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Matrix-assisted laser-desorption/ionization BIOTYPER: experience in the routine of a University hospital

E. Bessède[1,2,3], M. Angla-gre[1], Y. Delagarde[1], S. Sep Hieng[1], A. Ménard[1,2,3] and F. Mégraud[1,2,3]

1) CHU de Bordeaux, Hôpital Pellegrin, Laboratoire de Bactériologie, Bordeaux, 2) Université Victor Segalen Bordeaux 2, Laboratoire de Bactériologie and 3) INSERM U853, Bordeaux, France

Abstract

Matrix-assisted laser-desorption/ionization time-of-flight (MALDI-TOF) is positioned at the forefront of bacterial identification in the future. Its performance needed to be evaluated in a routine Bacteriology laboratory to determine its true benefits. A prospective study was carried out in the Bacteriology laboratory of the Pellegrin University Hospital in Bordeaux, France, from April to May 2009. Bacterial isolates from clinical samples were identified by conventional phenotypic bacteriological methods [Phoenix (Becton-Dickinson) or API strips (bioMérieux)] and in parallel with a mass spectrometer (Ultraflex III TOF/TOF and the BIOTYPER database from Bruker Daltonics). In case of a discrepancy between these results at the genus level, a 16S rRNA and/or *rpo*B gene sequencing was performed. Of the 1013 bacteria tested, 837 (82.6%) were correctly identified at the species level by MALDI-TOF mass spectrometry (MS) without extraction and 189 after extraction, i.e. 986 (97.3%) were correctly identified at the species level by MALDI-TOF MS, vs. 945 (93.2%) by phenotypic methods. Indeed, the extraction step was necessary for only 15% of the isolates. These results were even better when considering the genus, reaching almost 99% with MALDI-TOF MS and 98% with phenotypic methods. The performance of MALDI-TOF MS is very attractive considering its efficiency and rapidity, and the technique constitutes a precious tool for bacteriological identification in a routine laboratory.

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Corresponding author: F. Mègraud, Laboratoire de Bactèriologie, Hôpital Pellegrin, Place Amelie Raba Leon, 35076 Bordeaux, France
E-mail: francis.megraud@chu-bordeaux.fr

Introduction

Bacterial identification is still essentially based on methods developed at the end of the 19th Century by Koch, Pasteur and others. Recent progress, however, has led to automated techniques and, furthermore, molecular methods are becoming more popular. All of these routine methods allow a bacteriological identification within 8–24 h or more, which is quite long for urgent cases where antibiotherapy has to be established and adapted based on the species identification. Mass spectrometry (MS) was proposed for bacterial identification a long time ago [1], but only recently has progress been made allowing it to become a promising technique using matrix-assisted laser-desorption/ionization time-of-flight (MALDI-TOF) MS. Currently, MS is being used to determine bacteriological identification, based on the protein profile of each species of bacteria [2]. Good results have been obtained in different studies [3,4]. The present study aimed to use the simplest MALDI-TOF protocol to evaluate its accuracy in routine practice in a large University hospital in comparison with standard methods.

Materials and Methods

Bacterial isolates

One thousand and thirteen different isolates obtained in the Laboratory of Bacteriology at Pellegrin Hospital in Bordeaux, France, were included from April to May 2009. A large variety of clinical specimens were issued from the respiratory tract, ear, nose, throat, urine, biopsies, blood, pus, stools genital tract, and other diverse material, from which the bacteria were isolated at three of the six benches in our laboratory. Five people were involved in the study and participated

in the technical steps. Each clinical specimen was plated on an adequate agar plate (e.g. Bromocresol Purple lactose agar, Columbia agar + 5% sheep blood, Chocolate agar + Polyvitex, Columbia agar + 5% sheep blood and nalidixic acid; all from bioMérieux, Marcy l'Etoile, France), depending on the sample and in accordance with the recommendations of our own laboratory procedures. Each plate was incubated for 24 or 48 h in an aerobic, microaerobic or anaerobic atmosphere. In total, 624 Gram-negative rods, 366 Gram-positive cocci, 20 Gram-positive rods and three Gram-negative cocci were included. The detection of mycobacteria did not form part of our study because those identification tests are performed in a special laboratory. The present study also excluded *Campylobacter* sp. identification because our laboratory is the National Reference Center for *Campylobacter* and *Helicobacter* and a specific study was performed in parallel.

Phenotypic identification(s)

All of the bacteria isolated were identified using appropriate Phoenix galleries with the Phoenix automated microbiology system (Becton-Dickinson Diagnostics, Le Pont-De-Claix, France) depending on microscopic examination, including Gram staining, and catalase and oxidase activities. API strips (bioMérieux) were used for fastidious bacteria and anaerobic bacteria, and sometimes when the percentage of identification using the Phoenix system was <99%.

MS identification

Preparation of samples. A colony of each isolate directly issued from the primary agar plate was deposited on an MTP 384 target plate ground steel T F (Bruker Daltonics, Bremen, Germany) in a unique replicate and allowed to dry at room temperature, except for the Gram-positive cocci, which were deposited in two replicates: one before extraction and one after extraction. Each isolate was analyzed on the day of isolation. When the extraction protocol was perfomed, the manufacturer's recommendations were followed. Briefly, one colony was suspended in 300 μL of distilled water; 900 μL of ethanol was added and mixed. Then, the sample was centrifuged, the supernatant removed, and the pellets dried. Fifty microlitres of formic acid (70% in water) were added to the bacterial pellet; the components were mixed thoroughly, and 50 μL of acetonitrile was added. After centrifugation (13 000 **g** for 2 min), 1 μL of the supernatant containing the bacterial extract was transferred onto the target plate. In addition to the colony or the bacterial extract deposited, 1 μL of matrix solution (satured solution of a cyano-4-hydroxycinnamic acid in 50% acetonitrile) was added and was then crystallized by air-drying at room temperature for 5 min.

Measurements with the spectrometer. Measurements were performed with an Ultraflex III TOF/TOF mass spectrometer (Bruker Daltonics) equipped with a 200-Hz smart-beam I laser. The parameter settings were: delay: 80 ns; ion source: 1 voltage, 25 kV, ion source: 2 voltage, 23.4 kV; lens voltage: 6 kV; mass range: 0–20 137 kDa. Each run was validated with an *Escherichia coli* control sample provided by Bruker Daltonics where the presence of eight specific proteins insured that the spectrometer was set properly. Raw spectra of the strains were analyzed by MALDI BIOTYPER 2.0 software (Bruker Daltonics) using the default settings (all of the settings are potentially adjustable). The whole process from MALDI-TOF MS measurement to identification was performed automatically without any user intervention. Briefly, the software generated a list of peaks up to 100. The threshold for peak acceptance was a signal-to-noise ratio of 3. After alignment, peaks with a mass-to-charge ratio difference of <250 p.p.m. were considered to be identical. The peak lists generated were used for matches against the reference library, by directly using the integrated pattern matching algorithm of the software. All parameters were the same, regardless of the bacteria analyzed. Spectra were obtained in the positive linear mode after 1000 shots (size, 61 794 points; delay, 232 points). A score was attributed to each identification obtained by MALDI-TOF MS. When this score was >2.00, identification was considered correct at the species level; in the range 1.7–1.999, the identification was considered correct at the genus level; and <1.7, the identification was not similar enough to a spectrum to draw a conclusion. For bacteria other than Gram-positive cocci, when the score was <2.0, an extraction step was carried out and the extract was tested again.

Genotypic identification

16S rRNA gene sequencing [5,6] was performed as previously reported [7]. *rpo*B gene sequencing was also performed for *Staphylococcus* species [8]. The amplified primer-less sequences were compared with the GenBank database with the BLAST software at the National Center for Biotechnology Information computer server [9] (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the species identification was confirmed using the bioinformatics bacterial identification tool BIBI [10].

Identification was considered to be correct when there was a concordance between the identification found with the MALDI-TOF MS and that of current phenotypic methods. For discordant results at the species level, identifications were considered to be correct with MALDI-TOF MS if the score was >2. For discordant results at the genus level, 16S rDNA sequencing was performed and the molecular biology result was considered as the reference.

Results

MALDI-TOF MS identification

Of 1013 isolates (Fig. 1), 837 were identified at the species level (with score values ≥2) without extraction. For 176 isolates, an extraction step was performed. Among these, 149 isolates were identified at the species level (with score values ≥2); 16 were identified at the genus level (with score values in the range 1.7–1.999); seven isolates remained unidentified (score value <1.7); and four isolates were falsely identified (with score values ≥2). Among the 16 isolates not identified by MALDI-TOF MS (Table 1), four *Acinetobacter baumannii* were identified as *Acinetobacter* sp., and one *Acinetobacter johnsonii* as *Acinetobacter lwoffii*. Among the other strains, eight were correctly identified with the MALDI-TOF MS but with a score <2, in the range 1.789–1.976 (i.e. very close to 2). For two of these eight isolates, the phenotypic identification was not concordant either with sequencing at the species level. Lastly, the MALDI-TOF MS gave four false identifications: *Haemophilus salivarius* was identified as *Streptococcus salivarius*, *Bordetella parapertussis* was identified as *Bordetella bronchiseptica*, *Citrobacter freundii* was identified as *Pseudomonas aeruginosa*, and *Streptococcus australis* was identified as *Streptococcus parasanguinis* by sequencing and *Streptococcus mitis* by phenotypic methods. With MALDI-TOF MS, four isolates were incorrectly identified at the genus level and a further seven isolates were not identified at all, for a total of 11 unidentified or wrongly identified isolates. Among these 11 isolates, four were identified at the species level with phenotypic methods: *Haemophilus aphrophilus*, *P. aeruginosa*, *Streptococcus pneumoniae* and *Clostridium ramosum*.

Phenotypic identification

With regard to standard phenotypic methods, an identification was obtained for 1006 out of 1013 bacteria. Nine hundred and forty-five (93.2%) were correctly identified at the species level and 996 cases (98.4%) at the genus level. With regard to identification at the species level, the phenotypic methods failed for 52 isolates (5.13%). Among the most common errors, ten were coagulase negative staphylococci, six were *Pseudomonas* sp. and two were *Aeromonas* sp. for which the species were not well identified. Twelve Enterobacteriaceae and five *Corynebacteria* sp. were not correctly identified either. However, for the majority of the cases (35/52), standard methods were able to identify the correct genus. For 17 isolates, phenotypic methods failed concerning identification at the genus level; more precisely, they led to a false identification for ten isolates and, for the other seven isolates, no identification at all was found (Table 2). Among these 17 isolates, ten were correctly identified by MALDI-TOF MS.

Discussion

This large study based on MS, performed as part of routine laboratory identification, led to the several observations, as outlined below.

Deposit step

A single deposit was made per isolate and this may have negatively influenced the MALDI-TOF results. However, we considered that it was more valuable in terms of time and even cost to make a single deposit for routine diagnosis.

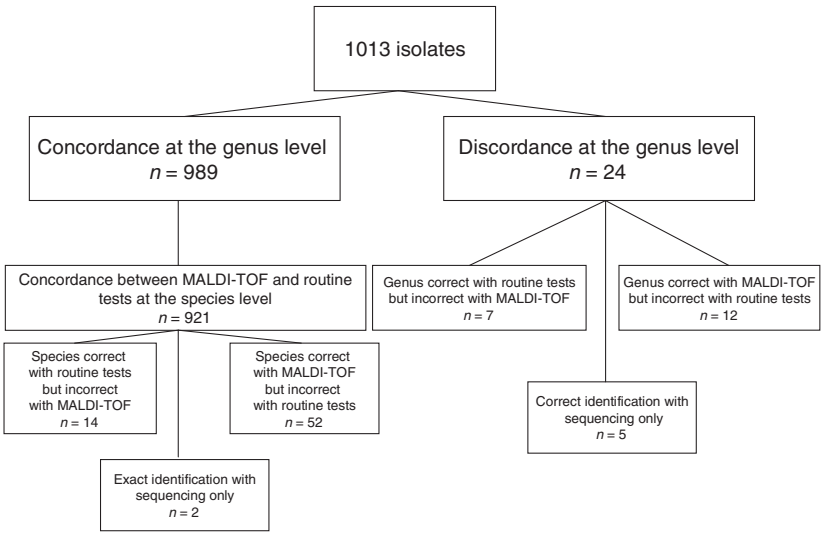

FIG. 1. Bacterial identification obtained with the different methods. MALDI-TOF MS, matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry.

TABLE 1. Unvalidated results of bacterial identification at the species or genus level using matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) after extraction vs. phenotypic methods

MALDI-TOF MS (score)	Routine phenotypic methods	16S rRNA gene sequencing
Isolates identified at the genus level		
Escherichia coli (1.975)	*E. coli*	NT
Acinetobacter sp. (2.228)	*Acinetobacter baumannii*	NT
Streptococcus parasanguinis (1.912)	*Streptococcus mitis*	*Streptococcus australis*
Acinetobacter sp. (2.175)	*Acinetobacter baumannii*	NT
Staphylococcus saprophyticus (1.907)	*S. saprophyticus*	NT
Streptococcus pyogenes (1.897)	*S. pyogenes*	NT
Bordetella bronchiseptica (2.02)	*Bordetella parapertussis*	*Bordetella parapertussis*
E. coli (1.896)	*E. coli*	NT
Acinetobacter lwoffii (2.312)	NI	*Acinetobacter johnsonii*
Staphylococcus saprophyticus (1.764)	*S. saprophyticus*	NT
Acinetobacter sp. (2.136)	*A. baumannii*	NT
Moraxella catarrhalis (1.901)	*Moraxella catarrhalis*	NT
Enterococcus faecalis (1.976)	*E. faecalis*	NT
Acinetobacter sp. (2.308)	*A. baumannii*	NT
Streptococcus pneumoniae (1.789)	*S. pneumoniae*	NT
Pseudomonas stutzeri (1.715)	*Pseudomonas oryzihabitans*	*P. stutzeri*
Isolates with an erroneous identification		
Corynebacterium jeikeium (2.098)	NI	*Lactobacillus rhamnosus*
Lactobacillus gasseri (2.139)	*Streptococcus* sp.	*Propionibacterium acnes*
Streptococcus salivarius (2.017)	*Haemophilus aphrophilus*	*H. aphrophilus*
Citrobacter freundii (2.093)	*Pseudomonas aeruginosa*	*P. aeruginosa*
Isolates for which no identification was found		
NI	NI	*Catenibacterium mutsuoka*
NI	*S. pneumoniae*	*S. pneumoniae*
NI	*Clostridium ramosum*	*C. ramosum**
NI	*Corynebacterium striatum*	*Corynebacterium phocae*
NI	*Bacteroides stercoris*	NT
NI	*Corynebacteria* sp.	*P. acnes*
NI	*Sphingobacterium* sp.	*Chryseobacterium* sp.*

NI, not identified; NT, not tested.
*Species not present in the database.

TABLE 2. Discrepancies observed for bacterial identification at the genus level using phenotypic methods vs. matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and 16S rRNA gene sequencing

Phenotypic methods	MALDI-TOF MS (score)	16S rRNA gene sequencing
Sphingobacterium sp.	NI	Chryseobacterium sp.*
Corynebacteria sp.	NI	*Propionibacterium acnes*
Streptococcus sp.	*Lactobacillus gasseri* (2.139)	*P. acnes*
Neisseria lactamica	*Gardnerella vaginalis* (2.382)	*G. vaginalis*
NI	*Aggregatibacter aphrophilus* (2.05)	*A. aphrophilus*
Corynebacteria sp.	*Bifidobacterium breve* (2.148)	*B. breve*
Gemella morbillorum	*Neisseria bacilliformis* (2.023)	*N. bacilliformis*
Gemella haemolysans	*Corynebacterium striatum* (2.291)	NT
Gemella haemolysans	*Staphylococcus saccharolyticus* (2.013)	*S. saccharolyticus*
Kocuria varians	*Turicella otitidis* (2.015)	NT
Corynebacteria sp.	*P. acnes* (2.03)	NT
NI	*Dermobacter hominis* (2.02)	*D. hominis*
NI	*Bacillus subtilis* (2.017)	*B. subtilis*
NI	*Neisseria elongata* (2.011)	*N. elongata*
NI	*Corynebacterium jeikeium* (2.098)	*Lactobacillus rhamnosus*
NI	*Acinetobacter lwoffii* (2.312)	*Acinetobacter johnsonii*
NI	NI	*Catenibacterium mutsuoka*

NI, not identified; NT, not tested.
*Species not present in the database.

Because excellent results were obtained, we consider that our deliberate choice was valid.

Extraction step

This step, which is without a doubt time-consuming, does not appear to be necessary in routine testing; only 15% of the isolates required an extraction. Among the 149 bacterial isolates for which an extraction was needed to achieve a score >2, the initial score was indeed very close to two for the majority (see Supporting information, Table S1). Finally, we consider that this step should not be performed routinely.

Discrepant results between MALDI-TOF and phenotypic methods

Errors with the phenotypic methods. As previously shown, the errors found with current phenotypic methods were generally at the species level because the genus was frequently correct. The percentage of errors found in Phoenix in the present study was similar to those found in various studies, in the range 83.9–99.3%, depending on the bacteria studied and methods used for the reference identification [11–13]. However, for all the Gram-positive cocci, the species determination remains quite difficult because neither the Phoenix, nor the API strips can lead to an identification with certainty, given that the scores obtained are not very high and that several identifications are often proposed. For these bacterial isolates, the identification is quite long, taking more than 24 h and sometimes requiring molecular methods. Therefore, the use of MALDI-TOF MS is of particular interest for such isolates with respect to obtaining a quicker result and a lower cost compared to current methods. Moreover, the

strains for which MALDI-TOF MS and sequencing results matched were bacteria that are usually difficult to identify with standard methods as a result of fastidious growth or because of specific incubation conditions (e.g. anaerobic atmosphere) [14]. This technique is undoubtedly of great help to identify rare species [15], or bacteria whose growth is difficult but which are present in the database. [16].

Errors with the MALDI-TOF MS. Different discrepancies can occur: (i) lack of identification at the species level and (ii) 'real' errors.

Using MALDI-TOF MS, in four cases, *A. baumannii* was identified only at the genus level as *Acinetobacter* sp. Similarly, for two strains of *S. pneumoniae*, one was identified with a very low score (1.789) and the other was not identified at all, whereas a result was obtained with phenotypic methods. There is no clear explanation for for this lack of success, except that there is such an important diversity in these species that the spectrum obtained does not always match those of the database. Moreover, for *Acinetobacter* sp., the failure may also be a result of the current limitations of Acinetobacter taxonomy (i.e. different taxa are grouped under the name *A. baumannii*).

Another problem with the MALDI-TOF MS was the low score obtained for eight bacterial isolates, even after extraction (Table 1). However, another parameter can be used as an identification criterion, which is the number of matching profiles corresponding to a given species. Indeed, for each species, several spectra corresponding to the same bacterial species are present in the Bruker Daltonics BIOTYPER database. Therefore, another possibility for validating the species identification would be to consider the number of times that the spectrum matches the bacterium under study. If this criterion had been used, these eight bacterial isolates would have been identified with the MALDI-TOF MS. This constitutes an interesting method for obtaininh a correct species identification and this approach would again shorten the identification process. Finally, MALDI-TOF MS is very accurate, more so than our usual methods to identify bacterial isolates [17].

Limitations as a result of the database

The main problem of the MALDI-TOF MS is the database which still needs to be expanded. Some isolates were not identified by MALDI-TOF MS because they are absent from the Bruker Daltonics database or they are present in insufficient numbers. Concerning anaerobic bacteria, the MALDI-TOF MS failed in a certain number of cases. These two points have been addressed in previous studies [4] indicating a weakness in the technique. This is problematic because anaerobic bacteria are difficult to identify under routine conditions and a solution via MALDI-TOF MS would be very welcome in this domain. Nevertheless, other studies have shown that, with an adapted database, the MALDI-TOF MS is of great interest for identifying anaerobic species within a short time [16]. Thus, all of these problems can be solved by adding more species and more isolates of the same species to the database.

Lastly, one major limit with the MALDI-TOF MS is the lack of information on antimicrobial susceptibility. There is certainly room for improvement here, again after having enlarged the Bruker Daltonics database. Indeed, the creation of sub-databases (e.g. to discriminate between methicillin-resistant and methicillin-susceptible *S. aureus*) should be feasible because their protein profiles are different [18,19], as well as there being the possibility of determining the resistance profile of different Enterobacteriaceae [20].

Integration of a spectrometer in laboratory routine

MS is undoubtedly the future tool of a bacteriology laboratory but its use in routine laboratory procedures will change work habits. To optimize its use and to give faster results concerning bacterial identification, ideally during agar plate reading, each colony suspected of medical interest (based on macroscopic and microscopic morphology) could be deposited on a target plate as a unique sample. After reading all of the plates, it would be possible to obtain identification of all bacteria collected and deposited on the target plate within 30–40 min for approximately 50 different strains. Ultimately, the bacteria for which susceptibility testing is necessary would be selected. Money would therefore be saved because antibiogram galleries, especially those with liquid medium, are quite expensive. Time would be saved, especially in comparison with phenotypic identification that requires a minimum of 24 h for the majority of isolates. Using spectrometry in such a way would constitute a real revolution in the standard bacteriology laboratory.

Conclusions

The present study, which evaluated the MS for routine identification of bacteria, showed that: (i) the extraction step is not necessary to obtain the greater majority of identifications, allowing one to save time and (ii) the number of spectrum matches can be used when the score is >1.7 and <2. Moreover, we evaluated the concordance of identification obtained with phenotypic methods vs. MALDI-TOF MS on more than 1000 isolates issued from clinical specimens, showing that: (i) the MALDI-TOF MS appears better than

our routine methods for bacterial identification and (ii) a better concordance was observed with sequencing than phenotypic methods.

The present study did not evaluate the cost of identification criterion, which has been considered positively in other studies.

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Transparency Declaration

The authors declare no conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Species for which an extraction was necessary to obtain a score>2.

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