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## SHORT COMMUNICATION

# MALDI-TOF MS Breakpoint Limitations for *Streptococcus mitis* Group Species Typing

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**SUMMARY**

***Background:*** **The most common laboratory procedures to differentiate *Streptococcus pneumoniae* from other alpha-hemolytic streptococci are based on the optochin susceptibility test, bile solubility, and the Quellung reaction; however, these tests are time consuming and could lead to misidentifications. Consequently, it has become a challenge to correctly identify the *Streptococcus mitis* group.**
***Methods:*** **The MALDI-TOF MS platform provides a quality and quick response for the identification at the species level for even the most challenging microorganism.**
***Results:*** **However, some microorganisms are promptly identified by mass spectrometry and laboratories should be cautious of this methodology limitation when reporting those identifications. These constraints include, among others, the differentiation of *S. pneumoniae* from the *S. mitis/oralis/pseudopneumoniae* group as demonstrated by the results of this work where a specificity of 89.4% was found for *S. pneumoniae* isolate identification.**
***Conclusions:*** **Standard protocol in analysis of peak spectra as well as database increases with new MSPs should be employed with the MALDI-TOF MS platform resulting in a good technique for identification of different *Streptococcus spp*.**
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## INTRODUCTION

In recent times, few technologies have revolutionized clinical microbiology as much as matrix assisted laser desorption ionization - time of flight mass spectrometry (MALDI-TOF MS). The ability to identify the most genetically or diverse microorganism from broth or plate culture media is gradually being replaced by biochemical, antigen-based methodologies and even some genetic and sequence-based technologies.
No new technology for microorganism identification is without problems and the same is true for MALDI-TOF MS. Among the most common errors are the inability to perform an accurate differentiation in those microorganisms that have a genotypic/protein profile similarity [1] and an absence of reliable data in the database. Using

the Bruker Biotyper database, *Streptococcus pneumoniae* could not be distinguished from *Streptococcus mitis* group species. *S. pneumoniae* is one of the microorganisms with greater clinical significance causing serious and life-threatening diseases. *S. mitis*, *S. oralis*, and *S. pseudopneumoniae* are common human oral cavity colonizers [2]. With the exception of few species, using standardized protocols, MALDI-TOF MS is a very powerful tool for species and subspecies identification and typing of pathogenic microbes. The use of databases which are pathogen-specific considerably improves the role of MALDI-TOF MS for typing [3]. The aim of this work was to evaluate the accuracy of the spectrum score of previously established discriminatory mass spectra to distinguish between SMG species specially focused in the rapid identification of *S. pneumoniae*.

## MATERIALS AND METHODS

Currently, (March, 2017), the standard MALDI Biotyper microorganism database (BDAL, Bruker Daltonics) contains 5627 main spectra (MSPs) of 380 microorganisms covering 2200 species.

The strategy followed in this study was: (*i*) To corroborate the reliability of the discriminatory peaks reported in previous studies [1,3-6], the spectra of 108 reference isolates contained in the Bruker Biotyper database were analyzed: *S. mitis* (n = 39), *S. oralis* (n = 38), *S. pneumoniae* (n = 30), *S. pseudopneumoniae* (n = 1), (*ii*) To assess the accuracy of the definitive selected peaks algorithm proposed for species identification, 173 invasive and non-invasive alpha-hemolytic streptococcal clinical isolates were tested, including 132 *S. pneumoniae* (127 optochin susceptible and 5 optochin resistant), 24 *S. mitis*, and 17 *S. oralis*.

*S. pneumoniae* identification was performed by optochin susceptibility and bile solubility tests [7]. Identification was confirmed by *gro*EL gene sequencing in 173 isolates, including the 5 optochin-resistant isolates. Serotyping was performed by a Single-Step Multiplex PCR Assay to determine 92 pneumococcal serotypes [8]. PCR results were confirmed by the Quellung reaction (Statem Serum Institute, Copenhagen, Denmark). Using multiplex-PCR, the 5 optochin-resistant isolates were non-encapsulated pneumococci.

Whole bacterial DNA was extracted from overnight blood-agar cultures using the NucliSENS easyMag automatic extraction platform (bioMérieux, Marcy-l'Étoile, France).

To confirm species identification the sequencing of a fragment of the *gro*EL gene, a reliable method for identifying streptococcal species [9], was performed on all isolates using the previously described primers set StreptogroELd/StreptogroELr for amplification and sequencing. The resulting sequences were compared with those contained in the leBIBI database (https://umr5558-bibiserv.univ-lyon1.fr/lebibi/lebibi.cgi).

Species identification was assigned if the *gro*EL sequence similarity was ≥ 99% with a sequence included in the database.

Bacterial extracts for MALDI-TOF MS identification (Bruker, Daltonics, Germany) was carried out by an ethanol-formic acid extraction method to generate high-quality spectra according to manufactures' recommendations.

Mass spectra acquisition was performed using a Microflex LT mass spectrometer (Bruker Daltonics, Germany) and the Flex Control software (version 3.3.108.2) with default parameter settings. Evaluation of the mass spectra was carried out using the Flex Analysis and MALDI Biotyper 3.1 software and library (version 3.1.66, Bruker, Daltonics, Germany). Bacterial identification as well as results interpretation was done according to the Bruker score system. The unknown spectra were acquired after analyte measurement under standard conditions (mass range 2000 - 20000 m/z and in linear positive mode of frequency 60 Hz) and compared against the 5,627 bacterial MSPs included in (BDAL, Bruker Daltonics) (March, 2017). Log (score) values of ≥ 2.0 ensured identification at species level, values between 2.0 and ≥ 1.7 assessed identification at genus level and values ≤ 1.7 were considered as unreliable identification.

Differences in the techniques employed for SMG species identification were evaluated by Fisher exact test using GraphPad Instat version 3.05 software (GraphPad Software Inc., La Jolla, CA, USA).

## RESULTS

To properly assess the ability of discriminatory peaks reported in previous studies for *S. pneumoniae, S. mitis, S. oralis*, and *S. pseudopneumoniae* differentiation [1,3-4]. The MSPs of 108 MG reference strains included in the BDAL database were evaluated. Overall, only 34/108 (31.5%) could be detected using the discriminatory peaks previously defined in the literature: *S. mitis* 11/39 (28.2%), *S. oralis* 15/38 (39.5%), *S. pneumoniae* 8/30 (26.7%), *S. pseudopneumoniae* 0/1 (0%).

The evaluation of the specificity of MALDI-TOF MS Biotyper 3.1 for SMG species identification was performed by the analysis of 132 *S. pneumoniae* isolates (including the 5 optochin-resistant isolates); overall the specificity was 99.2% for the isolates tested. Only one isolate characterized as *S. pneumoniae* by *groESL* sequencing was misidentified by MALDI-TOF MS as *S. oralis*.

On the other hand, the sensitivity of MALDI-TOF Biotyper as a powerful tool for SMG species identification was not evident. The results differed significantly comparing the score obtained after the first analysis mass spectra (peak score) or the peaks established for each species. Whereas the identification using the first score obtained of 118/132 (89.4%) at species level increased when the discriminatory peak evaluation was used p

< 0.0001. There were no mismatches for *S. mitis* or *S. oralis* with 100% specificity and sensitivity using the discriminatory peaks (5824 m/z and 6955 m/z) previously described in the literature.

## DISCUSSION

One of the limitations of MALDI typing is the lack of a verification protocol for data interpretation. Statistical analysis of peak patterns is essential in assessing the establishment of pathogenic-specific biomarker. That is why one of the major limitations is due to the methodology employed to analyze the protein profiles and peak patterns [10].

In fact, recent studies published by Lasch et al. [11] or Schirmeister et al. [12] have shown technical failures of MALDI-TOF MS typing results for not having breakpoints with enough discriminatory power to elucidate among *Staphylococcus aureus*, *Enterococcus faecium* or *Vibrio cholera* isolates.

However, if the statistical algorithm is correctly applied, it has been demonstrated that MALDI-TOF fingerprints and protein biomarkers can be employed fruitfully in a diagnosis of a particular microorganism as described by Williamson et al. in a conjunctivitis outbreak due to *S. pneumoniae* [13].

MALDI-TOF MS typing could be employed as a powerful technique for most of the microorganisms isolated in the clinical microbiology. The quick typing and identification of microbiological pathogens have significant public health and medical implications.

In this study, we specially focused our interest on the reliability of the MALDI-TOF to identify *S. pneumoniae* because of its clinical implications and the importance of an early diagnosis. The traditional *S. pneumoniae* identification methods include the optochin susceptibility test that takes 18 - 24 hours or the bile-solubility test that will probably need an overnight subculture to have enough colonies to guarantee the performance of the test. The correct identification using MALDI-TOFF will reduce this identification to one working day. According to the report published by Ikryannikova et al. [3] two discriminatory peaks were enough to distinguish among *S. mitis/oralis* and *S. pneumoniae* species (6940 and 9975 m/z). However, although our results did not differ in the global specificity and sensitivity of the discriminatory peaks used for *S. pneumoniae* identification which was close to 100%, it is true that if we had used the peaks established in the Ikryannikova study only the 22/132 (16.6%) of the species would have been identified. The main explanation for these poor results could be that the database was built using only 21 clinical isolates of eight different pneumococcal serotypes of the one hundred described up to date [14].

Special attention should also be given to another study published by Werno et al. [1] where another set of discriminatory peaks is purposed for *S. pneumoniae* characterization, 2937.5 m/z and 5877 m/z. In contrast with what happened with the report published by Ikryannikova, 124/132 (93.9%) strains would have been identified. Nevertheless, according to our experience, even though the 9975 m/z identification for *S. pneumoniae* is cost-ineffective, it should be added to those reported by Werno et al., not only because the aggregate of the peaks defined in both works allowed us the identification of all the strains defined as *S. pneumoniae*, but also because the 9975 m/z was the only peak found in 3/5 (60%) of the atypical pneumococci whose increase is an emerging problem [15].

Finally, it should be highlighted that the increase in the database with new MSPs, especially in those pathogens that are closely related such as SMG species, improves the ability of MALDI-TOF MS to be used as a replacement technique for typing and finally to be employed as a routine tool for identification and epidemiological surveillance in clinical microbiological laboratories.

**Declaration of Interest:**
The author declares no conflict of interest.

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