moreover, the experience level of the microbiologist conducting the analysis is a relevant factor for the successful identification of micro-organisms when using CPID.

The level of agreement of the tested methods (i.e. CPID, APID and MALDI-TOF) with 16S rDNA gene sequencing suggests that the results obtained by MALDI-TOF are closer to those obtained by 16S rDNA sequencing than to those obtained by phenotypic techniques. In fact, a better performance of MALDI-TOF was expected because this technique analyses highly abundant proteins, including ribosomal proteins (Fernández-Olmos et al., 2012b; Rettinger et al., 2012). Discordant results between MALDI-TOF and 16S rDNA gene sequencing can be explained, in part, with

### Table 1. Identification concordance between the evaluated methods and 16S rDNA sequencing stratified by number of isolates

<table>
<thead>
<tr>
<th>Species identified by 16S rDNA sequencing</th>
<th>No. of isolates</th>
<th>Method used*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CPID</td>
</tr>
<tr>
<td>Stenotrophomonas maltophilia</td>
<td>33</td>
<td>29</td>
</tr>
<tr>
<td>Achromobacter xylosoxidans</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Acinetobacter baumannii</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>Chryseobacterium indologenes</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Acinetobacter ursingii/oleivorans</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Acinetobacter gyllenbergii/haemolyticus</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Chryseobacterium bovis</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Pandoraea spororum/oxalativorans</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Achromobacter sp.</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Acinetobacter calcoaceticus</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Acinetobacter guillouiae</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Acinetobacter sp.</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Aquamicrobium luxatiensis</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Bordetella bronchiseptica/holmesii</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Chryseobacterium flavum/ginsengisoli</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Chryseobacterium gleum/arthrophaerae</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Chryseobacterium joostei</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Chryseobacterium letacus</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Cupriavidus metallidurans</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Myroides sp.</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Pseudomonas fluorescens group</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Rhizobium radiobacter/Agrobacterium sp.</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Sphingobacterium sp.</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Stenotrophomonas humi/X. axonopodis</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Total per method</td>
<td>76</td>
<td>38</td>
</tr>
</tbody>
</table>

*CPID, Conventional phenotypic identification; APID, automated phenotypic identification; M, MALDI-TOF.
the limitations of the database used, such as the lack of identification spectra of the reference strains in the equipment’s database (Fernández-Olmos et al., 2012b).

For *S. maltophilia*, the higher concordance rate of APID and MALDI-TOF (33/33) with 16S rDNA sequencing than that of CPID (29/33) is relevant because of the high isolation rates (33/76) of this organism compared with other UGNF-GNB, despite the impact and prognosis of *S. maltophilia* infections in CF patients still being unclear (Amin & Waters, 2012). Due to the high incidence of nosocomial infections caused by this micro-organism (Bosshard et al., 2006) and its intrinsic resistance to multiple classes of antimicrobials (Samonis et al., 2012), the correct identification of *S. maltophilia* is very important for the success of programs designed to treat or control nosocomial infections and for determining the rational use of antimicrobials.

The inability of phenotypic methods and MALDI-TOF to identify the *Pandoraea* genus is of concern and confirms data from other studies. CPID confused the *Pandoraea* genus with various other species of GNF-GNB, such as *Achromobacter* spp. (Fernández-Olmos et al., 2012a). Although the incidence and clinical significance of *Pandoraea* in CF patients are not well established, the genus deserves attention because it is associated with decreased lung function (Fernández-Olmos et al., 2012a). The fact that *Pandoraea* emerged from a reclassification of BCC species reinforces the need to better evaluate these isolates in CF patients. This genus is considered a chronic lung colonizer similar to *P. aeruginosa* and presents invasive potential, as demonstrated in CF patients who were previously colonized with *Pandoraea* and have progressed to bacteraemia (Martínez-Lamas et al., 2011). Decreased lung function associated with the invasive potential of *Pandoraea* was also observed in CF patients in Ireland, where the predominant species *Pandoraea pulmonicola* exhibits virulence factors comparable to those from *Burkholderia cenocepacia*; therefore, segregation of these patients is recommended (Costello et al., 2011).

Identification results obtained from *Acinetobacter* suggest some limitations of MALDI-TOF in identifying species of this genus, although its level of agreement with the sequencing method was higher than those obtained with the phenotypic methods. Six isolates produced spectra inconsistent with any spectra in the micro-organism database of VitekMS and could not be identified by MALDI-TOF. Three of them were *Acinetobacter* spp., according to 16S rDNA gene sequencing. This suggests either that the spectra obtained by analysing strains of different species of *Acinetobacter* are not distinct enough for accurately classifying these species or that the database provided by the MALDI-TOF equipment contains insufficient data for the correct differentiation of species within this genus. According to the manufacturer, the VitekMS database has only four identification spectra for *Acinetobacter* species: *A. baumannii* complex, *A. haemolyticus*, *A. junii* and *A. ursingii*. These findings suggest that molecular genomic techniques are still required for the correct identification of *Acinetobacter* spp. (Álvarez-Buylla et al., 2012). Indeed, DNA–DNA hybridization is still considered the gold standard for the identification of *Acinetobacter* spp. Some reports have shown that 16S rDNA gene sequencing is not sufficient to identify all the species belonging to this genus, while sequencing of other genes such as *recA*, *gyrB* and *rpoB* generated better results for the phylogenetic classification of *Acinetobacter* spp. (Bergogne-Bérézin et al., 2008). When using MALDI-TOF MS (Microflex LT, Bruker), Desai et al. (2012) observed some difficulty in the identification of species of the genus *Acinetobacter* with this method compared with the phenotypic identification techniques used as a reference. Because the taxonomy of *Acinetobacter* spp. is in constant flux, modifications in the databases of MALDI-TOF and phenotypic techniques should be made frequently to enable better identification of these species (Desai et al., 2012). There are no reports of *A. ursingii* associated with lung infections in CF patients. Out of 76 analysed strains, we have identified four strains of *A. ursingii* by APID and three strains by MALDI-TOF, suggesting that this species is easily distinguished from others not belonging to the *A. baumannii/calcoaceticus* complex.

The role of *Achromobacter* in CF patients is still unclear. Some studies have shown that chronic infection by this genus results in the rapid decline of lung function and inflammatory responses comparable to those caused by infection with *P. aeruginosa* (Hansen et al., 2010; Lambiase et al., 2011), while other studies failed to confirm these observations (Tan et al., 2002). This genus is considered opportunistic and has attracted attention as an emerging pathogen in patients with CF. We have obtained six strains in our study, confirming literature data that indicate 6–10% frequency in CF patients ( Ridderberg et al., 2012). Our data indicate that the use of MALDI-TOF was more discriminatory than other methods in identifying *Achromobacter*, mainly for the species *A. xylosoxidans*, which is most frequently involved in lung infections in CF patients, although the clinical significance of this infection is still not well established (Hansen et al., 2010).

*C. indologenes* is an uncommon human pathogen. Most infections caused by this organism are associated with hospitalized patients with underlying diseases, such as cancer and diabetes, or with the use of implantable devices (e.g. ventilators and catheters). Five species of *Chryseobacterium* are most commonly isolated from clinical specimens: *C. odoratum*, *C. multivorans*, *C. breve* and group IIb *Chryseobacterium* spp., which includes *C. indologenes* and *C. gleum* (Calderón et al., 2011). In hospital environments, *C. indologenes* is isolated from water systems and wet equipment, including ventilators, tubes and humidifiers (Sakurada, 2008). Our findings indicate that, among the UGNF-GNB, the number of isolated *C. indologenes* (5/76) was considerable. Specific studies on the prognosis, monitoring and clinical management of CF patients infected with this organism are lacking, but some data suggest that CF patients infected with *Chryseobacterium* spp. are more

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predisposed to co-infection with *P. aeruginosa* and species from the BCC (Lambias et al., 2007). In our study, MALDI-TOF identified 80% (4/5) of the *C. indologenes* isolates, showing a reasonable discriminatory power for the identification of this species.

Although we identified several other species in this study, such as *Aquamicrobium lusatiens*, *Bordetella bronchiseptica*, *Bordetella holmensis*, *Capriavidas metallidurans*, *Myroides* sp., *Rhizobium radiobacter*, *Agrobacterium* sp. and *Sphingobacterium* sp., no reports on the association between these species and CF or lung diseases have been published.

In conclusion, the use of MALDI-TOF for the identification of UGNF-GNB recovered from CF patients can be considered a useful tool, particularly for those microorganisms that have limited biochemical reactivity and that may lead to misidentifications when phenotypic, commercial or traditional bacterial identification techniques are used.

The use of the MALDI-TOF technique has the advantage of rapid microbial identification, which can inform the use of antimicrobials for patients with CF.

Although MALDI-TOF does not allow the correct identification of 1/4 of the species of UGNF-GNB in this study, this method has proved to be more accurate than the phenotypic methods currently used in most laboratories; moreover, a constantly updated database of the equipment could further improve the performance of this technique.

In this study, it was not possible to ascertain the technical performance of MALDI-TOF for some rare microorganisms because of the low number of isolates obtained. However, our results revealed data that provided important information on the performance of MALDI-TOF in identifying UGNF-GNB. Performing this assay with a larger number of samples is difficult because many of the analysed species are rarely isolated from clinical specimens. Routine identification of UGNF-GNB in patients with CF through MALDI-TOF can assist in understanding the role of these microorganisms in the progression of this disease.

### REFERENCES


