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RESEARCH ARTICLE

Matrix-assisted Laser Desorption Ionization-Time-of-Flight Mass Spectrometry (MALDI-TOF MS) as a Reliable Tool to Identify Species of Catalase-negative Gram-positive Cocci not Belonging to the *Streptococcus* Genus

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Abstract:

Objective:

To evaluate the performance of matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) by using 190 Catalase-negative Gram-Positive Cocci (GPC) clinical isolates.

Methods:

All isolates were identified by conventional phenotypic tests following the proposed scheme by Ruoff and Christensen and MALDI-TOF MS (Bruker Daltonics, BD, Bremen, Germany). Two different extraction methods (direct transfer formic acid method on spot and ethanol formic acid extraction method) and different cut-offs for genus/specie level identification were used. The score cut-offs recommended by the manufacturer ($\geq$ 2.000 for species-level, 1.700 to 1.999 for genus level and <1.700 no reliable identification) and lower cut-off scores ($\geq$1.500 for genus level, $\geq$ 1.700 for species-level and score <1.500 no reliable identification) were considered for identification. A minimum difference of 10% between the top and next closest score was required for a different genus or species.

MALDI-TOF MS identification was considered correct when the result obtained from MS database agreed with the phenotypic identification result.

When both methods gave discordant results, the 16S rDNA or *sodA* genes sequencing was considered as the gold standard identification method. The results obtained by MS concordant with genes sequencing, although discordant with conventional phenotyping, were considered correct. MS results discordant with 16S or *sod*A identification were considered incorrect.

Results:

Using the score cut-offs recommended by the manufacturer, 97.37% and 81.05% were correctly identified to genus and species level, respectively. On the other hand, using lower cut-off scores for identification, 97.89% and 94.21% isolates were correctly identified to genus and species level respectively by MALDI-TOF MS and no significant differences between the results obtained with two extraction methods were obtained.

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Conclusion:

The results obtained suggest that MALDI-TOF MS has the potential of being an accurate tool for Catalase-negative GPC identification even for those species with difficult diagnosis as *Helcococcus*, *Abiotrophia*, *Granulicatella*, among others. Nevertheless, expansion of the library, especially including more strains with different spectra on the same species might overcome potential "intraspecies" variability problems. Moreover, a decrease of the identification scores for species and genus-level identification must be considered since it may improve the MALDI-TOF MS accuracy.

INTRODUCTION

The Catalase-negative Gram-positive cocci (GPC) are among the microorganisms that are isolated more frequently in the microbiology laboratory. In most clinical laboratories, their identification is currently performed by conventional microbiological methods and, in some cases, by molecular methods as 16S rDNA gene and other housekeeping genes sequencing [1]. Although both methodologies achieve a reliable identification, these are time consuming and labor-intensive.

The correct identification of catalase negative GPC has clinical impact since for *e.g.* misidentification of certain species of *Enterococcus* (*E. gallinarum* or *E. casseliflavus*) could lead to failure of treatment if vancomycin is used. Likewise, misidentification of *Abiotrophia/Granulicatella* as *Streptococcus viridans* group might have a negative impact if low doses of penicillin are used for treatment.

The availability of robust diagnostic tools for catalase negative GPC identification has allowed to establish the role of several bacteria originally considered nonpathogenic for humans as responsible for infections in immuno-compromised or debilitated patients. For example, *Lactococcus* and *Vagococcus* were considered for a long time. *Enterococcus* variants [2], only through the use of new identification techniques have been recognized as new human pathogens.

Recently, many studies have evaluated the performance of MALDI-TOF MS for bacterial identification [3 - 8]. In this sense, correct identification for *Enterococcus* spp. by mass spectrometry at the species level has been reported by other authors [9, 10]. However, few works regarding Catalase-negative GPC identification by MALDI-TOF MS including a limited number of species [11, 12] or using an earlier database [11, 13] have been published.

MATERIALS AND METHODS

Bacterial Isolates

A collection of one hundred and ninety Catalase-negative GPC patients' clinical isolates that were recovered between 1998 and 2014 at the Hospital de Clínicas José de San Martín, Universidad de Buenos Aires, Argentina, were analyzed by MS by the Bruker Daltonics MicroFlex LT instrument using MALDI Biotyper software 3.1 (Bruker Daltonics, Bremen, Germany). This study included 96 *Enterococcus* spp. isolates and 94 isolates of catalase-negative GPC (excluding *Streptococcus* spp.) as *Leuconostoc*; *Aerococcus*; *Granulicatella/Abiotrophia*; *Globicatella*; *Helcococcus*; *Lactococcus*; *Gemella*; *Pediococcus*; *Facklamia*, *Vagococcus*; *Weisella*). All isolates had clinical relevance and were isolated from the following samples: 70% from respiratory specimens, 10% from blood, 10% from surgical samples, 5% from urine and 5% from other clinical sources.

All isolates, previously preserved at -70°C in stock medium, were subcultured twice consecutively on 5% sheep blood agar and incubated for 24-48 h in a 5% CO_2 atmosphere at 35ºC, prior to study.

Phenotypic and Molecular Identification

The standard biochemical identification was carried out following the proposed scheme by Ruoff and Christensen [14 - 16]. First, colony morphology on sheep blood agar plates and its microscopic morphology on the Gram stain were observed. The determination of physiological tests, such as: production of pyrrolidonyl arylamidase (PYR), production of leucineaminopeptidase (LAP), growth in broth containing 6.5% NaCl, vancomycin susceptibility; hemolysis on Trypticase soy agar with 5% sheep blood, motility, hydrolysis bile esculin, hippurate hydrolysis, and satellitism, test for growth at 10° and 45°C, among others was also performed. In addition, 16S rDNA gene or *sodA* (coding for the manganese-dependent superoxide dismutase) sequencing were also used in those species for which it is difficult to reach definitive identification by using conventional biochemical tests or to solve discrepancies between conventional

methodology and MALDI-TOF MS identification. Using the universal primers described by Weisburg *et al.* [17] and following the manufacturer's specifications for the Taq DNA polymerase (Promega), 16S rDNA PCR products were obtained. In the case of *sodA* gene, the methodology described by Poyart *et al.* [18] was used. Sequencing of the PCR products was performed on both DNA strands using ABIPrism 3100 BioAnalyzer equipment at Macrogen Inc. sequencing facility, South Korea. The sequences were analyzed by BLAST analysis using two databases (GenBank and EMBL-Bank) to perform the sequence comparison.

Sample Preparation for MALDI-TOF MS

Two methods: i) direct transfer formic acid method on spot and ii) ethanol formic acid extraction have been performed to prepare bacteria for identification by MALDI-TOF MS. For the direct transfer-formic acid method, MALDI-TOF target plates were inoculated into the spots by picking a freshly grown overnight colony and overlaid with 1 µl of 70% formic acid (Sigma-Aldrich). Each spot was allowed to dry and subsequently overlaid with 1 µl of matrix solution (a-cyano-4hydroxycinnamic acid in 50% acetonitrile, 2.5% TFA). For the ethanol formic extraction method, a loopful of bacteria was transferred directly into a tube with 300 µl of distilled water and mixed thoroughly to suspend the cell effectively in the water. and 900 µl ethanol (100%) was added later. The cell suspension was centrifuged at 13,000 rpm for 2 min, and the supernatant was decanted. After a second centrifugation in the same conditions as previously specified, residual ethanol was removed from the pellet using a pipet. The pellet was air dried and thoroughly resuspended in 50 µl 70% formic acid and an equal volume of acetonitrile. After centrifugation at 13,000 rpm for 2 min, 1 µl of the supernatant was transferred to the MALDI TOF target plate and allowed to dry at room temperature before being overlaid with 1 µl of HCCA matrix solution [12].

MALDI-TOF MS Analysis

Mass spectra were acquired using the MALDI-TOF MS spectrometer in a linear positive mode (Microflex LT mass spectrometer, Bruker Daltonics, Germany). Biotyper library version 3.0 and MALDI Biotyper software version 3.1 were used for bacterial identification with default parameters setting (positive linear mode; laser frequency 60 Hz; ion source 1 voltage, 20 kV; ion source 2 voltage, 16.7 kV; lens voltage, 7.0 kV; mass range, 2,000 to 20,000 Da). For each spectrum, 240 laser shots in 40-shot steps from different areas of the sample spot were accumulated and analyzed (automatic mode, default setting). The Bruker Bacterial Test Standard (BTS) (Bruker Daltonics, Germany), an extract of *Escherichia coli* supplemented with RNase A and myoglobin, was used for instrument calibration according to the instructions of the manufacturer. Each specimen was run in duplicate.

With the purpose of improving the available database for *Globicatella*, due to the fact that in the MALDI Biotyper database, *G. sanguinis* is not included, the MALDI-TOF MS analysis of the spectra obtained for eigth *G. sanguinis* clinical isolates was performed. The 24 spectra obtained from the eight spots for each *G. sanguinis* strains were analyzed by FlexAnalysis (version 3.0, Bruker Daltonics). Finally, a minimum of 20 accurate spectra were downloaded in MALDI Biotyper sofware (version 3.0, Bruker Daltonics) to create a main spectrum profile (MSP) of each strain according to the manufacturer's recommendations.

MALDI-TOF MS Data Interpretation

Different cut-offs for genus/specie level identification were used in order to depict the identification results. The score cut-offs recommended by the manufacturer were used to determine species-level identification ≥ 2.000, genus level identification, 1.700 to 1.999 and <1.700 unreliable identification. Additionally, based on previous studies [4, 7, 19] and on our own results [20], lower cut-off scores for identification were used as following: ≥1.500 for genus level, ≥ 1.700 for species-level and score <1.500 was considered as no reliable identification. Moreover, a 10% difference between the first two diagnostic species having the best matches in the database was required to give species identification. If these conditions were not met, identification was considered correct only at the genus level [19].

MALDI-TOF identification was considered correct when the result obtained from MS database agreed with the phenotypic identification result.

When both methods gave discordant results, we carried out the 16S rDNA or *sodA* sequencing as the gold standard identification method. The results obtained by MS concordant with identification by 16S rDNA or *sodA* sequencing were considered correct. MS results discordant with 16S rDNA or *sodA* identification were considered incorrect.

Statistical Analysis

Population parameters of both extraction methods were compared using the z test [21].

RESULTS AND DISCUSSION

Using the direct transfer formic acid method and lower cut-off scores for identification, 186 isolates (97.89%) to the genus level and 179 isolates (94.21%) to the species level were correctly identified by MALDI-TOF MS (Tables **1** and **2**). On the other hand, the results obtained using the ethanol formic acid-acetonitrile extraction methods were: correct identification of 185 isolates (97.3%) to the genus level and 177 isolates (93.1%) to the species level. These results clearly showed no significant differences between the results obtained with both the methods of extraction (with confidence levels of 95% and 99%).

Table 1. Agreement of MALDI-TOF MS with standard biochemical or molecular identifications for *Enterococcus* spp. using different identification cutoff scores.

Conventional or molecular identification method	No of isolates	Genus ID (score >1.5)	Species ID (score >1.7)	No ID	Genus ID (score >1.7)	Species ID (score >2.0)	No ID
Enterococcus faecalis	20	20	20		20	20	
Enterococcus faecium	20	20	20		20	20	
Enterococcus raffinosus	11	11	11		11	9	
Enterococcus gallinarum	7	7	7		7	7	
Enterococcus casseliflavus	11	11	11		11	10	
Enterococcus avium	11	11	11		11	11	
Enterococcus hirae	7	7	7		7	7	
Enterococcus mundtii	2	2	2		2	2	
Enterococcus devriesei	1	1	1		1	1	
Enterococcus durans	5	5	5		5	5	
Enterococcus malodoratus	1	1	1		1	1	
SubTotal n (%)	96	96 (100%)	96 (100%)	0 (0%)	96 (100%)	93 (95.83%)	0 (0%)

Table 2. Agreement of MALDI-TOF MS with standard biochemical or molecular identifications for related genera using different identification cutoff scores.

Conventional or molecular identification method[a]	No of isolates	Genus ID[b] (score >1.5)	Species ID (score >1.7)	NO ID[c]	Genus ID (score >1.7)	Species ID (score >2.0)	NO ID
Leuconostoc mesenteroides	8	8	8		8	7	
Leuconostoc pseudomesenteroides	3	3	3		3	1	
Leuconostoc lactis	4	4	4		4	3	
Aerococcus viridans	11	9	9	2	8	1	2
Aerococcus urinae	5	5	5		5	5	
Abiotrophia defectiva	5	5	5		5	2	
Granulicatella adiacens	10	10	9		9	9	1
Granulicatella elegans	2	1	1	1	1	1	1
Globicatella sanguinis	5	5	5		5	3	
Helcococcus kunzii	7	7	7		7	7	
Lactococcus lactis	9	9	9		9	9	
Lactococcus garviae	1	1	1		1	0	
Gemella morbillorum	3	3	3		3	3	
Gemella haemolysans	5	4	4	1	4	3	1
Gemella sanguinis	2	2	2		2	2	
Pediococcus acidilactici	1	1	1		1	1	
Pediococcus pentosaceus	4	4	4		4	2	
Facklamia languida	1	1	1		1		
Facklamia hominis	1	1	1		1	1	
***Vagococcus* sp.**	6	6	0		6	0	
Weissella viridescens	1	1	1		1	1	

(Table 4) contd.....

Conventional or molecular identification method[a]	No of isolates	Genus ID[b] (score >1.5)	Species ID (score >1.7)	NO ID[c]	Genus ID (score >1.7)	Species ID (score >2.0)	NO ID
SubTotal n (%)	94	90 (95.75%)	83 (88.29%)	4 (4.25%)	89 (94.6%)	61 (64.89%)	5 (5.32%)
Total n (%)	190	186 (97.89%)	179 (94.21%)	4 (2.10%)	185 (97.37%)	154 (81.05%)	5 (2.63%)

[a]Table format adapted from reference [3]; [b]ID: identification; [c]NO ID: not reliable identification

Using a lower score (≥ 1.7) for the species-level identification when analyzing the included *Enterococcus* isolates, the species-level identification increased from 93 *Enterococcus* isolates (95.83%) to 96 isolates (100%) respectively. Moreover, in the case of related genera, the use of lower scores showed a significant increase in the identification rate to species level from 64.89% (61 isolates) to 88.29% (83 isolates). No misidentification results were observed from changing the genus or species score cut-offs (Tables **1** and **2**).

In accordance with Schulthess *et al.* [12], the Bruker MALDI Biotyper system using the direct transfer-formic acid sample preparation method was shown to be a highly reliable tool for the identification of GPC. Furthermore, in agreement with our results, these authors found no significant difference between the two extraction methods used for sample preparation.

Here, we included 94 isolates of catalase-negative GPC (excluding *Streptococcus* spp.). In a previous work, Alatoom *et al.* [11] included 48 isolates of related genera to *Enterococcus* and *Streptococcus*. However, species as *Leuconostoc mesenteroides*, *Leuconostoc pseudomesenteroides*, *Leuconostoc lactis*, *Gemella haemolysans*, *Gemella sanguinis, Gobicatella sanguinis*, *Vagococcus* spp., among others, were not included in previous works. Similarly, Schulthess B *et al.* study [12] included 33 isolates of related genera with lower diversity of species: *Aerococcus viridans* and a few other genera as *Abiotrophia*, *Gemella*, *Granulicatella*, *Lactococcus* and *Leuconostoc*.

MALDI-TOF MS had an excellent performance in identifying all *Enterococcus* species including those of uncommon isolation and difficult identification by conventional biochemical tests as *Enterococcus malodoratus* and *Enterococcus devriesei* and also those species most biochemically inert such as *Enterococcus hirae* and *Enterococcus durans*. Our results are in agreement with those published by other authors [9, 10] showing the reliability of MALDI-TOF MS in the identification of *Enterococcus* species.

Regarding *Aerococcus* spp., using the cut-off scores recommended by the manufacturer, only 1 of the 11 *Aerococcus viridans* isolates was identified to the species level. On the other hand, all *Aerococcus urinae* isolates tested were correctly identified to species level, regardless of the score cut-offs used.

Helcococcus kunzii, a fastidious species is difficult to identify due to its phenotypic similarity to *A. viridans* which shares the morphology in liquid medium, sensitivity to vancomycin, production of pyrrolidonyl arylamidase and growth in broth NaCl 6.5%. Esculin hydrolysis was correctly identified at species level by MALDI-TOF MS with both of the extraction procedures used. Alatoom *et al.* [11] also reported a correct identification of *H. kunzii* to the species level when they used the protein extraction method. In contrast, when they used the direct colony method, the *H. kunzii* isolates were only identified to genus level [11].

When *Lactococcus* spp. was tested, all *L. lactis* isolates were correctly identified to species level with high scores (2.116 to 2.511) opposed to only one *L. garviae* isolate, which was identified with a mean score of 1.849.

With a single *Gemella* isolate (*G. haemolysans*), a reliable identification was not obtained; the remaining *Gemella* isolates were identified to the species level.

MALDI-TOF MS exhibited a reliable performance in identifying *Globicatella sanguinis* (all isolates were identified to the species level). Since the only species that is included in the Bruker database is *Globicatella sulfidifaciens*, this result was successfully achieved due to the previous incorporation of 8 clinical spectra of *G. sanguinis* strains in the database.

The identification of *Vagococcus* spp. isolates tested by MALDI-TOF MS as *V. fluvialis* could not be reliably confirmed since the correct identification at the species level of this genus cannot be achieved either by the traditional methods of identification or by the 16S rDNA gene or the *sodA* gene sequencing. In addition, the Bruker database only contained 4 spectra of *V. fluvialis*; the spectra of other species were not included. The *sodA* sequence analysis of the six *Vagococcus* isolates tested revealed only 81-83% of sequence identity with *Vagococcus salmoninarum*. These results indicate that in this particular case, the identification is reliable only to genus level. The exposed results could be explained in part due to the small number of *Vagococcus' sodA* sequences available in the databases.

MALDI-TOF MS allows us to identify some *L. lactis* isolates with an unusual biochemical profile (positive

arabinose fermentation), being misidentified by conventional identification schemes as *Enterococcus faecium*. Moreover, an *Aerococcus viridans* isolate that hydrolyzed arginine and therefore was erroneously identified as *Aerococcus sanguinicola* by standard biochemical tests was solved by MALDI-TOF. Further testing of these isolates by 16S rDNA gene sequencing confirmed that the Biotyper identification was correct.

In conclusion, our results showed that the extraction preparation method would not be necessary since it did not expose a significant increase in the percentage of identification when was compared with the direct transfer formic acid method. Furthermore, MALDI- TOF MS allows us to recognize isolates with atypical biochemical as *L. lactis* or *A.viridans.* All the results exposed above suggest that MALDI-TOF MS has the potential of being an accurate tool for catalase negative GPC identification even for species with difficult diagnosis as *Helcococcus*, *Abiotrophia*, *Granulicatella*, among others. Nevertheless, expansion of the library, especially including more strains with similar spectra and a decrease of the identification scores for species- and genus-level identifications may improve MALDI-TOF MS accuracy.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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