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ORIGINAL ARTICLE

MALDI-TOF MS performance to identify gram-positive cocci clinical isolates in Porto Alegre/RS, Brazil

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SUMMARY

Until recently, gram-positive cocci identification has mainly relied on conventional and time-SCoNuming phenotypic methods. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) has emerged as a rapid alternative for bacterial identification and our study aimed to compare the performance of this methodology with the golden standard method for gram-positive cocci identification. *Staphylococcus* spp (n=386), *Enterococcus* sp (n=46), *Streptococcus* spp (n=18) clinical isolates and reference strains were studied. MALDI-TOF MS methodology yielded agreement identification of 440 strains out of 450 (97.8%) identified by conventional phenotypic method. The species with more disagreements on identification was *S. epidermidis* (n=5). Some *S. haemolyticus* isolates displayed two distinct genus identification at the first acquisition by MALDI-TOF MS. Two E. gallinarum were misidentified as *E. faecium* and one streptococci isolate was erroneously identified by MALDI-TOF (*S. gordonii* as *S. mitis/oralis*). Our data suggest that MALDI-TOF MS is fast and reliable, and can be implemented in a clinical microbiology laboratory setting, mostly due to accuracy for gram-positive cocci identification.

INTRODUCTION

Gram-positive cocci identification has been always a challenge for microbiology laboratories around the world. The emergence of *Staphylococcus* coagulase-negative (SCoN) as pathogens and antimicrobial resistance reservoirs had required the application of reliable identification methods, either for establishing epidemiological trends as for confirming treatment failures in order to clearly understand pathogenicity mechanisms.[1,2,3] Likewise, *Enterococcus* sp became an important pathogen due to their increasing role as opportunistic agents and their special ability to acquire resistance to antimicrobial drugs, including vancomycin.[4] In addition, *Streptococcus* spp are an heterogeneous group that have been linked to infective endocarditis, invasive pyogenic infections, septic arthritis, subacute bacterial endocarditis, pharyngitis, impetigo, and pneumococcal pneumonia, the major cause of worldwide mortality for this genus.[5,6,7,8,9]

Until recently, bacterial identification has mainly relied on conventional and time-SCoNuming phenotypic methods, commercial or automated systems that often do not distinguish the variable expression of some staphylococcal phenotypic characteristics, and gene sequencing identification techniques.[1,3,10] Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) has emerged as a rapid alternative for bacterial identification, based on the ionization of co-crystallized sample material (microbial cells protein composition) by short laser pulses.[11] The ions are accelerated and their time of flight is measured in a vacuum flight tube, yielding a bacterial mass spectrum.12 Paradox between the fact that conventional methodology could discriminate among small phenotypic differences in SCoN and automated systems could not may be solved or attenuated by this alternative instrument, since its principle is based on specific protein profiles, closely similar to a molecular identification method as 16S rDNA sequencing.[13]

The aim of the present study was to compare the MALDI-TOF MS methodology and conventional phenotypic methods to identify gram-positive cocci isolates recovered from clinical specimens.

MATERIAL AND METHODS

Bacterial strains

Staphylococcus spp (n=386), *Enterococcus* sp (n=46) and *Streptococcus* spp (n=18) recovered from different clinical samples between 2002 and 2010, as well as reference strains, were studied. These isolates were obtained from patients attending at three hospital facilities in Porto Alegre, south Brazil and by American Type Culture Collection (ATCC). The isolates were subcultured on 5% blood sheep agar (bioMeriéux, Marcy l'Etoile, France) and incubated at 35°C for 24h. Then, they were submitted either to phenotypic methods as to analysis by MALDI-TOF MS methodology.

Conventional phenotypic method

All the clinical isolates were submitted to conventional phenotypic tests by the methodology proposed by Bannerman and Peacock[14] and Antunes[15] for staphylococci, Teixeira[16] for enterococci, and Spellerberg and Brandt,[17] Beck, Frodl and Funke[18] for streptococci bovis group. For identification of staphylococci, the following characteristics were tested: catalase, colony morphology and pigmentation, Gram stain, hemolysis, susceptibility to novobiocin (5µg), polymyxin B (300U), fosfomycin (200µg) and deferoxamine (100µg), enzyme activity of arginine arylamidase, ornithine decarboxylase and urease, and acid production from trehalose, mannitol, mannose, xylose, cellobiose, arabinose, maltose, lactose, sucrose and raffinose. For the enterococcal isolates, the following phenotypic characteristics were evaluated: catalase, colony morphology and pigmentation, hydrolysis of esculin in presence of 40% bile, growth in 6.5% NaCl, motility, and acid production from mannitol, sorbose, arginine, arabinose, sorbitol, raffinose, sucrose, pyruvate and methil-alfa-D-glucopyranoside. The species belonging to the *Streptococcus bovis* group analyzed in the present study (*S. gallolyticus* and *S. infantarius*) were tested for Lancefield group D reaction (Oxoid, Hampshire, United Kingdom), hydrolysis of esculin in presence of 40% bile, growth in 6.5% NaCl and acid production from mannitol, methil-alfa-D-glucopyranoside and trehalose. All tests results were obtained after incubations of 24h, 48h and 72h at 35°C.

Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS)

The sample preparation for mass spectrometry was carried out as follow. Each steel slide contained three acquisitions groups, and each acquisition group contained 16 spots, being able to perform 48 different isolates. An amount of a freshly grown 24-hour-old colony was placed directly onto a steel target sample spot in a thin film. This film was then overlaid with 1µl of a saturate matrix solution of α-cyano-4-hydroxycinnamic acid and dried at room temperature. The slide was then inserted into the MALDI-TOF MS instrument (bioMeriéux, Marcy l'Etoile, France). The mass spectra generated were analyzed and compared with a reference spectra database.

Data analysis

Descriptive measures were evaluated from MALDI-TOF results. Identification agreement between conventional phenotypic method and MALDI-TOF were computed for the accuracy determination of the method. Misidentification rates were evaluated by type error as major error (disagreement at genus level) and minor error (disagreement at species level).

Identification criteria used in our study were based on the reliability provide by the instrument, which was based in probability: one microorganism with ≥90% indicated conclusive identification if same genus, two microorganisms with ≥90% indicated inconclusive identification if same genus, one or two microorganisms with ≥90% indicated unreliable identification if different genus. When the instrument was not able to provide any identification, it was considered technique limitation.

RESULTS

The accuracy of MALDI-TOF MS for bacterial identification and the misidentified isolates were provided, in detail, in the Table1 and Table 2, respectively. All *Staphylococcus aureus* isolates were correctly identified by MALDI-TOF MS methodology. The method accuracy for SCoN isolates was 98% and all *S. haemolyticus* and *S. hominis* species were correctly identified. *S. epidermidis* identification matched at the species level in 89 of 94 isolates tested (identification agreement of 94.7%). Five strains were discordant, all presenting minor error: two *S. epidermidis* was erroneously identified as *S. warneri*, and strains misidentified as *S. hominis* subs. *hominis, S. intermedius* and *S. saprophyticus*. MALDI-TOF MS failed to identify three *S. epidermidis* isolates in the first acquisition set; however, all were correctly identified in further analysis. The new methodology yielded correct identification at species level for all *S. haemolyticus* isolates (n=106), although ten strains had to be tested again. Interestingly, four isolates had the wrong genus identification (major error) in the first acquisition by MALDI-TOF MS, with three identifications SCoNisting of *Corynebacterium aurimucosum* and one of *Enterococcus faecalis*. Moreover, six isolates could not be identified in the first analysis, only being identified in the further acquisition.

Table 1 - MALDI-TOF MS accuracy analysis for gram-positive cocci identification.

Taxon (No.)	Agreement identification between MALDI-TOF MS and conventional phenotypic method – No (%)	
	Genus level	Specie level
Staphylococcus spp. (386)	384 (99.5)	379 (98.2)
S. aureus (43)	43 (100)	43 (100)
S. epidermidis (94)	94 (100)	89 (94.7)
S. haemolyticus (106)	106 (100)	106 (100)
S. hominis (26)	26 (100)	26 (100)
S. saprophyticus (95)	94 (99)	94 (99)
Others SCoN[a] (22)	21 (95.5)	21 (95.5)
Enterococcus sp. (46)	46 (100)	44 (95.7)
E. faecalis (28)	28 (100)	28 (100)
E. faecium (8)	8 (100)	8 (100)
Others[b]	10 (100)	8 (80)
Streptococcusc spp. (18)	18 (100)	17 (94.4)
Micrococacceae (386)	384 (99.5)	379 (98.2)
Streptococcaceae (64)	64 (100)	61 (95.3)

[a] *S. capitis (5), S. caprae (1), S. cohnii (3), S. intermedius (1), S. lugdunensis (1), S. sciuri (1), S. warneri (9) and S. xylosus (1);*
[b] *E. casseliflavus (2), E. gallinarum (6) and E. hirae (2);*
[c] *S. agalactiae (1), S. gallolyticus (7), S. gordonii (1), S. infantarius (2), S. mitis/S. oralis (3), S. mutans (1), S. parasanguis (1), S. salivarisus subs salivarius (1) and S. sanguis (1).*

Table 2 - Disagreement identifications between conventional method and MALDI-TOF MS

Bacterial isolates (No.)	Identification provided by MALDI-TOF MS (No.)	Type of disagreement
Staphylococcus epidermidis (5)	*S. warneri (2)*	Minor Error
	S. hominis subs hominis (1)	Minor Error
	S. intermedius (1)	Minor Error
	S. saprophyticus (1)	Minor Error
Staphylococcus saprophyticus (1)	*No identification*	No identification
Staphylococcus warneri (1)	*Staphylococcus warneri / Prevotella buccalis*	Unreliable identification
Enterococcus gallinarum (2)	*E. faecium (2)*	Minor Error
Streptococcus gordonii (1)	*Streptococcus mitis / Streptococcus oralis (1)*	Minor Error

Among *S. saprophyticus* isolates, identification matched at the species level was 98.9%; one strain could not be identified. Similarly, all the twenty-six strains of *S. hominis* were correctly identified in the species-level (100%), with one isolate not identified in the first accquisition. Others SCoN analyzed in this study (*S. capitis, S. caprae, S. cohnii, S. intermedius, S. lugdunensis, S. sciuri* and *S. xylosus*) showed identification agreement at the species-level between the conventional phenotypic method and MALDI-TOF MS. Discordant results was yielded by one *S. warneri* isolate, which produced two bacterial mass spectrum with identical probability (*Staphylococcus warneri* 99.9% / *Prevotella buccalis* 99.9%).

For enterococcal isolates, identification agreement was 95.7%. All *E. faecalis, E. faecium, E. casseliflavus* and *E. hirae* isolates was correctly identified by MALDI-TOF methodology. However, correct genus but incorrect species identification was provided for two of six *E. gallinarum* strains. These isolates were misidentified as *E. faecium*, therefore, classified as minor error (Table 2).

A group of miscellaneous *Streptococcus* species was analyzed by MALDI-TOF MS methodology. A total of 17 out of 18 streptococci isolates were identified at species level, with agreement identification rate of 94.4%. One *S. gordonii* strain was misidentified as *Streptococcus mitis/Streptococcus oralis* group (minor error) and all *S. gallolyticus* isolates (n=7) matched exactly at subspecie-level by the methodology of study.

DISCUSSION

An identification technique for clinically relevant bacteria has to be fast, accurate and reliable. However, conventional phenotypic methods for gram-positive cocci are relatively problematic for use in routine laboratories, with bioquimical tests that require more than 72 hours for species-level identification.[19] Currently, automated identification systems and miniaturized methods are commonly used in laboratories for rapid identification of microorganisms, but the accuracy for some gram-positive cocci has been found to be lower than the one of the reference method. It could be probably due ambiguous reactions, phenotypic variation, atypical biochemical characteristics and slow growth rates, leading to misidentification within the short incubation times used by automated instrument.[19, 20]

The MALDI-TOF MS methodology, when implemented in the clinical laboratories, has been reported to be efficient, cost-effective and rapid in microorganisms identification. It enabled wide bacterial species range identification and, under routine laboratory conditions, showed a high accurate identification rate at the species level.[21, 22]

In this study, MALDI-TOF methodology yielded agreement identification of 440 (97.8%) of 450 gram-positive cocci isolates identified by conventional phenotypic method. The better performance of the new methodology was to staphylococci, followed for enterococci and streptococci isolates. Among SCoN, correctly identification rate at the species level was 98% (336/343), with 6 isolates misidentified and 1 not identified. These results were like previous reports, which included SCoN clinical isolates.23 Dubois et al and Carpaij et al, in recent studies, demonstrated that MALDI-TOF, compared to molecular identification of clinical and reference strains, is an accurate method for important SCoN species determination (accuracy of 99.3% and 100%, respectively), a method that could be implemented for identification of this gram-positive cocci group in routine clinical microbiology.[24, 25]

The misidentified *S. epidermidis isolates* (identified as *S. warneri, S. hominis* subs. hominis, *S. intermedius* and *S. saprophyticus*) could be discriminated by conventional biochemical tests, as bound coagulase (positive results for *S. intermedius*) novobiocin susceptibility (among these species, only *S. saprophyticus* is resistant) and desferrioxamine susceptibility (*S. epidermidis* and *S. hominis* are susceptible, but *S. warneri* is not).[19] Although *S. epidermidis* and *S. hominis* species show similar phenotypic profile, fosfomycin disks can be used as feasible test to differentiate this strains (*S. epidermidis* is susceptible to fosfomycin and *S. hominis* is resistant).[15]

The MALDI-TOF MS system identified one *S. warneri* isolate with mass spectrum of two unrelated bacteria - *Staphylococcus warneri* 99.9% / *Prevotella buccalis* 99.9%. *Prevotella buccalis* is an anaerobic gram-negative bacilli isolated from the human oral microbiota.[26] Phenotypically, both microorganisms can be differentiated by prior samples processing, without any complementary tests.

Some *S. haemolyticus* isolates displayed two distinct genus identification at the first acquisition by MALDI-TOF MS. *S. haemolyticus* isolates was identified incorrectly as Corynebacterium aurimucosum, microorganism belong the normal human flora; some species are common contaminants and occasional causes of prosthetic joint infection.[27, 28] Similarly, the methodology inaccurately identified one isolate as *E. faecalis*. These major errors found at the first acquisition results were after solved by repeating the MALDI-TOF MS test. Phenotypically, characteristics of these bacterial genera easily could be evaluated by initial assessment such as gram staining and colony morphology, allowing the tracking of misidentified results and after acquisition for new analysis.

Forty-four of forty-six enterococci isolates that were identified by MALDI-TOF had concordant identification with phenotypic methods. These results were similar to previous

studies.[22] However, two minor errors were found for *E. gallinarum* isolates. These strains were misidentified as *E. faecium*, showing results not particularly acceptable (correctly identification at specie-level of 66.7%), although number of isolates investigated was low, similarly of other study.[29] In study of comparative genomics of enterococci strains, Palmer et al reported the divergence between *E. faecium* and *E. faecalis* species and identified the genetic caracteristics the motile enterococcal species *E. casseliflavus* and *E. gallinarum*. In opposition to *E. faecalis,* wide phylogenetic divergence was observed among *E. faecium* strains.[30] This is of special interest for bacteria identification, because the MALDI-TOF is a methodology based on proteomic profile, allowing biomarkers separation by mass/charge ratio.[11] Wherefore, genomics variation among the same species distinct strains could affect the bacterial identification, since would induce changes in the microorganism protein profile. Moreover, *E. faecium* and *E. gallinarum* share similar biochemical characteristics (*Enterococcus* group II). The divergent phenotypic test between them is that *E. gallinarum* is motile and MGP (methil-alfa-D-glucopyranoside) positive, while *E. faecium* is not.[16, 31] Despite of misidentified *E. gallinarum* isolates, all *E. faecium* were correctly identificated by MALDI-TOF. This enterococci species is an emergent and challenging nosocomial pathogen, mainly vancomycin-resistant *E. faecium* (VREfm), isolated from hospitalized patients with bacteremia and endocarditis and frequently linked with multiple drugs resistance, compromising the antimicrobial therapy.[32-35] Griffin et al demonstrated that the new methodology has the ability to report an accurate and rapid diagnostic test to reduce this pathogen spread in colonized patients, preventing hospital acquired infections outbreaks.[36]

One streptococci isolate was erroneously identified by MALDI-TOF (*S. gordonii* as *S. mitis/oralis*). These strains belong to Viridans and Mitis group, which comprise a significant proportion of the oropharyngeal tract normal flora.[37] Although the present study has small number of streptococci isolates (n=18), previous reports showed that Vitek MS database is more specific for *S. viridans* identification.[38] However, duo high level of the similarity between this group, analysis with a larger number of isolates should be performed to define the MALDI-TOF MS accuracy for the *S. viridans* identification.

CONCLUSION

MALDI-TOF MS has the potential of being an accurate tool for majority of gram-positive cocci identification. In addition, the methodology is simple, inexpensive, fast, and reliable and can be implemented in a clinical microbiology laboratory setting, improving efficiency, turnaround time and cost-effectiveness for bacterial identification.

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