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Note

Evaluation of matrix-assisted laser desorption ionization-time-of-flight mass spectrometry for species identification of Nonfermenting Gram-Negative Bacilli

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ABSTRACT

Matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) to identify 396 Nonfermenting Gram-Negative Bacilli clinical isolates was evaluated in comparison with conventional phenotypic tests and/or molecular methods.
MALDI-TOF MS identified to species level 256 isolates and to genus or complex level 112 isolates. It identified 29 genera including uncommon species.

Most of the studies published about Nonfermenting Gram-Negative Bacilli (NFGNB) identification by MS refer to species mainly isolated from respiratory secretions of patients suffering from cystic fibrosis (CF) (Vanlaere et al., 2008; Degand et al., 2008; Marko et al., 2012; Fernández-Olmos et al., 2012, Lambiase et al., 2013). This study assesses the ability of MALDI-TOF MS to identify other species of NFGNB, including uncommon species.

We evaluated MALDI-TOF MS performance identification of 396 NFGNB clinical isolates recovered between 2009 and 2013 from clinical samples at the Hospital de Clínicas José de San Martín, Universidad de Buenos Aires, Argentina. The analysis was performed using Bruker Daltonics MicroFlex LT instrument, Biotyper software 3.1 (Bruker Daltonics, Bremen, Germany). This study included 29 different genera of NFGNB. All isolates from clinically significant samples were previously identified using standard biochemical tests following the identification scheme proposed by Wauters and Vaneechoutte (2011). In addition, 16S rRNA gene sequencing (Weisburg et al., 1991) and *recA* gene (1040 bp) sequencing (Mahenthiralingam et al., 2000) were used on 91 isolates and 28 *Burkholderia cepacia* complex (BCC) isolates, respectively.

Additionally, 20 *Acinetobacter baumannii* and 34 non-*baumannii* *Acinetobacter* spp. isolates were characterized by sequence analysis of *rpoB* using the primers previously described (Gundi et al., 2009).

All PCR reactions were carried out with *Taq* DNA polymerase based on manufacturer's specifications (Promega). Sequencing of PCR products was performed on both DNA strands using ABIPrism 3100 BioAnalyzer equipment at Macrogen Inc., South Korea. The sequences were analyzed using the BLAST v2.0 software (http://www.ncbi.nlm.nih.gov/BLAST/).

A colony growth was smeared on MALDI target and overlaid with 1.5 μl of formic-acid (70%), air-dried and overlaid with 1 μl of matrix (α-cyano-4-hydroxycinnamic acid). Mass spectra were acquired using the MALDI-TOF MS spectrometer in a linear positive mode (Microflex, Bruker Daltonics). A bacterial test standard was used for instrument calibration. Each specimen was run in duplicate and mass spectra were analyzed in an *m/z* range of 2000 to 20,000.

For result interpretation, the score cutoffs recommended by the manufacturer were used to determine species-level identification (≥2.000), genus/family/group-level identification, 1.7 to 1.999, and <1.700, unreliable identification. In addition, a 10% difference between the first two best matches in the database was required to give species identification. If this condition were not met, identification was considered correct at the genus or group level if the first two matches belonged to the same genus or group of bacteria. In this study,

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misidentification was defined as a discrepancy between the identification result given by MALDI-TOF MS and the result obtained by biochemical conventional test and/or by 16S rRNA or *recA* or *rpoB* sequencing (Saffert et al., 2011). MALDI-TOF identification was considered correct when the result obtained from MS database agreed with the phenotypic or molecular identification result.

In the case of *Pseudomonas fluorescens* complex (*P. fluorescens*, *Pseudomonas lundensis*, *Pseudomonas libanensis*, *Pseudomonas koorensis*)

Table 1
Agreement of MALDI-TOF MS with standard biochemical or molecular identifications.

Conventional or molecular identification method	No. of isolates	No. of isolates in agreement with			
		Species level	Genus/complex level	Misidentified	Unreliable identification
Achromobacter spp.	48		48[a]		
Acinetobacter spp.					
A. baumannii	20	19		1[b]	
A. johnsonii	6	5	1		
A. lwoffii	3	3			
A. pittii	11	11			
A. ursingii	3	2		1[c]	
A. junii	3				
A. radioresistans	1	3			
A. guilloiae	1	1			
A. haemolyticus	2	1			
A. oleivorans	1	2		1[d]	
A. soli	1			1[e]	
Alcaligenes faecalis	6	6			
Bordetella bronchiseptica	3	3			
Bordetella holmesii	2	2			
Bordetella parapertussis	1	1			
Bordetella hinzii	3	3			
Bordetella trematum	3	3			
Brevundimonas diminuta	8	8			
Brevundimonas vesicularis	1	1			
Burkholderia cepacia complex					
B. cenocepacia	7	7			
B. cepacia	6	2		4[f]	
B. lata	1		1[g]		
B. contaminans	12	4	7[h]	1	
B. multivorans	1	1			
B. vietnamiensis	1	1			
Burkolderia gladioli	6	5			1
Chryseobacterium gleum	4	4			
Chryseobacterium indologenes	3	1	1[i]	1	
Comamonas kerstersii	10	10			
Comamonas testosteroni	1	1			
Cupriavidus pauculus	1	1			
Cupriavidus respiraculi	1	1			
Delftia acidovorans	6	6			
Elizabethkingia meningoseptica	13	9		4[j]	
Empedobacter brevis	1	1			
Inquilinus limosus	4	2			2
Kerstersia gyorium	2	2			
Myroides odoratimimus	6	6			
Ochrobactrum antrophi	7	2	5[k]		
Oligella urethralis	10	10			
Pseudomonas oryzihabitans	3	3			
Pandoraea apista	1	1			
Pandoraea pulmonicola	1	1			
Pandoraea sputorum	2	2			
Pannonibacter phragmitetus	6	6			
Pseudomonas aeruginosa	24	24			
Pseudomonas mendocina	4	4			
Pseudomonas stutzeri	13	13			
Pseudomonas putida group	34		34[l]		
Pseudomonas fluorescens group	15		15[m]		
Ralstonia mannitolilytica	2			2[n]	
Ralstonia pickettii	2	2			
Rhizobium radiobacter	5	5			
Stenothrophomonas maltophilia	25	25			
Shewanella algae	9	1		8[o]	
Shewanella putrefaciens	4	4			
Sphingobacterium multivorum	9	9			
Sphingobacterium spiritivorum	1	1			
Sphingomonas paucimobilis	2	1			1
Wautersiella falsenii	2	2			
Weeksella virosa	1	1			
Wohlfahrtiimonas chitiniclastica	1	1			
Total (% agreement to level)	396	256 (64.65%)	112 (28.28%)	24 (6.06%)	4 (1.01%)

and of *Pseudomonas putida* complex (*P. putida*, *Pseudomonas monteilii*, *Pseudomonas mosselii*, *Pseudomonas plecoglossicida*, *Pseudomonas fulva*), neither phenotypic identification nor 16S rRNA sequencing can discriminate with high efficiency the species within these complexes. In this case, sequencing of several genes is required to achieve species level identification (Yamamoto and Harayama, 1998). As the main objective of the present study was to evaluate the performance of MALDI-TOF MS in the identification of NFGNB clinical isolates, when MALDI-TOF MS gave different species designations for species included in the mentioned complexes, it was only considered a correct identification for genus or group level. The same consideration was applied in the case of *Achromobacter* spp. because species within the genus (*Achromobacter xylosoxidans*; *Achromobacter rhulandii*; *Achromobacter piechaudii*; *Achromobacter denitrificans*; among others), are closely related and cannot adequately be differentiated by biochemical tests or by 16S rRNA gene sequencing and MLST sequence should be run to approach species (Ridderberg et al., 2012; Spilker et al., 2012).

Statistical analysis to calculate the efficiency of MALDI-TOF MS and reference methods (conventional phenotypic/molecular methods) was carried out. Confidence intervals for identification were calculated with DAG Stat (Mackinnon, 2000).

The agreement of MALDI-TOF MS with standard biochemical or molecular identification methods is shown in Table 1.

Over a total of 396 NFGNB clinical isolates, MALDI-TOF MS correctly identified to species level, 256 isolates (64.65%) and to genus or complex level, 112 isolates (28.28%), while 24 isolates (6.06%) were misidentified and 4 (1.01%) isolates were not identified. Efficiency at species level and genus level was 0.65 (95% CI: 0.60–0.69) and 0.93 (95% CI: 0.90–0.95), respectively.

As regards the identification of 28 BCC isolates, the results of MALDI-TOF MS were 100% consistent with those of *recA* sequencing for identification at the complex level. The Biotyper database does not contain any *Burkholderia contaminans* mass spectra, the most frequent BCC species isolated from CF and non-cystic fibrosis patients in our country (Martina et al., 2013). After the inclusion of some *B. contaminans* spectra of the reference strains, only 4 out of 12 of *B. contaminans* clinical isolates, were correctly identified by MALDI-TOF to species level. Since most of the isolates were only identified at genus level, more spectra need to be included in the database to increase the spectra profiles and allow the identification to BCC species.

The two *Ralstonia mannitolilytica* isolates were misidentified as *Ralstonia pickettii*. On the other hand, *R. pickettii* isolates were correctly identified at the species level. Other members of the *Burkholderiaceae* family as *Pandoraea* and *Cupriavidus* were also correctly identified to the species level. *Pandoraea* species are considered as emerging pathogens in CF patients (Bittar and Rolain, 2010; Costello et al., 2011). However, due to their limited biochemical reactivity, this pathogen is commonly misidentified as *Ralstonia* or *Burkholderia* species (Coenye et al., 2000; Pimentel and MacLeod, 2008) when conventional identification phenotypic methods are applied. In CF, proper identification of the organisms isolated from respiratory secretions is very important for assessing the therapeutic conduct to follow, since these organisms may exhibit different degrees of pathogenicity (Costello et al., 2011; Caraher et al., 2008).

All *A. baumannii* isolates except one (misidentified as *Acinetobacter nosocomialis*) were correctly identified at species level. Among the 34 isolates of non-*A. baumannii* species, MALDI-TOF MS identification at species level was obtained in the 87.5% of the isolates.

The ability of MALDI-TOF MS for the differentiation of species within the *Acinetobacter calcoaceticus–A. baumannii* complex has been emphasized by other authors (Hsueh et al., 2014; Šedo et al., 2013). However, Alvarez-Buylla et al. (2012) showed that molecular techniques are still needed to identify the different species of clinical interest within this genus.

Regarding *Flavobacteriaceae*, MALDI-TOF MS identified to species level 100% of *Myroides odoratimimus*, *Empedobacter brevis*, *Wautersiella falsenii*, and *Weeksella virosa* isolates. However, on the other hand, some *Elizabethkingia meningoseptica* isolates were misidentified as *Elizabethkingia miricola*. Nevertheless, both species can be differentiated phenotypically by urea hydrolysis by *E. miricola* (Wauters and Vaneechoutte, 2011). In relation to reaching identification of *W. falsenii* by conventional tests is difficult because this microorganism has phenotypic characteristics resembling those of *Chryseobacterium* spp. and *Empedobacter* spp. (indole-positive) and a biochemical profile resembling that of CDC group II-h, but urease-positive (Kämpfer et al., 2006).

Although MALDI-TOF MS did not demonstrate a good efficiency on *Chryseobacterium indologenes* isolate identification, all *Chryseobacterium gleum* isolates were identified to species level. The differentiation between *C. gleum* and *C. indologenes* by conventional biochemical tests is difficult because they can only be differentiated by acidification of ethylene glycol and by growth at 42 °C; both tests are positive for *C. gleum* (Wauters and Vaneechoutte, 2011).

To our knowledge, this is the only work that has evaluated the ability of MALDI-TOF MS to identify *Pannonibacter phragmitetus* isolates. It resembles *Rhizobium radiobacter* in their phenotypic characteristics being difficult to differentiate both species by conventional methodology identification.

MALDI-TOF MS had an excellent performance in identifying all *Bordetella* species tested (*Bordetella bronchiseptica*, *Bordetella hinzii*, *Bordetella trematum*, *Bordetella holmesii*, *Bordetella parapertussis*).

Kerstersia spp., initially described by Coenye et al. (2003), phenotypically resembled *Alcaligenes faecalis*, but the latter is oxidase positive and has a fruity odor. Additionally, members of the genus *Kerstersia* are difficult to separate from other members of *Alcaligenaceae*, like *Bordetella* spp. (mainly from oxidase-negative *Bordetella* species). However, MALDI-TOF correctly identifies *Kerstersia gyiorum* isolates included in this study. Recently,

Notes to Table 1:

[a] Regarding to *Achromobacter* spp. identification, 44 isolates were identified as *Achromobacter xylosoxidans*; 3 as *Achromobacter rhulandii* and 1 as *Achromobacter denitrificans*.

[b] Only one *Acinetobacter baumannii* isolate was erroneously identified as *Acinetobacter nosocomialis*.

[c] One *Acinetobacter ursingii* misidentified strain was identified as *Acinetobacter johnsonii*.

[d] The only *Acinetobacter oleivorans* isolate was misidentified as *Acinetobacter johnsonii*.

[e] The only *Acinetobacter soli* isolate was misidentified as *Acinetobacter baylyi*.

[f] The four misidentified strains were identified as three *B. pyrroc inia* and one *B.cenocepacia* strain.

[g] MALDI-TOF could not distinguish between two species: *B. lata*/*B. cepacia*.

[h] MALDI-TOF could not distinguish between *B. contaminans* and *B. cepacia* in seven cases (there was a difference smaller than 10% between the first and second identification scores); in one case *B. contaminans* strain was misidentified as *B. cepacia*.

[i] One *Chryseobacterium indologenes* misidentified strain was identified as *Chryseobacterium joostei* and the other could not be identified to species level.

[j] The four misidentified strains were identified as *Elizabethkingia miricola*.

[k] Five strains of *Ochrobactrum antrophi* were only identified to genus level as *Ochrobactrum* sp.

[l] The 34 isolates with phenotype consistent with *Pseudomonas putida* group were identified by MALDI-TOF as follows: *Pseudomonas monteilii* 12; *Pseudomonas putida* 8; *Pseudomonas plecoglossicida* 6; *Pseudomonas putida* group 3; *Pseudomonas mosselii* 3; *Pseudomonas fulva* 2.

[m] The 15 isolates with phenotype consistent with *Pseudomonas fluorescens* group were identified by MALDI-TOF as follows: *Pseudomonas koreensis* 5; *Pseudomonas clororaphis* 4; *Pseudomonas libanensis* 3; *Pseudomonas lundensis* 2; *Pseudomonas fluorescens* 1.

[n] The two *Ralstonia mannitolilytica* isolates were misidentified as *Ralstonia pickettii*.

[o] Eight *Shewanella algae* isolates were misidentified by MALDI-TOF as *Shewanella putrefaciens*.

also other authors have demonstrated the utility of MALDI-TOF as well (Pence et al., 2013).

Phenotypic differentiation of *Comamonas kerstersii* from *Comamonas testosteroni* is difficult and these isolates are reported as *Comamonas* spp. Susceptibility to deferoxamine, non-use of testosterone, a pyrrolidonylarylamidase negative test, growth at 42 °C and a positive tyrosine hydrolysis differentiate *C. kerstersii* from other *Comamonas* species. However, these tests require a prolonged incubation for reading, instead, MALDI-TOF MS identifies both species with high efficiency.

Regarding the genus *Shewanella*, MALDI-TOF MS identified with high efficiency all *Shewanella putrefaciens* isolates. Conversely, *Shewanella algae*, the most frequently species isolated from clinical specimens, was misidentified as *S. putrefaciens*. Although Bohme et al. recognize the value of MALDI-TOF to identify *Shewanella* spp. as a food spoilage agent, such work does not mention MALDI-TOF ability to identify to species level (Böhme et al., 2010).

According to these results, MALDI-TOF had a good performance in the identification of 29 genera of NFGNB including uncommon species such as *C. kerstersii*, *K. gyiorum*, *W. falsenii*, *Wohlfahrtiimonas chitiniclastica*, *P. phragmitetus*, *Pandoraea* spp., *Cupriavidus* spp., *Bordetella* spp., among others. Nevertheless, expansion of the library, especially including more strains with similar spectra may improve MALDI-TOF MS accuracy.

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