Original

Mª Fátima López-Fabal[1]
José Luís Gómez-Garcés[1]
José Luís López-Hontangas[2]
Nuria Sanz[1]
Carmen Muñoz[1]
Marta Regodón[1]

Vitek MS matrix-assisted laser desorption ionization-time of flight mass spectrometry for identifying respiratory bacterial pathogens: a fast and efficient method

[1]Servicio de Microbiología. Hospital Universitario de Móstoles. Madrid. Spain
[2]Servicio de Microbiología. Hospital Universitario La Fe. Valencia. Spain

ABSTRACT

Mass spectrometry has become a reference resource for identifying microorganisms in clinical microbiology services. One hundred and fifty one clinical isolates were selected from respiratory specimens routinely identified as *Streptococcus pneumoniae* (43), *Haemophilus influenzae* (64) and *Moraxella catarrhalis* (44). These identifications were compared with other phenotypical methods and mass spectrometry (MALDI -TOF -MS Vitek). Result discrepancies were assessed by 16S rRNA sequencing.

Thirty-eight of the 43 strains of *S. pneumoniae* (86%) were identified as such using phenotypical methods and spectrometry. In 5 cases, MALDI-TOF identified 4 of them as *Streptococcus pseudopneumoniae* and 1 as *S. mitis/oralis*. Forty-eight of the 64 strains were identified as *H. influenzae* (75%) using biochemical identification systems and automated identification systems, whereas MALDI-TOF-MS Vitek identified 51 strains (79%) as such. Conventional methods and spectrometry identified all the 40 strains tested (100%) as *M. catarrhalis*. All strains with discrepant results were sequenced, and in all cases, the identification obtained by spectrometry was confirmed. The results obtained in this study show that mass spectrometry provides identification of these bacteria faster and in a more reliable way than those based on conventional phenotypical methods.

La espectrometría de masas en la identificación de patógenos respiratorios bacterianos: un método rápido y eficaz

RESUMEN

La espectrometría de masas se ha convertido en un recurso de referencia para la identificación de microorganismos en los servicios de Microbiología Clínica. Se estudiaron 151 aislamientos clínicos procedentes de muestras respiratorias que se identificaron rutinariamente como *Streptococcus pneumoniae* (43), *Haemophilus influenzae* (64) y *Moraxella catarrhalis* (44). Estos resultados se compararon con otros métodos fenotípicos y espectrometría de masas (MALDI-TOF Vitek-MS). Las discrepancias en los resultados se valoraron mediante secuenciación del ARNr 16S.

Treinta y ocho de las 43 cepas de *S. pneumoniae* (86%) fueron identificadas como tales tanto por métodos bioquímicos como por espectrometría. En 5 casos MALDI-TOF identificó 4 como *Streptococcus pseudopneumoniae* y 1 como *S. mitis/oralis*. Cuarenta y ocho de las 64 cepas fueron identificadas como *H. influenzae* (75%) al utilizar galerías comerciales y sistemas automáticos, mientras que MALDI-TOF identifica como tales a 51 cepas (79%). Los métodos convencionales y la espectrometría identificaron como *M. catarrhalis* las 40 cepas estudiadas (100%). Todas las cepas con resultados discrepantes fueron secuenciadas, confirmándose en todos los casos la identificación obtenida por espectrometría. Los resultados obtenidos en este estudio demuestran que la espectrometría de masas ofrece una identificación de estas bacterias más rápida y fiable que la basada en métodos convencionales.

INTRODUCTION

Classic cultivable bacterial pathogens in acute respiratory infections mainly include *Streptococcus pneumoniae, Haemophilus influenzae* and *Moraxella catarrhalis*. Their identification in respiratory secretions, bronchial aspirates, etc., includes a serial of conventional phenotypic tests that usually allow diagnosis within 24-48 hours. In order not to delay the identification response too long, these phenotypic schemes are limited to using specific techniques such as sensitivity to optochin, bile solubility, growth on chocolate agar and lack of growth on blood agar plates or detecting butyrate esterase and oxidase respectively for each of the three microorganisms discussed[1,2]. Therefore, the ability to identify species closest to but other than the above, such as *S. pseudopneumoniae, H. parainfluen-*

Correspondencia:
José Luís Gómez-Garcés
Servicio de Microbiología. Hospital Universitario de Móstoles. C/ Rio Júcar s/n.
28935-Mostoles (Madrid). Spain
Telephone number: +34916648600
E-mail: jlgarces@salud.madrid.org

zae, and other genera and species close to *Moraxella catarrhalis*, is usually not covered by the usual routine and only takes place in specially selected strains for epidemiological or clinical interest.

Mass spectrometry, using matrix-assisted laser soft ionization desorption/ionization techniques including mass analyzer known as "time of flight" (MALDI-TOF) has been incorporated less than ten years ago in the microbiology laboratory especially aimed at characterizing microbial protein in order to make a rapid and reliable diagnosis[3,4] although the proof-of-concept that mass spectrometry could identify crude bacteria was established over 30 years ago[5]. Although these proteomic techniques are not yet fully implemented, it is clear that they offer attractive opportunities in securing and advancing microbial diagnosis. However, using them has uncovered some limitations, mainly in differentiating bacterial species that are very close structurally and genomically, as in the case of *S. pneumoniae* and other alpha haemolytic streptococci that share the same habitat[6].

This paper is aimed at assessing this proteomic test, compared with others that show different phenotypic and genotypic characteristics, for characterizing the three classic bacterial pathogens isolated from respiratory samples.

MATERIAL AND METHODS

Microorganisms. Forty-three alpha haemolytic streptococci, 64 gram-negative small coccobacilli and 44 gram negative diplococci isolated from respiratory secretions from patients at our hospital during 2012, were presumptively identified as *S. pneumoniae, H. influenzae* or *M. catarrhalis* respectively, based on the results obtained by Gram staining, catalase production, sensitivity to optochin, growth on blood agar / chocolate agar and oxidase and butyrate esterase production.

Isolates. Respiratory samples were cultured on blood agar and chocolate agar (bioMérieux, France) and incubated at 35°C in atmosphere of 5% of CO_2 for isolating and identifying the said organisms.

Sensitivity to optochin. Blood agar plates were inoculated with alpha haemolytic colonies obtained in the previous cultures and a 5 µg optochin disk was placed in the centre on the inoculated area. These subcultures were incubated for 24 hours at 35°C in an atmosphere of 5% CO_2. Optochin sensitivity was defined as a zone of inhibition ≥ 14 mm in diameter.

Bile solubility test. A 0.5 mL of 2% deoxycholate was added to 0.5 mL suspensions of each isolate prepared in phosphate-buffered saline (PBS) and incubated at 35ºC for 2 h. A positive test was indicated by visible clearing of suspension (Keith 2006)

Biochemical identification systems. RapID STR (bioMérieux, France) and RapID NH (Remel, USA) systems for streptococci and for *Haemophilus* and *Moraxella* respectively were inoculated, incubated and interpreted in accordance with the manufacturer's criteria.

Latex agglutination. Slidex agglutination with pneumo-Kit (bioMérieux, France) for identification of *S. pneumoniae* from previous cultures were performed following the manufacturer's instructions

Immunochromatographic detection of antigens. Immunochromatographic devices NOW *S. pneumoniae* antigen test (Binax, USA) for the detection of cell wall polysaccharide of pneumococci were inoculated from previous cultures. Although this test is designed for urine or other sterile fluids, it was carried out following the manufacturer's instruction to suit the purpose of this study.

Oxidase production. Tetramethyl phenylenediamine dihydrochloride disks (Sigma-Aldrich, Switzerland) were impregnated with the contents of a loop of culture from blood agar plates. The violet colour after 2 minutes indicated a positive reaction.

Production of tributyrin esterase. TRIBU strips (Sigma, USA) were impregnated with the contents of a loop of culture from blood agar plates. A yellow product of acidification indicated a positive result.

VITEK-2. Identification cards ID-GPC (bioMérieux, France) for streptococci and ID-NH (bioMérieux, France) for *Haemophilus* and *Moraxella* were used for the identification of microorganisms according to manufacturer standards.

Identification by MALDI-TOF MS. Plate preparation, mass spectra generation and the combined process were performed according to the standards previously described for the Vitek MS system (bioMérieux, France)[7], using an Axima Assur-

Table 1 Identification of 5 streptococci by different methods.

Strain	RapID STR	VITEK-2	MALDI-TOF	Sequencing	Latex Aglutinat	Inmunochromatografic	Bile test
10S	*S. pneumoniae*	*S. pneumoniae*	*S. pseudopneumoniae*	*S. pseudopneumoniae*	*S. pneumoniae*	*S. pneumoniae*	Not soluble
14S	*S. pneumoniae*	*S. constellatus*	*S. mitis/oralis*	*S. mitis/oralis*	*S. pneumoniae*	*S. pneumoniae*	Not soluble
33S	*S. pneumoniae*	*S. pneumoniae*	*S. pseudopneumoniae*	*S. pseudopneumoniae*	*S. pneumoniae*	*S. pneumoniae*	Not soluble
39S	*S. pneumoniae*	*S. pneumoniae*	*S. pseudopneumoniae*	*S. pseudopneumoniae*	*S. pneumoniae*	*S. pneumoniae*	Not soluble
41S	*S. pneumoniae*	*S. pneumoniae*	*S. pseudopneumoniae*	*S. pseudopneumoniae*	*S. pneumoniae*	*S. pneumoniae*	Not soluble

ance mass spectrometer with version 1.0.0 of the acquisition software and MS-ID database. According to the manufacturer's instructions, a confidence value between 60 and 99% for a given species was considered as a good identification (ID).

If a unique pattern ID is not recognized, a list of possible organisms with confidence values < 60 % was given, considered low discrimination (LD) for each of the identified species. When a single result is obtained with a value <60%, the organism is considered to be outside the limits of the identification system database and recorded as "no ID"[7].

Sequencing. A suspension of 0.5 McFarland bacterial strains under study was prepared as an extraction of nucleic acids from 500µL of the suspension in the automatic extractor Easy Mag (bioMérieux, France).

Between 5 and 50 ng of extracted DNA were amplified by chain reaction of the polymerase in standard conditions, with the previously described oligonucleotides B27F and U1492R[8].

The amplification products were sequenced using Thermo Sequenase Dye Primer Cycle Manual Sequencing Kit (USB Corporation, USA) using primers 16S- NF 5'CACTGGAACTGAGACACGGT -3 'and 16S- NR 5'GTCTATTCCTTTGAGTTTTA -3' internal to the above, designed in our laboratory, which amplify a 586 bp fragment.

Sequencing reactions were loaded on a Long-Read Tower Sequencer (Siemens Healthcare Diagnostics, USA) and the sequences obtained were compared with those in Microbial Genomes BLAST. Identification criteria used was a degree of homology ≥ 97%.

RESULTS

S. pneumoniae. Forty-three strains were presumptively identified as *S. pneumoniae* because of their Gram morphology, optochin susceptibility and catalase test. This identification was confirmed in all cases using biochemical identification systems, the immunochromatographic test and latex agglutination. The VITEK-2 system identified 39 strains as *S. pneumoniae* and the remaining strains as *S.* no *pneumoniae*.

Using the spectrometry system, 38 strains (86%) were identified as *S. pneumoniae*, and the other 5 strains were identified as *Streptococcus* related to *S. pneumoniae* but belonging to other species (4 as *S. pseudopneumoniae* and 1 as *S. mitis / oralis)* that also were not soluble in bile. These five strains were sequenced, confirming the results of mass spectrometry (table 1).

Furthermore, these last 5 strains were subcultured on plates with sheep blood with an optochin disk and simultaneously incubated for 18-24 hours at 35°C in an atmosphere with and without CO_2. Another 5 strains positively identified as *S. pneumoniae* were tested as controls. In an air environment, all strains showed an inhibition halo ≥ 14 mm in response to the 5 µg of optochin, whereas, when incubated in a 5 % CO_2 atmosphere, only the controls reached 14 mm in diameter (table 2).

H. influenzae. Sixty-four strains were presumptively identified as *H. influenzae* considering their Gram morphology and growth on chocolate agar and lack of growth on blood agar. When using biochemical galleries, 48 were identified as *H. influenzae* and the remaining 16 as *H. parainfluenzae*. Using VITEK-2, 48 were assigned to *H. influenzae*, 12 were identified as *H. parainfluenzae* and 4 as *H. haemolyticus*.

The spectrometry system identified 51 strains as *H. influenzae*, 12 as *H. parainfluenzae* and the remaining strain as *H. haemolyticus*.

Regarding the 8 strains with no concordance with the three methods, sequencing confirmed the identifications provided by the spectrometry (table 3).

M. catarrhalis. Forty strains were initially identified as *M. catarrhalis*, due to their Gram morphology, catalase test, production of oxidase and a positive test in tributyrin esterase. The VITEK-2 system did not identify any of these strains as such, while mass spectrometry and biochemical galleries provided a good identification as *M. catarrhalis*.

Table 2 **Optochin disk inhibition halo after 18-24 h incubation at 35°C in CO_2 an ambient atmosphere.**

	CO_2			Ambient		
Optochin inhibition halo	<8mm	8-13mm	≥ 14 mm	<8 mm	8-13mm	14 mm
S. pneumoniae	-	-	5	-	-	5
S. pseudopneumoniae / S. mitis/oralis	2	3	-	-	-	5

Table 3 **Identification of 8 *Haemophilus* spp. by different methods.**

Strain	RapID NH	VITEK-2	MALDI-TOF	Sequencing
5 H	*H. parainfluenzae*	*H. haemolyticus*	*H. influenzae*	*H. influenzae*
21H	*H. parainfluenzae*	*H. influenzae*	*H. influenzae*	*H. influenzae*
22H	*H. parainfluenzae*	*H. influenzae*	*H. influenzae*	*H. influenzae*
23H	*H. parainfluenzae*	*H. influenzae*	*H. influenzae*	*H. influenzae*
26H	*H. influenzae*	*H. haemolyticus*	*H. influenzae*	*H. influenzae*
37H	*H. influenzae*	*H. haemolyticus*	*H. influenzae*	*H. influenzae*
43H	*H. influenzae*	*H. parainfluenzae*	*H. influenzae*	*H. influenzae*
45H	*H. parainfluenzae*	*H. influenzae*	*H. parainfluenzae*	*H. parainfluenzae*

DISCUSSION

Final classification of streptococci, including *S. pneumoniae*, continues to be a problem,

further aggravated by the fact that many of them share the same habitat. For example, using routine phenotypic discrimination techniques between *S. pneumoniae* and other alpha haemolytic streptococci, belonging to the *mitis/oralis* complex, does not always provide satisfactory or conclusive results[9]. Although optochin susceptibility is the most frequently used diagnostic test in clinical microbiology laboratories to differentiate pneumococci from other alpha haemolytic streptococci, the conditions for its use remain uncertain. This is because while some highly prestigious manuals[1] recommend incubating in CO_2, the disk manufacturer recommends incubation in ambient atmosphere. This distinction is interesting because of the recognition of *S. pseudopneumoniae* as a distinct species, but still close to *S. pneumoniae*, although with possibly different specifications as a respiratory pathogen, and whose behaviour on an optochin disc is variable. In this series, as well as in others[10], there are 5 strains in which the inhibition halo was less than 14 mm when incubated in a CO_2 atmosphere. However, when incubated in an ambient atmosphere, they clearly exceeded this limit. The 5 strains were then sequenced and classified as *S. pseudopneumoniae* The rest of the pneumococci, however, showed diameters over 14 mm, both in an ambient atmosphere and CO_2 supplemented environment. The possibility of establishing these misdiagnoses can sometimes affect the valuation of sensitivity parameters or resistance of these species[11].

Biochemical identification systems do not safely distinguish between *S. pneumoniae* and other streptococci either, and automated systems usually show an even weaker differentiation for these microorganisms[10]. Antigenic determination, a useful tool for an initial diagnostic approach, does not guarantee a correct identification of these microorganisms because, even though its sensitivity is excellent, its specificity is extremely poor[12].

Such as other authors suggest[13], our results confirm that distinction between *S. pneumoniae* and nonpneumococal isolates is accurately performed by Vitek MS.

A final phenotypic differentiation between *H. influenzae* and other *Haemophilus* species by conventional testing is only possible by porphyrin test that requires 24 hours more[14]. In our series, we did not use the porphyrin test so as not to delay the report of results, thus limiting any possible differentiation between the species. As a result of the commercial galleries used, 48 of the 64 strains were identified as *H. influenzae*, and the remaining 16 were identified as *H. parainfluenzae*. Using the automated VITEK-2 system, 48 were also assigned to *H. influenzae*, although they did not fully correspond with the strains listed as such by the galleries, 12 were identified as *H. parainfluenzae* and 4 as *H. haemolyticus*.

These results are consistent with various articles published, which indicate that the identification provided by the miniaturized galleries or automated methods differ, in approximately 20% of the cases, from the results obtained by conventional methods[15]. The spectrometry system identified 51 strains as *H. influenzae*, 12 as *H. parainfluenzae* and the remaining strain as *H. haemolyticus*. In total, 8 strains showed different results with the three methods. Sequencing confirmed the identifications provided by the spectrometer.

In some cases the accuracy of identifying species within the *Haemophilus* genus is important since *H. influenza* is currently known to induce respiratory infections in contrast to *H. haemolyticus* or *H. parainfluenzae* and thus accurate identification could reducing unnecessary antibiotic treatment[16] as well as to provide an important tool when it is used for epidemiological purposes. This reliability seems to be achieved more accurately with spectrometry rather than other methods[15,16].

On the other hand, the routine identification of *M. catarrhalis* strains showed no obstacles in our series, simply demonstrating the production of oxidase and esterase tributin, which is fully consistent with the results obtained with identification galleries and spectrometry, whereas VITEK cards were invalid for these diagnoses.

Among the numerous papers published recently regarding the ability of mass spectrometry systems, using matrix-assisted laser soft ionization desorption/ionization techniques including the mass analyzer known as "time of flight" (MALDI-TOF), to identify microorganisms commonly found in clinical microbiology laboratories[5,17-19] it is possible that the correct identification of respiratory bacterial pathogens are those that provide more controversial results. However, in our series, although limited by the low number of strains, the spectrometry technique gave excellent results for identifying and differentiating *M. catarrhalis, S. pneumoniae, H. influenzae* and related species, without the need of a prior protein extraction and confirming these results in strains of questionable identification through amplification and sequencing.

In summary, our findings indicate that Vitek MS is a suitable tool for the swift and reliable identification of classic cultivable bacterial pathogens in acute respiratory infections.

REFERENCES

1. Spellerberg B. & Brandt C. *Streptococcus*. In *Manual of Clinical Microbiology,* Edited by American Society for Microbiology, Washington DC, 10th ed. 2011: pp. 331-349.
2. Speeleveld E, Fosspre JM, Gordts B, Landuyt HW. Comparison of three rapid methods, tributyrine, 4-methylumbelliferyl butyrate, and indoxyl acetate, for rapid identification of *Moraxella catarrhalis*. J Clin Microbiol 1994;32: 1362-3.
3. Bizzini A, Durussel C, Bille J, Greub G, Prod'hom G. Performance of matrix-assisted laser desorption ionization-time of flight mass spectrometry for identification of bacterial strains routinely isolated in a clinical microbiology laboratory. J Clin Microbiol 2010; 48:1549 –54.
4. Dubois D, Segonds C, Prere MF, Marty N, Oswald E. Identification of clinical *Streptococcus pneumoniae* isolates among other alpha and non-hemolytic streptococci by use of the Vitek MS matrix-assisted laser desorption ionization-time of flight mass spectrometry system. J Clin Microbiol 2013; 51:1861-7.

5. Seng P, Drancourt M, Gouriet F, La Scola B, Fournier PE, Rolain JM et al. Ongoing revolution in bacteriology: routine identification of bacteria by matrix-assisted laser desorption ionization time-of flight mass spectrometry. Clin Infect Dis 2009; 49:543–51.
6. Ikryannikova LN, Filimonova AV, Malakhova MV, Savinova T, Filimonova O, Ilina EN et al. Discrimination between *Streptococcus pneumoniae* and *Streptoccocus mitis* based on sorting of their MALDI mass spectra. Clin Microbiol Infect 2013; 19:1066-71
7. Dubois D, Grare M, Prere MF, Segonds C, Marty N, Oswald E. Performances of the Vitek MS matrix-assisted laser desorption ionization-time of flight mass spectrometry system for rapid identification of bacteria in routine clinical microbiology. J Clin Microbiol 2012; 50:2568-76
8. Weisburg WG, Barns SM, Pelletier DA, Lane DJ. 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol 1991; 173:697-703.
9. Facklam RR. What happened to the streptococci: overview of taxonomic and nomenclature changes. Clin Microbiol Rev 2002; 15: 613–30.
10. Arbique JC, Poyart C, Trieu-Cuot P, Quesne G, Carvalho Mda G, Steigerwalt AG, et al. Accuracy of phenotypic and genotypic testing for identification of *Streptococcus pneumoniae* and description of *Streptococcus pseudopneumoniae* sp. nov. J Clin Microbiol 2004; 42:4686-96.
11. Song JH, Baek JH. Incorrect identification of *Streptococcus pneumoniae* and its effect on antimicrobial resistance rates. Int J Antimicrob Agents 2009; 33:92-9.
12. Keith ER, Podmore RG, Anderson TP, Murdoch DR. Characteristics of *Streptococcus pseudopneumoniae* isolated from purulent sputum samples. J Clin Microbiol 2006; 44:923-7.
13. Branda JA, Markham RP, Garner CD, Rychert JA, Ferraro MJ. Performance of the Vitek MS v2.0 system in distinguishing *Streptococcus pneumoniae* from nonpneumococcal species of the *Streptococcus mitis* group. J Clin Microbiol. 2013; 51:3079-82.
14. Haag AM, Taylor SN, Johnston KH, Cole RB. Rapid identification and speciation of Haemophilus bacteria by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.J Mass Spectrom 1998; 33:750-6.
15. Frickmann H, Christner M, Donat M, Berger A, Essig A, Podbielski A et al. Rapid discrimination of *Haemophilus influenzae*, *H. parainfluenzae*, and *H. haemolyticus* by fluorescence in situ hybridization (FISH) and two matrix-assisted laser-desorption-ionization time-of-flight mass spectrometry (MALDI-TOF-MS) platforms. PLoS One 2013; 8:e63222
16. Bruin JP, Kostrzewa M, van der Ende A, Badoux P, Jansen R, Boers SA, et al. Identification of *Haemophilus influenzae* and *Haemophilus haemolyticus* by matrix-assisted laser desorption ionization-time of flight mass spectrometry. Eur J Clin Microbiol Infect Dis 2014; 33:279-84.
17. Ferreira L, Vega S, Sánchez-Juanes F, González M, Herrero A, Muñiz MC, et al. Identifying bacteria using a matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer. Comparison with routine methods used in clinical microbiology laboratories. Enferm Infecc Microbiol Clin 2010; 28:492-7.
18. Jordana-Lluch E, Martró Català E, Ausina Ruiz V. Mass spectrometry in the clinical microbiology laboratory. Enferm Infecc Microbiol Clin. 2012; 30:635-44.
19. Mellmann A, Cloud J, Maier T, Keckevoet U, Ramminger I, Iwen P,et al. Evaluation of matrix-assisted laser desorption ionization-time-of-flight mass spectrometry in comparison to 16S rRNA gene sequencing for species identification of non-fermenting bacteria. J Clin Microbiol 2008; 46:1946-54.